Joint Danube Survey 3

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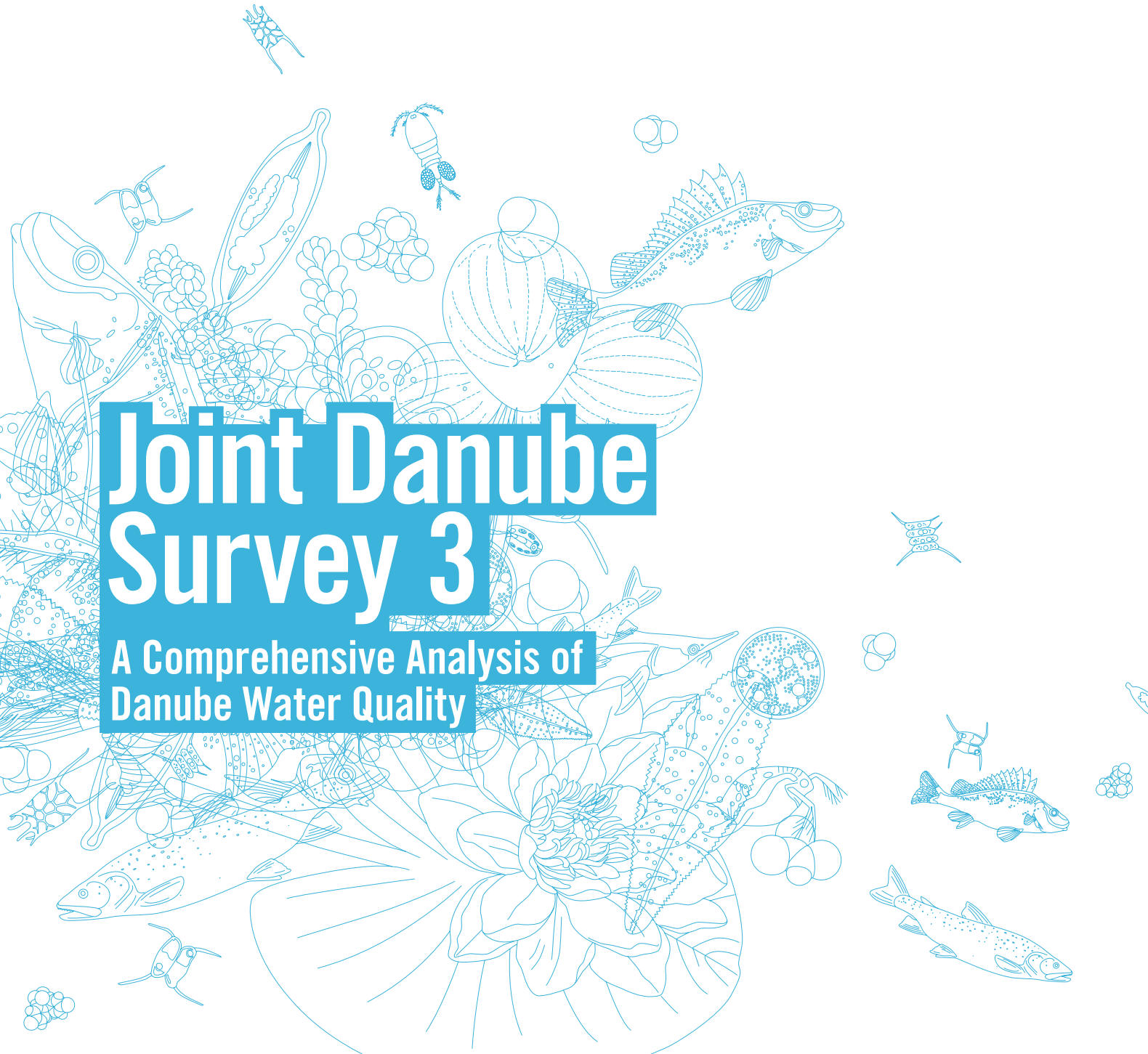

Joint Danube Survey 3

A Comprehensive Analysis of Danube Water Quality

Editors: Igor Liška, Franz Wagner, Manfred Sengl, Karin Deutsch and Jaroslav Slobodník

This Final Report contains an overview of the scientific findings of the Joint Danube Survey 3 (JDS3).
For a number of chapters, more detailed information can be found on the attached CD-ROM,
where full versions of the scientific papers are presented.
Map showing the JDS3 sampling sites is presented at the end of the report.

The authors wish to thank all those who supported and assisted in carrying out the JDS3, including national Delegations to the ICPDR, Core Team members, national teams and supporting laboratories, as well as sponsors from the private sector. Their support was crucial in carrying out the JDS3.

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1 ICPDR's efforts towards ensuring a healthy and clean Danube: Joint Danube Survey 3

Igor Liška

The adoption of the Convention on Cooperation for the Protection and Sustainable Use of the Danube River (Danube River Protection Convention) was driven by the strong intention of the Danube countries to intensify their water management cooperation in the field of water protection and water use. One of the key elements of that cooperation was collection and distribution of reliable information on water quality. To achieve this Transnational Monitoring Network was developed by the Danube countries producing information about water quality on annual basis since 1996.

This monitoring activity provided the necessary basis for harmonized water quality assessment throughout the whole basin, which not only gave an overview of water quality trends in the basin and of loads of substances discharged into the Black Sea but it fostered achieving of compatibility between water quality assessment approaches in the Danube countries. With the view of obtaining a complex outlook on the state of the Danube the yearly assessment of water quality has been supplemented by periodic investigative surveys which are carried out every six years in synchronicity with the river basin management planning period according to the EU Water Framework Directive (WFD).

The first Joint Danube Survey was carried out in 2001. For the first time comparable data about the entire course of the river have been provided covering over 140 different biological, chemical and bacteriological parameters. These data were used as an essential information source for the first analysis of the Danube River Basin District according to WFD Article 5. Six years later the second Joint Danube Survey has created a comprehensive and homogeneous database on the status of the aquatic ecosystem of the Danube and its major tributaries. For the first time the fish survey on the whole Danube was carried out bringing a unique dataset and contributing also to methodological harmonization between EU and non-EU countries. JDS2 also introduced the first ever systematic survey of hydromorphological parameters in the entire navigable longitudinal Danube stretch using a single method. The survey confirmed earlier ICPDR conclusions of a generally improving trend for water quality along the main Danube River. It also reinforced specific problems, especially at a number of tributaries and downstream of large cities. It appeared as well that a number of specific problem areas such as pollution by WFD priority substances as well as the newly emerging contaminants need further more extensive examination, particularly in some tributaries. JDS2 has proved to be a valuable tool for improving the databases for water quality assessments and it has confirmed the need to carry out such investigative monitoring exercise on a regular basis. Information produced by the two Joint Danube Surveys helped the ICPDR Contracting Parties to implement the Danube River Protection Convention and the EU Water Framework Directive and the concept of JDS has become an integral part of TNMN. The findings of JDS2 contributed to the first Danube River Basin District Management Plan.

That is why the signatories of the Danube Declaration, which was adopted at the ICPDR Ministerial Meeting in 2010 appreciated the very valuable results of the previous Joint Danube Surveys in 2001 and 2007 and requested the ICPDR to prepare a third Joint Danube Survey (JDS3) to be held in 2013. JDS3 was also addressed in the Action Plan of the EU Strategy for the Danube River Basin under the Priority Area 4 "To restore & maintain the quality of waters".

The general objective of the JDS3 was to undertake an international longitudinal survey that would produce comparable and reliable information on water quality for the whole of the length of the Danube River including the major tributaries on a short-term basis. The outcomes of the JDS3 should cover the information gaps as necessary for the implementation of the EU Water Framework Directive (WFD).

The specific objectives and added values of the JDS3 were identified by the Monitoring and Assessment Expert Group (MA EG) of the ICPDR as follows:

- Support to the revision of Danube River Basin District Management Plan by 2015;
- Assessment of methods for large rivers;
- Monitoring of new candidate priority substances;
- Identification and prioritization of Danube River Basin District specific substances;
- Trend analysis for Danube River Basin District relevant substances;
- Highlighting the link between surface water and groundwater pollution;
- Investigation of quality of sediments;
- Harmonization of sampling methods for WFD biological quality elements;
- Investigation of invasive alien species;
- Improvement of hydromorphological assessment with the view of developing a harmonized approach for the Danube;
- Interlinking hydromorphology and biology (habitat quality);
- Interlinking chemistry – biology – microbiology;
- Support to future Intercalibration exercise in the Danube River Basin District;
- Specific investigations (zooplankton, microbiology, ecotoxicology (bioassays));
- Testing new methods;
- Training/learning by doing;
- Public awareness raising.

Top specialists from the Danube countries took part in the survey and they worked in close cooperation with a large number of national experts making this way JDS3 a good opportunity for harmonization of monitoring methods throughout the basin as well as for testing new methods. This report shall serve not only as a review of the water quality in the Danube but also as a methodological training tool for widespread distribution in Danube countries. Such international cooperation towards improvement of monitoring practices aims to increase the reliability and accuracy of information on water quality throughout the entire basin. Availability of correct and reliable data is a basic prerequisite for setting any programmes of measures for ensuring the environmental objectives of EU WFD.

Joint Danube Surveys were not only an important source of information on water quality for the ICPDR but they were also an excellent tool for raising the public awareness and understanding about the Danube among the people who live in the basin. This has been achieved through the events and press conferences held along the Danube during the survey and this process will continue through follow-up presentations of the results after publishing of this survey report. The ICPDR is aware that only through the active involvement and interest of people can the goal of a clean and healthy Danube River Basin be achieved and the third Joint Danube Survey was a good opportunity for the public and stakeholders not only to learn about the status of the Danube water but to continue in fulfilling the JDS motto: "Watch Your Danube".

Organizing the third Joint Danube Survey was only possible thanks to financing and in-kind contributions by the ICPDR Contracting Parties. Financial, logistical, scientific and/or laboratory support was also received from the EC JRC in Ispra, from the FP7 Integrated Project SOLUTIONS, from the NORMAN Association (Network of reference laboratories, research centres and related organizations for monitoring of emerging environmental substances), TZW Karlsruhe/IAWD, Medical University Vienna and viaDonau in Vienna. The additional financial support was received from Donauchemie and Coca-Cola. DANUBEPARKS - the Danube River Network of Protected Areas organized monitoring of riparian bird species.

Gratitude goes to all ICPDR Contracting Parties, institutions, governmental officials, experts, stakeholders and other "friends of the Danube" for their commitment, enthusiasm and contributions ensuring that JDS3 could have successfully taken place.

2 Survey preparation

Jaroslav Slobodnik

2.1 Survey programme

The Third Joint Danube Survey (JDS3) was undertaken from 13 August to 25 September 2013. 68 sites were sampled by the JDS3 Core Team along a 2581 km stretch of the Danube, 15 of which were located in the mouths of tributaries or side arms. Samples from the first two stations Böfinger Halde and Kelheim in Germany were collected using cars, the remaining 2354 km were sampled by ships.

Sampling at the JDS3 stations included five different sample types – surface water, sediment, biology, suspended particulate matter (SPM) and biota (fish and mussels) – each with a different determinand list. Water samples for general physico-chemical and chemical analyses and phytoplankton were taken in the middle of the river and tributaries, whereas water samples for microbiological determinations were taken at all three (left, middle, right) profiles. Sediment samples from left and right bank were mixed and wet sieved on-board to obtain 63 um fraction. Whenever possible SPM samples were collected by the on-board centrifuge at the sampling site, however, in some cases a stretch of the river between two sampling sites had to be sampled instead due to time constraints. SPM samples from the first two sampling sites were collected by a centrifuge installed in a car.

Sampling of benthic invertebrates was carried out at the left and right bank of the Danube only. Additionally, a dredging of the bottom material for collection of benthic invertebrates took place in the middle of the river. Phytobenthos samples were taken from left and right bank both on the Danube itself and tributaries. Macrophytes were also collected at all sites within the 3 km stretches on the left and right bank.

Soil samples, leaves of riverbank plants from the flooded zone and riparian macrophytes were collected for the follow up analysis of metals.

Ground water and bank filtrate samples from seven pre-selected sites near the Danube were taken by local drinking water suppliers and delivered to the survey ships in 2 l polypropylene bottles.

Large volume water samples of 500 l were taken, after separating SPM and water phase by a centrifuge on board of the laboratory ship Argus, at 22 selected sites aiming to obtain sufficient material for the follow up Effect Directed Analysis (EDA) by a battery of bioassays and chemical target and non-target screening. Sample volume of 1000 l were taken at two sites (downstream Novi Sad, downstream Giurgiu/Ruse) for more detailed in-depth analysis and NORMAN Collaborative Trial on non-target screening involving 18 laboratories (downstream Giurgiu/Ruse).

Both raw water samples and water extracts obtained by large volume sampling were distributed to selected laboratories equipped with different types of LC-HR-MS(MS) and GC-MS equipment for screening of non-target substances. Such “digital sample banking” was carried out with the goal to allow for re-processing of the information and subsequent identification of contaminants present in samples in future without the need for additional sampling.

An “active” passive sampler system was installed on board Argus equipped with a battery of passive samplers for hydrophobic and polar compounds. During the sampling the survey laboratory ship moved downstream along a defined stretch and collected samples which contain water pollutants

integrated in time and space along that stretch. Samplers were exchanged every 4 - 6 days in order to cover the pre-defined river stretches.

A fish survey was performed at 32 sampling sites on the Danube out of which six sites were not included in the JDS3, however, matching sites from the previous JDS2 in order to observe long-term trends. The sampling was logistically kept as a parallel activity using separate vessels Messschiff IV in the Austrian reach of the Danube and Wien for the rest of the survey, along with two electrofishing boats. At each site, one of these boats secured sampling of shallow water areas during the day and night sampling whereas the other sampled the river bottom with an electrified bottom trawl net. The selection of sampling sites and time schedule of the survey were harmonised with the programme of sampling for chemical and biological analyses. Fish muscles, fish liver and whole fish specimen of pre-selected species were collected whenever available and stored in the freezer for further processing and chemical analyses. Fish otolithes were sampled for the follow up isotope analysis and fish blood samples were prepared/analysed directly on-board for assessment with two different ecotoxicological assays.

EC JRC performed an extended analysis of persistent organic pollutants and mercury in SPM samples from selected 23 sites and in six fish tissue and liver samples. The sites were selected in a way to ensure highest possible overlap with the sites analysed by JRC in the JDS2.

The hydromorphological survey included collection of background hydromorphological data for each station. A detailed hydromorphological characterisation of each JDS3 site was performed to support the interpretation of biological results. A joint ornithological survey for selected Danube stretches was carried out prior to the JDS3 to support the hydromorphological investigation, especially the occurrence of steep bank and sediment bar breeders as indicators for intact river reaches.

Detailed information on the actual sampling programme is shown in Table 1.

2.1.1 Survey preparation / Cruise Manual

Preparations for the JDS3 as regards the definition of survey objectives; selection of parameters to be measured; identification of sampling and analysis methods; identification of sampling sites and selection of experts was carried out by the Monitoring and Assessment Expert Group (MA EG) of the ICPDR. During the preparatory phase, the JDS3 Cruise Manual was developed, containing a detailed description of the tasks to be accomplished during preparation of the survey, sampling and analyses programme and reporting. A set of Standard Operational Procedures (SOPs) was developed, describing in detail sampling procedures and on-board analyses.

A series of meetings was held before the survey to agree upon logistical issues, equipment preparation and the methods to be used. Consumables, sample containers, chemicals and smaller equipment were purchased and delivered to the survey ships in July and August 2014. A significant part of the equipment was loaned by the JDS3 cooperating laboratories.

2.1.2 JDS3 Core Team

Members of the JDS3 Core Team and Reserve Team were nominated by the Danube states (Contracting Parties of the ICPDR) and selected by the ICPDR MAEG. Core Team members, responsible for sampling and on-board analyses, were on-board of the three ships (Istros, Argus and Wien) during the survey.

JDS3 Core Team			
Igor Liska	JDS3 Project Manager	Igor Stanković	Macrophytes expert
Jaroslav Slobodnik	Technical Coordinator	Mary Craciun	Chemistry expert
Bela Csanyi	Core Team Leader	Florentina Dumitrache	Chemistry expert
Momir Paunovic	Deputy Core Team Leader	Peter Tarabek	Chemistry expert
Thomas Huber	Benthic invertebrates expert – Upper Danube	Jan Busovsky	Hydromorphology expert
Patrick Leitner	Benthic invertebrates expert – Upper Danube	Radoslav Cuban	Hydromorphology expert
Jozsef Szekeres	Benthic invertebrates expert – Middle Danube	Peter Matok	Hydromorphology expert
Claudia Nagy	Benthic invertebrates expert – Lower Danube	Stefan Jakwerth	Microbiology expert
Jarmila Makovinska	Phytobenthos expert	Stoimir Kolarevic	Microbiology and ecotoxicology expert
Martin Dokulil	Phytoplankton expert	Georg Reischer	Microbiology expert
Ulrich Donabaum	Phytoplankton expert		

Fish Team			
Vinzenz Bammer	Fish Team Leader	Agnes Irma Gyorgy	Fish expert – bottom trawling
Michael Schabuss	Fish expert	Zoltan Szaloky	Fish expert – bottom trawling
Horst Zornig	Fish expert	Andras Weiperth	Fish expert – bottom trawling
Lachezar Pehlivanov	Fish expert		

Special guests from SOLUTIONS project	
Bjoern Deutschmann	Fish expert - ecotoxicology
Sandor Sipos	Fish expert - ecotoxicology
Tobias Schulze	Large volume sampling expert

Reserve Team	
Mila Kirilova Alexandrova-Ihtimanska	Benthic invertebrates expert
Katarina Holubova	Hydromorphology expert

JDS3 Managerial and Administrative Team		
ICPDR Executive Secretary	Ivan Zavadsky	Political back-up of the JDS3 project
Information expert	Alexander Hoebart	JDS3 website, database and data collection templates
GIS expert	Zoran Major	Map preparation
Public awareness expert	Benedikt Mandl	Public awareness, liaison with the ICPDR PP EG
Financial officer	Anna Koch	Financial accounting support
Administration & editing	Valeriya Gyosheva	Administration & editorial support, ICPDR intern
Administration	Sasa Tomasevic Balazs Nemeth	Administration support, ICPDR interns

2.1.3 JDS3 National Teams

National Teams either joined the JDS3 ships upon entering the territory of their country or carried out their parallel sampling at the same sites using own ships and cars. They cooperated with the JDS3 Core Team in collecting and processing the samples. Participation of National Teams was not only a

great help in accomplishing the ambitious technical programme of the survey but it was a unique opportunity for exchange of experience and harmonisation of the sampling and analytical methodologies throughout the Danube Basin. Such activity was essential to the implementation of the WFD and represented a particular support to the intercalibration activities. The network of National JDS3 Coordinators helped the Core Team with all necessary logistical arrangements in their home countries.

JDS3 National Coordinators		
Country	National Coordinator	Deputy National Coordinator
Germany	Manfred Sengl	
Austria	Helena Mühlmann	Franz Wagner
Czech Republic	Pavla Wildova	
Slovakia	Emilia Misikova-Elexova	
Hungary	Eniko Becsakne Tornay	
Croatia	Dagmar Surmanovic	
Serbia	Dušan Dobričić	
Bulgaria	Mina Asenova	
Romania	Gabriel Chiriac	
Ukraine	Oleksandr Bon	Yurii Nabyvanets
Moldova	Gabriel Gilca	Svetlana Stirbu

JDS3 Fish Team National Coordinators	
Germany	Jörg Brandner
Austria	Gerald Zauner
Slovakia	Vlado Kovac
Hungary	Tibor Erős Balázs Tóth
Croatia	Milorad Mrakovčić
Romania	Oliver Dumitrascu Istvan Gergely Radu Suciu
Bulgaria	Luchezar Pehlivanov

2.1.4 JDS3 Determinands

Altogether more than 800 individual parameters were investigated within the JDS3. This number includes parameters determined on-board during the survey and also the chemical, microbiological, ecotoxicological, radiological, isotope analysis and biological parameters analysed after the cruise. Special care was taken to include analysis of all biological quality elements needed for the assessment of the status of the Danube River according to the WFD and priority substances selected by the MAEG as important for the Danube basin based on the information from previous monitoring efforts and surveys. A specific focus was given to the identification of Danube River Basin Specific Pollutants (RBSPs). Water samples were therefore screened for presence of more than 650 target organic pollutants whereas additional several hundreds of non-target substances were tentatively identified for future evaluations.

2.1.5 JDS3 Laboratories

A large proportion of the laboratory services required for the JDS3 were secured through in-kind contributions by the ICPDR Contracting Parties, who provided their top laboratory facilities.

Supplementary analyses were contracted to the JDS3 laboratories. Leading national laboratories from Germany, Austria, Czech Republic, Slovakia, Croatia and Serbia performed the chemical analyses. The post-survey biological analyses were provided as in-kind contributions by several institutions in Austria (benthic invertebrates, phytoplankton, macrophytes, fish), Slovakia (phytobenthos), Hungary (benthic invertebrates, fish), Croatia (macrophytes) and Serbia (benthic invertebrates). An in-depth microbiological survey including specific ecotoxicological and antibiotic resistance pattern analyses, ornithological survey, strontium isotopes and radioactive contamination survey was carried out by a number of Austrian research institutes and universities. The EC JRC in Ispra, Italy, provided valuable support to the JDS3 through analyses of a wide range of priority substances, emerging pollutants, nutrients and microbiology parameters in various matrices.

Numerous NORMAN Association (www.norman-network.net) and SOLUTIONS (www.solutions-project.eu) laboratories were involved in sampling and chemical/ecotoxicological analyses of JDS3 samples.

For a full list of laboratories involved, see Table 2.

2.1.6 The ships

The survey was carried out using three ships kindly provided and financed by Romania (Istros), Serbia (Argus) and Austria (Wien). Technical information on the ships is as follows:

	Istros	Argus	Wien
Type of boat	Motor boat - accommodation and sample/material storage facilities, dining room	Motor boat, mounted grab - a research vessel used for water quality surveys, equipped with sampling devices, in-built field instrumentation and laboratory desks	Motor boat with electrofishing equipment and accommodation facilities
Captain	Ilie Suhov	Jovica Golubovic	Otto Bohdal
Cruising speed	18 km/h	25 km/h	22 km/h
Dimensions	Length: 32 m; width: 6.8 m; draught: 1.4 m	Length: 33.0 m; width: 4.5 m; draught: 1.3 m; height: 5 m	Length: 20.4 m; width: 3.8 m; draught: 1.2 m
Crew	7 persons	5 persons	1 person

The Argus was used for sampling and on-board laboratory analyses, while the Istros provided accommodation for the Core Team and National Team members as well as storage. Three small boats from the ships were used for parallel biological and chemical on-shore sampling. The fish team on Wien followed a separate sampling schedule using additional two electrofishing boats.

An additional ship (Meßschiff IV) offered as in-kind contribution by viadonau (Vienna, Austria) has been used to support the fish survey in the Austrian reach of the Danube.

2.1.7 The survey

2.1.7.1 Sampling

A small proportion of the chemical, biological, microbiological and ecotoxicological determinations were carried out directly on board the Argus; the majority of samples were transported under controlled conditions to the JDS3 laboratories for analysis.

Measurements made directly on-board during the survey included:

- General physico-chemical parameters – conductivity, dissolved oxygen, pH, water temperature, transparency;
- Microbiological parameters – Intestinal Enterococci (MU/SF Microtiterplates), Escherichia coli (Colilert), Total Coliforms (Colilert);
- Phytoplankton chlorophyll-a;
- Comet assay.

Water samples for analysis of heavy metals were filtered through 0.45 µm pore size membrane filters using a filtration device.

Sediment samples were taken from the left and right banks of the river (1-1.5 m depth) with a sampling net and mixed. This was followed by on-board grain size fractioning with wet sieving in order to get a less than 63 µm fraction for later analysis in the JDS3 laboratories.

SPM samples were collected from the middle of the river by pumping and centrifugation of water starting at a JDS3 station and continuing when sailing until the sufficient amount of the sample material had been recovered.

Mussels, fish tissue and fish liver collected from selected sites were collected and preserved (deep frozen) for analysis of trace metals and persistent organic pollutants on the list of WFD priority substances. Fish blood samples were taken directly on-board for the immediate Comet assay and follow up micronucleus assay.

Sediments and SPM samples were first freeze-dried in the laboratory of Umweltbundesamt GmbH in Vienna, Austria and then distributed for analysis to the individual laboratories. Fish muscles and liver samples were freeze-dried in the laboratory of EC JRC whereas whole fish samples were freeze-dried in the Water Research Institute, Prague, Czech Republic. All remaining sediment, SPM and whole fish samples were stored in the JDS3 Central Storage Facility at the Water Research Institute in Bratislava, Slovakia for future analysis.

Sampling of benthic invertebrates was conducted using two parallel techniques – AQEM Kick & Sweep sampling and deep water dredging in the middle of the river. Phytobenthos sampling was accompanied by direct on-site biomass determination by fluorescence detection. Macrophytes were collected at ca. 3 km stretches on both sides of the Danube and its tributaries. The electric fishing CEN-standardised methodology was used for fish survey purposes in the lithoral zone and a novel bottom trawling method has been applied on the river trans-sections.

Large volume water samples of 500 l were concentrated on cartridges filled out with three different sorbents after separating SPM and water phase by a centrifuge on board of the laboratory ship Argus.

The passive sampler system installed on board Argus was equipped with a battery of passive samplers for hydrophobic and polar compounds. Additionally, sediment samples were collected at representative sites towards the end of each stretch for assessment of contaminant concentrations that partition in the water phase (freely dissolved concentration).

Isotopes $^{87}Sr/^{86}Sr$ were analysed in river water and fish otoliths.

The hydromorphological survey included collection of background hydromorphological data for each station such as historical, topographical and navigation maps, satellite images, hydrologic and morphometric, land use data as well as basic data on harbours and daily traffic density for certain reaches. A continuous longitudinal survey of 10 rkm stretches was carried out to obtain an overview of the hydromorphological conditions of the Danube from Kelheim (rkm 2,415) to the Danube Delta (rkm 0). A detailed hydromorphological characterisation of each JDS3 site was performed to support the interpretation of biological results. This included sampling of the river bed material at each sampling locality for sediment characterisation; flow velocity and discharge measurements; integrative suspended particular matter measurements and water level slope/water level fluctuation measurements.

Samples of water, sediments and biota were stored in the refrigerators and/or freezers on-board of the survey ships and each 2-4 days transported to the laboratories for further analysis in cooling boxes either in a car with cooling capacity or via a courier service. In general, polypropylene bottles were used for storage of samples to be analysed for metals and general physico-chemical parameters and glass or polycarbonate bottles were used for samples to be analysed for organic micropollutants. Variations of sample temperature during the transport were controlled by adding special temperature-reading microchips to the cooling boxes. The laboratories were instructed to store the samples at cool or in a frozen state until analysis. Analyses of field blank samples were included among the JDS3 samples as a part of the quality control procedures.

Detailed description of the sampling procedures was provided in the Standard Operational Procedures (SOPs) developed for each sampled matrix and parameter, prior to the survey.

2.1.7.2 Hydrological conditions

A brief information on hydrological conditions during the survey is provided in Chapter 3.

2.1.8 Financial arrangements

The overall JDS3 budget exceeded 2 million EUR, most of which was financed by the ICPDR Contracting Parties (Germany, European Commission, Romania, Austria, Serbia, Slovakia, Hungary, Croatia, Czech Republic and Bulgaria) through financial and/or in-kind contributions. Financial support was also received from the Donauchemie AG (Austria) and Coca Cola HBC / Coca Cola Company. Additional in-kind contribution came from the Contracting Parties through the participation of National Teams. A significant in-kind contributions exceeding 400,000 EUR came from the NORMAN Association and SOLUTIONS project. The Austrian FWF-project P25817-B22 and P 23900-B22 contributed to JDS3 activities with more than 230,000 EUR. Substantial contribution came also from EC JRC in Ispra (Italy) providing chemical and microbiological analysis as well as shipping of samples and from TZW Karlsruhe in cooperation with IAWD in terms of chemical analyses of surface and ground water samples. DANUBEPARKS – the Danube River Network of Protected Areas organized monitoring of riparian bird species.

2.1.9 Public awareness

During the JDS3, press conferences were organised along the route of the cruise: the official public launch was organised in Regensburg (Germany) with consecutive press events in Vienna (Austria), Gabcikovo (Slovakia), Budapest (Hungary), Vukovar (Croatia), Belgrade (Serbia), Ruse (Bulgaria) and Vilkovo (Ukraine). The closing press conference was held in Tulcea (Romania).

A special website dedicated to the JDS3 (www.danubesurvey.org) was created providing important information about the survey, profiles of Core Team members and interesting scientific findings.

Numerous leaflets and fact sheets with relevant information about the JDS3 (such as the route, experts and institutions involved etc.) were developed in English as well as some national languages and distributed at respective press conferences.

2.1.10 Reporting

The JDS3 report is available on the website of the ICPDR (www.icpdr.org). The data and relevant metadata from the JDS3 were collected in the specifically developed Data Collection Templates (DCTs) allowing for their direct upload into the ICPDR database system. The existing Water Quality Database is being extended for the JDS3 component. Special care was taken to ensure the traceability of the quality assurance/quality control aspects of each result including into DCTs large amount of relevant metadata with detailed method description.

The database is being extended for storage of hydromorphology parameters, modules for phytoplankton and macrophytes and new types of microbiological (e.g. antibiotic resistance bacteria, metagenomics, etc.) and ecotoxicological (bioassays) parameters were updated. The “Danufishbase” at the Federal Agency for Water Management (BAW) in Austria, developed for the JDS2, was used for control, analysis and administration of fish data collected during the JDS3. The screening data of

target emerging substances and non-target pollutants will be stored in parallel in the NORMAN EMPODAT and NORMAN MassBank databases, respectively (http://www.norman-network.net/?q=node/24).

All database components (chemistry, biology, hydromorphology, ecotoxicology) are being integrated in order to facilitate future data processing and modelling efforts.

2.2 Acknowledgements

Special thanks are owed to NORMAN Association (www.norman-network.net) and SOLUTIONS (EU FP 7 project, www.solution-project.eu) for providing large amounts of special chemical and ecotoxicological analyses, the Austrian FWF-project P25817-B22 and P 23900-B22 for generous co-financing of the microbiological survey, JRC Ispra for the great logistical support at shipping of samples around Europe, trace-level chemical and microbial metagonomics analyses of organic substances of JDS3 samples, to DANUBEPARKS (www.danubeparks.org) for organizing monitoring of riparian bird species and to TZW Karlsruhe in cooperation with IAWD for carrying out chemical analyses of surface and ground water samples. Gratitude goes to Donauchemie AG (Austria) and Coca Cola HBC / Coca Cola Company for financial contribution to the survey.

Table 1: List of sampling locations and samples collected during the JDS3

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS1	L	Böfinger Halde	DE		1/2	2581		1	1	1	1	1	1	1								1		13/08/13
JDS1	R	Böfinger Halde	DE		1/2	2581					1	1		1										13/08/13
JDS2	L	Kelheim – gauging station	DE		2	2415		1	1	1	1	1	1	1		1								13/08/13
JDS2	M	Kelheim – gauging station	DE		2	2415		1							1			1	1					13/08/13
JDS2	R	Kelheim – gauging station	DE		2	2415		1			1	1		1										13/08/13
JDS3	L	Geisling power plant (upstream, downstream)	DE		2	2355	2354	1			1	1		1							1	1	1	14/08/13
JDS3	M	Geisling power plant (upstream, downstream)	DE		2	2355	2354	1	1	1	1		1											14/08/13
JDS3	R	Geisling power plant (upstream, downstream)	DE		2	2355	2354	1			1	1		1							1			14/08/13
JDS4	L	Deggendorf	DE		2	2285		1			1	1		1							1		1	15/08/13
JDS4	M	Deggendorf	DE		2	2285		1	1	1	1		1											15/08/13
JDS4	R	Deggendorf	DE		2	2285		1			1	1		1										15/08/13
JDS5	L	Mühlau	DE		2	2258		1			1	1		1									1	16/08/13
JDS5	M	Mühlau	DE		2	2258		1	1	1	1		1							1				16/08/13
JDS5	R	Mühlau	DE		2	2258		1			1	1		1							1	1		16/08/13
JDS6	L	Jochenstein	DE	AT	3	2204		1			1	1		1							1	1	1	17/08/13
JDS6	M	Jochenstein	DE	AT	3	2204		1	1	1	1		1		1	1			1					17/08/13
JDS6	R	Jochenstein	DE	AT	3	2204		1			1	1		1										17/08/13
JDS7	L	Upstream dam Abwinden-Asten	AT		3	2120		1			1	1		1							1	1		18/08/13
JDS7	M	Upstream dam Abwinden-Asten	AT		3	2120		1	1	1	1		1											18/08/13
JDS7	R	Upstream dam Abwinden-Asten	AT		3	2120		1			1	1		1										18/08/13
JDS8	L	Oberloiben	AT		3	2008		1			1	1		1								1		18/08/13
JDS8	M	Oberloiben	AT		3	2008		1	1	1	1		1		1		1							18/08/13
JDS8	R	Oberloiben	AT		3	2008		1			1	1		1										18/08/13
JDS9	L	Klostemeuburg	AT		4	1942		1			1	1		1										19/08/13
JDS9	M	Klostemeuburg	AT		4	1942		1	1	1	1		1			1		1	1					19/08/13

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS9	R	Klostemeuburg	AT		4	1942		1			1	1		1										19/08/13
JDS10	L	Wildungsmauer	AT		4	1895		1			1	1		1								1		20/08/13
JDS10	M	Wildungsmauer	AT		4	1895		1	1	1	1		1		1					1				20/08/13
JDS10	R	Wildungsmauer	AT		4	1895		1			1	1		1								1		20/08/13
JDS11	L	Upstream Morava (Hainburg)	AT		4	1881		1			1	1		1								1		21/08/13
JDS11	M	Upstream Morava (Hainburg)	AT		4	1881		1	1	1	1		1											21/08/13
JDS11	R	Upstream Morava (Hainburg)	AT		4	1881		1			1	1		1										21/08/13
JDS12	L	/Morava (rkm 0.08)	AT	SK		1880		1				1		1										21/08/13
JDS12	M	/Morava (rkm 0.08)	AT	SK		1880		1		1			1											21/08/13
JDS12	R	/Morava (rkm 0.08)	AT	SK		1880		1				1		1										21/08/13
JDS13	L	Bratislava	SK		4	1869		1			1	1		1							1			21/08/13
JDS13	M	Bratislava	SK		4	1869		1	1	1	1		1		1	1				1				21/08/13
JDS13	R	Bratislava	SK		4	1869		1			1	1		1										21/08/13
JDS14	L	Gabcikovo resevoir	SK	HU	4	1852	1848	1			1	1		1									1	22/08/13
JDS14	M	Gabcikovo resevoir	SK	HU	4	1852	1848	1	1	1	1		1											22/08/13
JDS14	R	Gabcikovo resevoir	SK	HU	4	1852	1848	1			1	1		1										22/08/13
JDS15	L	Medvedov/Medve	SK	HU	4	1806		1			1	1		1										23/08/13
JDS15	M	Medvedov/Medve	SK	HU	4	1806		1	1	1	1		1		1									23/08/13
JDS15	R	Medvedov/Medve	SK	HU	4	1806		1			1	1		1							1	1		23/08/13
JDS16	L	/Moson Danube Arm – end (rkm 0.1)	HU		4	1794		1			1	1		1										23/08/13
JDS16	M	/Moson Danube Arm – end (rkm 0.1)	HU		4	1794		1		1	1		1											23/08/13
JDS16	R	/Moson Danube Arm – end (rkm 0.1)	HU		4	1794		1			1	1		1										23/08/13
JDS17	L	Klizska Nema	SK	HU	4	1790		1			1	1		1							1			23/08/13
JDS17	M	Klizska Nema	SK	HU	4	1790		1	1	1	1		1							1				23/08/13
JDS17	R	Klizska Nema	SK	HU	4	1790		1			1	1		1								1		23/08/13
JDS18	L	/Vah (rkm 0.8)	SK			1766		1				1		1										24/08/13

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS18	M	/Vah (rkm 0.8)	SK			1766		1		1			1							0				24/08/13
JDS18	R	/Vah (rkm 0.8)	SK			1766		1				1		1										24/08/13
JDS19	L	Iza/Szony	SK	HU	5	1761		1			1	1		1										24/08/13
JDS19	M	Iza/Szony	SK	HU	5	1761		1	1	1	1		1			1				0				24/08/13
JDS19	R	Iza/Szony	SK	HU	5	1761		1			1	1		1							1	1		24/08/13
JDS20	L	Szob	HU		5	1707		1			1	1		1										25/08/13
JDS20	M	Szob	HU		5	1707		1	1	1	1		1		1	1			1	0				25/08/13
JDS20	R	Szob	HU		5	1707		1			1	1		1							1	1		25/08/13
JDS21	L	Budapest upstream – Megyeri Bridge	HU		5	1660		1			1	1		1							1	1		25/08/13
JDS21	M	Budapest upstream – Megyeri Bridge	HU		5	1660		1	1	1	1		1			1		1						25/08/13
JDS21	R	Budapest upstream – Megyeri Bridge	HU		5	1660		1			1	1		1										25/08/13
JDS22	L	Budapest downstream – M0 bridge	HU		5	1632		1			1	1		1			1				1	1		26/08/13
JDS22	M	Budapest downstream – M0 bridge	HU		5	1632		1	1	1	1		1		1	1								26/08/13
JDS22	R	Budapest downstream – M0 bridge	HU		5	1632		1			1	1		1										26/08/13
JDS23	L	/Rackeve-Soroksar Danube Arm – rkm 59	HU		5	1586		1				1		1										28/08/13
JDS23	M	/Rackeve-Soroksar Danube Arm – rkm 59	HU		5	1586		1		1			1											28/08/13
JDS23	R	/Rackeve-Soroksar Danube Arm – rkm 59	HU		5	1586		1				1		1										28/08/13
JDS24	L	Dunafoldvar	HU		5	1560		1			1	1		1										28/08/13
JDS24	M	Dunafoldvar	HU		5	1560		1	1	1	1		1			1								28/08/13
JDS24	R	Dunafoldvar	HU		5	1560		1			1	1		1							1	1		28/08/13
JDS25	L	Paks	HU		5	1533		1			1	1		1							1	1		29/08/13
JDS25	M	Paks	HU		5	1533		1	1	1	1		1											29/08/13
JDS25	R	Paks	HU		5	1533		1			1	1		1										29/08/13
JDS26	L	Baja	HU		5	1481		1			1	1		1										29/08/13
JDS26	M	Baja	HU		5	1481		1	1	1	1		1											29/08/13
JDS26	R	Baja	HU		5	1481		1			1	1		1							1	1		29/08/13

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS27	L	Hercegszanto	HU		5	1434		1			1	1		1							1	1		30/08/13
JDS27	M	Hercegszanto	HU		5	1434		1	1	1	1		1		1	1	1		1	1				30/08/13
JDS27	R	Hercegszanto	HU		5	1434		1			1	1		1										30/08/13
JDS28	L	Upstream Drava	HR	RS	6	1384		1			1	1		1										31/08/13
JDS28	M	Upstream Drava	HR	RS	6	1384		1	1	1	1		1		1					1				31/08/13
JDS28	R	Upstream Drava	HR	RS	6	1384		1			1	1		1							1			31/08/13
JDS29	L	/Drava (rkm 1.4)	HR			1379		1				1		1										31/08/13
JDS29	M	/Drava (rkm 1.4)	HR			1379		1		1			1				1							31/08/13
JDS29	R	/Drava (rkm 1.4)	HR			1379		1				1		1										31/08/13
JDS30	L	Downstream Drava (Erdut/Bogojevo)	HR	RS	6	1367		1			1	1		1										31/08/13
JDS30	M	Downstream Drava (Erdut/Bogojevo)	HR	RS	6	1367		1	1	1	1		1				1							31/08/13
JDS30	R	Downstream Drava (Erdut/Bogojevo)	HR	RS	6	1367		1			1	1		1							1	1		31/08/13
JDS31	L	Ilok/Backa Palanka	HR	RS	6	1300		1			1	1		1										01/09/13
JDS31	M	Ilok/Backa Palanka	HR	RS	6	1300		1		1	1		1		1									01/09/13
JDS31	R	Ilok/Backa Palanka	HR	RS	6	1300		1			1	1		1							1	1		01/09/13
JDS32	L	Upstream Novi-Sad	RS		6	1262		1			1	1		1										02/09/13
JDS32	M	Upstream Novi-Sad	RS		6	1262		1		1	1		1				1	1						02/09/13
JDS32	R	Upstream Novi-Sad	RS		6	1262		1			1	1		1								1		02/09/13
JDS33	L	Downstream Novi-Sad	RS		6	1252		1			1	1		1										03/09/13
JDS33	M	Downstream Novi-Sad	RS		6	1252		1	1	1	1		1		1	1	1			1				03/09/13
JDS33	R	Downstream Novi-Sad	RS		6	1252		1			1	1		1							1	1		03/09/13
JDS34	L	Upstream Tisa (Stari Slankamen)	RS		6	1216		1			1	1		1										03/09/13
JDS34	M	Upstream Tisa (Stari Slankamen)	RS		6	1216		1		1	1		1											03/09/13
JDS34	R	Upstream Tisa (Stari Slankamen)	RS		6	1216		1			1	1		1										03/09/13
JDS35	L	/Tisa (rkm 1.0)	RS			1215		1				1		1										03/09/13
JDS35	M	/Tisa (rkm 1.0)	RS			1215		1		1			1				1							03/09/13

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS35	R	/Tisa (rkm 1.0)	RS			1215		1				1		1										03/09/13
JDS36	L	Downstream Tisa/Upstream Sava (Belegis)	RS		6	1200		1			1	1		1							1	1		04/09/13
JDS36	M	Downstream Tisa/Upstream Sava (Belegis)	RS		6	1200		1	1	1	1		1		1	1	1			1				04/09/13
JDS36	R	Downstream Tisa/Upstream Sava (Belegis)	RS		6	1200		1			1	1		1										04/09/13
JDS37	L	/Sava (rkm 7.0)	RS			1170		1				1		1										04/09/13
JDS37	M	/Sava (rkm 7.0)	RS			1170		1		1			1				1	1						04/09/13
JDS37	R	/Sava (rkm 7.0)	RS			1170		1				1		1										04/09/13
JDS38	L	Upstream Pancevo/Downstream Sava	RS		6	1159		1			1	1		1										06/09/13
JDS38	M	Upstream Pancevo/Downstream Sava	RS		6	1159		1	1	1	1		1		1									06/09/13
JDS38	R	Upstream Pancevo/Downstream Sava	RS		6	1159		1			1	1		1							1	1	1	06/09/13
JDS39	L	Downstream Pancevo	RS		6	1151		1			1	1		1										06/09/13
JDS39	M	Downstream Pancevo	RS		6	1151		1	1	1	1		1			1	1							06/09/13
JDS39	R	Downstream Pancevo	RS		6	1151		1			1	1		1							1	1	1	06/09/13
JDS40	L	Upstream Velika Morava	RS		6	1107		1			1	1		1							1		1	07/09/13
JDS40	M	Upstream Velika Morava	RS		6	1107		1	1	1	1		1		1									07/09/13
JDS40	R	Upstream Velika Morava	RS		6	1107		1			1	1		1										07/09/13
JDS41	L	/Velika Morava	RS			1103		1				1		1										07/09/13
JDS41	M	/Velika Morava	RS			1103		1	1	1			1				1							07/09/13
JDS41	R	/Velika Morava	RS			1103		1				1		1										07/09/13
JDS42	L	Downstream Velika Morava	RS		6	1097		1			1	1		1							1	1	1	07/09/13
JDS42	M	Downstream Velika Morava	RS		6	1097		1	1	1	1		1											07/09/13
JDS42	R	Downstream Velika Morava	RS		6	1097		1			1	1		1									1	07/09/13
JDS43	L	Banatska Palanka/Bazias	RS	RO	7	1071	1073	1			1	1		1										08/09/13
JDS43	M	Banatska Palanka/Bazias	RS	RO	7	1071	1073	1	1	1	1		1			1								08/09/13
JDS43	R	Banatska Palanka/Bazias	RS	RO	7	1071	1073	1			1	1		1							1		1	08/09/13
JDS44	L	Irongate reservoir (Golubac/Koronin)	RS	RO	7	1040		1			1	1		1								1		09/09/13

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS44	M	Irongate reservoir (Golubac/Koronin)	RS	RO	7	1040		1	1	1	1		1		1		1							09/09/13
JDS44	R	Irongate reservoir (Golubac/Koronin)	RS	RO	7	1040		1			1	1		1							1		1	09/09/13
JDS45	L	Irongate reservoir (Tekija/Orsova)	RS	RO	7	954		1			1	1		1							1			09/09/13
JDS45	M	Irongate reservoir (Tekija/Orsova)	RS	RO	7	954		1	1	1	1		1											09/09/13
JDS45	R	Irongate reservoir (Tekija/Orsova)	RS	RO	7	954		1			1	1		1										09/09/13
JDS46	L	Vrbica/Simijan	RS	RO	8	926		1			1	1		1										10/09/13
JDS46	M	Vrbica/Simijan	RS	RO	8	926		1	1	1	1		1		1									10/09/13
JDS46	R	Vrbica/Simijan	RS	RO	8	926		1			1	1		1							1	1	1	10/09/13
JDS47	L	Upstream Timok (Rudujevac/Gruia)	RS	RO	8	849		1			1	1		1								1		12/09/13
JDS47	M	Upstream Timok (Rudujevac/Gruia)	RS	RO	8	849		1	1	1	1		1		1									12/09/13
JDS47	R	Upstream Timok (Rudujevac/Gruia)	RS	RO	8	849		1			1	1		1							1		1	12/09/13
JDS48	L	/Timok (rkm 0.2)	RS	BG		845		1				1		1							1		1	12/09/13
JDS48	M	/Timok (rkm 0.2)	RS	BG		845		1		1			1											12/09/13
JDS48	R	/Timok (rkm 0.2)	RS	BG		845		1				1		1								1		12/09/13
JDS49	L	Pristol/Novo Selo Harbour	RO	BG	8	834		1			1	1		1									1	13/09/13
JDS49	M	Pristol/Novo Selo Harbour	RO	BG	8	834		1	1	1	1		1			1								13/09/13
JDS49	R	Pristol/Novo Selo Harbour	RO	BG	8	834		1			1	1		1							1	1		13/09/13
JDS50	L	Downstream Kozloduy	BG	RO	8	685		1			1	1		1							1	1	1	14/09/13
JDS50	M	Downstream Kozloduy	BG	RO	8	685		1	1	1	1		1		1									14/09/13
JDS50	R	Downstream Kozloduy	BG	RO	8	685		1			1	1		1										14/09/13
JDS51	L	/Iskar (rkm 0.3)	BG			637		1				1		1							1			14/09/13
JDS51	M	/Iskar (rkm 0.3)	BG			637		1		1			1											14/09/13
JDS51	R	/Iskar (rkm 0.3)	BG			637		1				1		1										14/09/13
JDS51a	M	Upstream Olt	RO	BG		606		1																15/09/13
JDS51b	M	/Olt (rkm 0.4)	RO			605		1																15/09/13
JDS52	L	Downstream Olt	RO	BG	8	602		1			1	1		1							1		1	15/09/13

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS52	M	Downstream Olt	RO	BG	8	602		1	1	1	1		1		1									15/09/13
JDS52	R	Downstream Olt	RO	BG	8	602		1			1	1		1								1		15/09/13
JDS53	L	Downstream Zimnicea/Svishtov	RO	BG	8	550		1			1	1		1							1		1	15/09/13
JDS53	M	Downstream Zimnicea/Svishtov	RO	BG	8	550		1	1	1	1		1		1	1	1			1				15/09/13
JDS53	R	Downstream Zimnicea/Svishtov	RO	BG	8	550		1			1	1		1								1		15/09/13
JDS54	L	/Jantra (rkm 1.0)	BG			537		1				1		1										16/09/13
JDS54	M	/Jantra (rkm 1.0)	BG			537		1		1			1											16/09/13
JDS54	R	/Jantra (rkm 1.0)	BG			537		1				1		1										16/09/13
JDS55	L	Downstream Jantra	RO	BG	8	532		1			1	1		1								1		16/09/13
JDS55	M	Downstream Jantra	RO	BG	8	532		1	1	1	1		1			1	1	1						16/09/13
JDS55	R	Downstream Jantra	RO	BG	8	532		1			1	1		1							1		1	16/09/13
JDS56	L	/Russenski Lom	BG			498		1				1		1										16/09/13
JDS56	M	/Russenski Lom	BG			498		1		1			1											16/09/13
JDS56	R	/Russenski Lom	BG			498		1				1		1										16/09/13
JDS57	L	Downstream Ruse/Giurgiu	BG	RO	8	488		1			1	1		1							1	1	1	18/09/13
JDS57	M	Downstream Ruse/Giurgiu	BG	RO	8	488		1	1	1	1		1		1	1	1	1						18/09/13
JDS57	R	Downstream Ruse/Giurgiu	BG	RO	8	488		1			1	1		1							1	1		18/09/13
JDS58	L	/Arges	RO			432		1				1		1									1	18/09/13
JDS58	M	/Arges	RO			432		1		1			1											18/09/13
JDS58	R	/Arges	RO			432		1				1		1										18/09/13
JDS59	L	Downstream Arges, Oltenita	RO	BG	8	429		1			1	1		1									1	19/09/13
JDS59	M	Downstream Arges, Oltenita	RO	BG	8	429		1	1	1	1		1			1	1							19/09/13
JDS59	R	Downstream Arges, Oltenita	RO	BG	8	429		1			1	1		1							1	1		19/09/13
JDS60	L	Chiciu/Silistra	RO	BG	8	378		1			1	1		1									1	19/09/13
JDS60	M	Chiciu/Silistra	RO	BG	8	378		1	1	1	1		1		1	1	1			1				19/09/13
JDS60	R	Chiciu/Silistra	RO	BG	8	378		1			1	1		1							1	1		19/09/13

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS61	L	Giurgeni	RO		9	235		1			1	1		1							1	1		20/09/13
JDS61	M	Giurgeni	RO		9	235		1	1	1	1		1											20/09/13
JDS61	R	Giurgeni	RO		9	235		1			1	1		1									1	20/09/13
JDS62	L	Braila	RO		9	167		1			1	1		1									1	21/09/13
JDS62	M	Braila	RO		9	167		1	1	1	1		1		1	1				1				21/09/13
JDS62	R	Braila	RO		9	167		1			1	1		1							1	1	1	21/09/13
JDS63	L	/Siret (rkm 1.0)	RO			154		1				1		1							1	1		22/09/13
JDS63	M	/Siret (rkm 1.0)	RO			154		1		1			1				1							22/09/13
JDS63	R	/Siret (rkm 1.0)	RO			154		1				1		1										22/09/13
JDS63a	M	Upstream Prut	RO			137		1																22/09/13
JDS64	L	/Prut (rkm 1.0)	RO	MD		135		1				1		1										22/09/13
JDS64	M	/Prut (rkm 1.0)	RO	MD		135		1		1			1				1							22/09/13
JDS64	R	/Prut (rkm 1.0)	RO	MD		135		1				1		1										22/09/13
JDS65	L	Reni	RO	UA	9	130		1			1	1		1										22/09/13
JDS65	M	Reni	RO	UA	9	130		1	1	1	1		1		1	1	1		1	1				22/09/13
JDS65	R	Reni	RO	UA	9	130		1			1	1		1							1	1		22/09/13
JDS66	L	Vilkova – Chilia arm/Kilia arm	RO	UA	10	18		1			1	1		1							1	1		24/09/13
JDS66	M	Vilkova – Chilia arm/Kilia arm	RO	UA	10	18		1	1	1	1		1											24/09/13
JDS66	R	Vilkova – Chilia arm/Kilia arm	RO	UA	10	18		1			1	1		1										24/09/13
JDS67	L	Sulina – Sulina arm	RO		10	31		1			1	1		1										25/09/13
JDS67	M	Sulina – Sulina arm	RO		10	31		1	1	1	1		1		1	1	1			1				25/09/13
JDS67	R	Sulina – Sulina arm	RO		10	31		1			1	1		1							1	1		25/09/13
JDS68	L	Sf.Gheorghe – Sf.Gheorghe arm	RO		10	107		1			1	1		1							1	1	1	25/09/13
JDS68	M	Sf.Gheorghe – Sf.Gheorghe arm	RO		10	107		1	1	1	1		1											25/09/13
JDS68	R	Sf.Gheorghe – Sf.Gheorghe arm	RO		10	107		1			1	1		1							1	1		25/09/13
		Number of samples						202	51	68	160	136	68	136	26	23	22	7	6	13	49	46	28	

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
		Passive sampling stretches																						
V1	M	Regensburg – Passau	DE			2375	2225																	
V2	M	Passau – Bratislava	DE	SK		2225	1852																	
V3	M	Bratislava – Budapest	SK	HU		1852	1632																	
V4	M	Budapest – Vukovar	HU	HR		1648	1297																	
V5	M	Vukovar – Belgrade	HR	RS		1297	1154																	
V6	M	Belgrade – Turnu-Severin	RS	RO		1154	930																	
V7	M	Turnu-Severin – Ruse	RO	BG		930	495																	
V8	M	Ruse – Braila	BG	RO		495	170																	
V9	M	Braila – Tulcea	RO			170	71																	
		Fish sampling stretches/cross-sections[6]																						
JDS2		Kelheim	DE			2420									1									14/8/13
JDS2-5		Niederalteich	DE			2278									1									16/8/13
JDS6		Jochenstein	DE	AT		2215	2204								1									16/8/13
JDS2 -9		Ybbs Persenbeug	AT			2072	2061								1									17/8/13
JDS8		Oberloiben	AT			2010	2000								1									18/8/13
JDS10		Wildungsmauer – Heinburg	AT			1894	1880								1									22/8/13
JDS13		Bratislava	SK			1876	1869								1									23/8/13
JDS2-17		Cunovo	SK			1852									1									24/8/13
JDS15		Medvedov/Medve	HU	SK		1807	1800								1									25/8/13
JDS20		Szob	HU			1705	1700								1									26/8/13
JDS22		Budapest downstream	HU			1632	1627								1									27/8/13
JDS27		Hercegszanto	HU			1446	1441								1									29/8/13
JDS28		Upstream Drava, Aljmas	HR	RS		1380									1									30/8/13
JDS31		Ilok/Backa Palanka	HR	RS		1303	1300								1									31/8/13
JDS33		Novi Sad downstream	RS			1252	1250								1									2/9/13

Station code	Location code	Location name	Country code	Country code	Danube type	River-km: start	River-km: other/end	Surface water	SPM	Sediments[1]	Benthic invertebrates[2]	Phytobenthos	P hytoplankton	Macrophytes	Fish	SPM – JRC	SW – LVSPE 500 L	Ground water[4]	Fish tissue/liver[3]	Whole fish[5]	Soil: flooded zone	Leaves flooded zone	Riparian macrophytes	Date of sampling
JDS36		Downstream Tisa/Upstream Sava (Belegis)	RS			1202									1									3/9/13
JDS38		Upstream Pancevo/Downstream Sava	RS			1163									1									4/9/13
JDS2-54		Grocka	RS			1132									1									6/9/13
JDS40		Upstream Velika Morava	RS			1107									1									7/9/13
JDS44		Irongate reservoir (Golubac/Koronin)	RO	RS		1046	1036								1									8/9/13
JDS46		Vrbica/Simijan[7]	RO	RS		1027	1022								1									9/9/13
JDS47		Upstream Timok (Rudujevac/Gruia)	RO	RS		850	840								1									11/9/13
JDS50		Downstream Kozloduy	BG	RO		690	680								1									13/9/13
JDS2-72		Downstream Iskar	BG	RO		634	624								1									14/9/13
JDS52		Downstream Olt	RO	BG		602	597								1									15/9/13
JDS53		Downstream Zimnicea/Svishtov	BG	RO		557	545								1									16/9/13
JDS57		Downstream Ruse/Giurgiu	RO	BG		485	495								1									18/9/13
JDS60		Chiciu/Silistra	BG	RO		383	373								1									19/9/13
JDS62		Braila	RO			172	163								1									21/9/13
JDS65		Reni	RO	UA		136	132								1									22/9/13
JDS2-93a		Chilia Arm-Valcov	RO			60									1									23/9/13
JDS67		Sulina – Sulina arm	RO			21									1									24/9/13
		Number of sampling sites													**32**									

[1] Sediment samples taken left and right and mixed prior to wet sieving;
[2] Benthic invertebrates samples taken left and right with Kick&Sweep AQEM methodology and middle (5 stripes in deep water) by dredging;
[3] Fish tissue/liver – dissected Abramis brama;
[4] Ground water provided by local operators at Regensburg, Vienna, Budapest, Novi Sad, Belgrade, Slivo Polje and Giurgiu.
[5] Whole fish samples – 3-5 specimen Abramis brama whenever available; Aspius aspius, Carassius gibelio at JDS60; Abramis brama and C. Gibelio at JDS67.
[6] Fish sampling sites included 6 locations from the JDS2 (in blue colour), which were not in the official programme of the JDS3

Table 2: List of JDS3 cooperating laboratories

(*In alphabetic order*)

No.	Laboratory name	Country
1	Austrian Agency for Health and Food Safety, Vienna	AT
2	Bavarian Environment Agency, Augsburg	DE
3	Bundesamt für Wasserwirtschaft, Institut für Gewässerökologie, Fischereibiologie und Seenkunde, Mondsee	AT
4	Croatian Waters, Central Water Management Laboratory, Zagreb	HR
5	Danube Research Institute, Hungarian Academy of Sciences, Goed	HU
6	DVGW-Technologiezentrum Wasser (TZW), Karlsruhe	DE
7	DWS-Hydro-Oekologie Vienna	AT
8	Environmental Institute, Kos	SK
9	European Commission, DG Joint Research Centre, Institute for Environment and Sustainability, Unit H.01 Water Resources, Ispra	
10	Federal Institute for Materials Research and Testing, Berlin	DE
11	Floodplain Ecology and River Basin Management, Vienna	AT
12	French National Institute for Industrial Environment and Risks, Verneuil-en-Halatte	FR
13	Helmholtz Centre for Environmental Research – UFZ, Leipzig	DE
14	Institute for Biological Research 'Sinisa Stankovic', Belgrade	RS
15	Medical University Vienna, Institute for Hygiene and Applied Immunology, Water Hygiene	AT
16	Medical University, Graz	AT
17	Norwegian Institute for Water Research, Oslo	NO
18	Povodi Moravy, s.p., Brno	CZ
19	RECETOX, Masaryk University, Brno	CZ
20	RWTH Aachen University, Inst. for Environmental Research (Biology V), Department of Ecosystem Analysis, Aachen	DE
21	Systema Bio- und Managementconsulting GmbH, Vienna	AT
22	Umeå University, Department of Chemistry, Umea	SE
23	Umweltbundesamt GmbH, Vienna	AT
24	University of Belgrade, Faculty of Biology	RS
25	University of Natural Resources and Applied Life Science, Low-Level Counting-Laboratory Arsenal, Vienna	AT
26	University of Natural Resources and Applied Life Sciences, Institute for Hydrobiology and Aquatic Ecology Management, Department Water – Atmosphere – Environment, Vienna	AT
27	University of Natural Resources and Life Sciences, Vienna, Dept. of Chemistry, Division of Analytical Chemistry, VIRIS Laboratory – Biological Migration Studies, Tulln	AT
28	University of Vienna, Faculty of Life Sciences, Department of Limnology and Hydrobotany, Vienna	AT
29	Vienna University of Technology, Institut of Chemical Engineering, Vienna	AT
30	Wassercluster, Lunz	AT
31	Water Research Institute, Bratislava	SK
32	Water Research Institute, Brno	CZ
33	Zweckverband Landeswasserversorgung Betriebs- und Forschungslabor, Langenau	DE
34	Jaroslav Černi Institute for the Development of Water Resources (JCI)	RS
35	Department of Biology, University of Josip Juraj Strossmayer, Osijek	HR
36	Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš	RS

3 Hydromorphology

Ulrich Schwarz, Katarina Holubova, Radoslav Cuban, Peter Matok, Jan Busovsky

3.1 Introduction

Considering the River basin analysis (2005) and first River Basin management plans (2009) across Europe hydromorphological alterations were recognised as significant water management issues which is also reflected in the updated River basin analysis (2013) and upcoming update of the Danube river basin district management plan (2015) elaborated under ICPDR coordination for the Danube. The most significant pressures were defined by longitudinal continuity interruptions (dams, weirs) and morphological alterations, lateral connectivity interruptions (loss of floodplains, bank reinforcements) and hydrological alterations. These alterations may cause the decline of species biodiversity, a reduced species abundance, altered population composition as well as the hindrance of species migration and the corresponding decline of naturally reproducing fish populations (in particular sturgeon species for the Danube river itself). Alterations of sediment quantity and composition as well as sediment accumulation and erosion upstream and downstream of dams have also to be considered.

The JDS3 Hymo assessment (longitudinal survey) and detailed JDS site analysis serve as a Danube river wide investigation of hydromorphological conditions, an evaluation tool of the current hydromorphological conditions as well as the assessment of hydromorphological alterations based on the deviation from near to natural conditions which were defined by authors for JDS3 purposes. Further it delivers basic information/data for the development of restoration measures and increase knowledge of the hydromorphological conditions of the Danube. The hydromorphological assessments which were performed in the frame of JDS3 are based on a methodology which was elaborated for this purpose. The results provide information based on the applied methodology, which does not replace any national methodology in any Danube riparian country. The results can therefore by nature differ from assessments which were performed based on different national methodologies.

After the first overall hydromorphological assessment of the Danube during JDS2 in 2007 (ICPDR 2008) a methodology which was oriented on the CEN standard (CEN "Water quality – Guidance standards on the assessment of hydromorphological features in rivers" (EN14614:2004 (CEN 2004) and CEN "Water quality – Guidance standard on determining the degree of modification of river hydromorphology" EN 15843:2010 (CEN 2010)) was further extended and applied during JDS3 to 10 rkm segments. In addition a detailed in-situ measurement and sampling of hydromorphological parameters was possible for all of the 68 JDS3 sites. The SOP (Standard Operational Procedure) for the hydromorphological analysis defines the two different approaches for the continuous longitudinal assessment and the detailed site survey. The first one will assess the hydromorphological situation along the whole Danube while the latter one provides substantial supporting data and information for the interpretation of biological results at a particular sampling site and allows the comparison and validation of the assessment by detailed field measurements by using a specific site assessment

approach (CEN based national SK approach developed by VÚVH). To fulfil the main task the so called WFD 3Digit approach, a selection of relevant parameters applied for the near to natural[1] based assessment of the morphological, hydrological and continuity components required by WFD (Annex II and V) parameters of the continuous assessment were used during JDS3.

The first time measured hydromorphological parameters for each site in detail raised the quality and reliability of hydromorphological assessment significantly and support directly the assessment of the biological elements for water bodies under the WFD. The strongest link is given to the physical habitat description of fish, macrozoobenthos and macrophytes, by providing data on substrate composition, flow velocities, discharges and the width-depth variability of sites by detailed cross sections.

The JDS3 hydromorphological survey delivers a sound based data set supporting the required hydromorphological risk assessment by the countries, underlined by in-situ measurements and provides for the first time detailed physical habitat data for 68 JDS sites allowing more specific analysis and correlation between Biological Quality Elements (BQE) and Hydromorphological Quality elements (namely for morphology and flow regime). The assessment was based on a concise methodology, applicable for the whole 2,400 rkm long Danube river stretch assessed during the survey and should supplement, but not substitute, the national hydromorphological assessments required by WFD.

During entire JDS3 relatively steady low flow conditions prevailed in the Danube. Also not all of the methodological parameters could be measured in situ in all river sections due to different reasons.

3.2 Methods

The preparation, survey and elaboration of results were a process taking over two years and included a collection of a lot of background data and several working steps. Based on the experiences of JDS2 the following working steps can be distinguished:

1. A various set of background maps and data was collected prior the survey and provided to the core team members such as current and older navigation maps or high resolution aerial images in form of so called “Fact sheets” for all JDS sites.
2. Method development (both for continuous assessment and site survey) and preparation of the survey equipment and operation.
3. Survey, site sampling (measurements and sediment samples), assessments and photo documentation.
4. Databases, analysis with resulting graphs, maps and reports.

To manage collection of all data during JDS3 there were always two HYMO experts working on board of the ship and three experts involved in preparing the methods, data and evaluation of the results.

In general two major survey and assessment methodologies can be distinguished:

1. Continuous longitudinal hydromorphological assessment of 10 rkm segments (it is important to indicate that the CEN oriented method used in the JDS assessment are based on principle of “arithmetic mean” value both for WFD 3Digit and for the overall assessments).This approach was also applied for transboundary stretches where the arithmetic mean values integrate conditions from both banks and do not reflect the specific situation from each river bank.

[1] for the entire document the near to natural conditions should be seen as those defined by authors for JDS3 purposes

2. Detailed site analysis by field work data, measurements, samples and assessment.

For the continuous assessment all the data is qualitative and obtained by high resolution image analysis, maps and field observations, where ever possible during low water conditions.

3.2.1 Continuous longitudinal hydromorphological assessment of 10 rkm segments

The assessment is based on a 10rkm segmentation of the whole Danube from Kelheim to the delta (about 2,420 rkm) allowing assessment values for channel, left/right banks, left/right floodplain (forming the base dataset for the WFD 3Digit assessment) as well as the overall assessment.

The assessment of the hydromorphology is based on comparing the deviation from near to natural conditions which were defined by authors for JDS3 purposes (see extended version on the attached CD) based on the given Danube typology developed in 2003 by Sommerhäuser et al. (see Table 3 below).While some parameters were derived from various historical sources (such as planform, floodplain extent, land use), other parameters are only defined as presence or absents (degree) of human alterations, namely the amount of artificial bank material.

Table 3: The 10 River Section Types

Danube Section Type	Description
Section Type 1: Upper course of the Danube	Upper course of the Danube (rkm 2786: confluence of Brigach and Breg – rkm 2581: Neu Ulm)
Section Type 2: Western Alpine Foothills Danube	Western Alpine Foothills Danube (rkm 2581: Neu Ulm – rkm 2225: Passau) with two sub-sections split in Regensburg
Section Type 3: Eastern Alpine Foothills Danube	Eastern Alpine Foothills Danube (rkm 2225: Passau – rkm 2001: Krems)
Section Type 4: Lower Alpine Foothills Danube	Lower Alpine Foothills Danube (rkm 2001: Krems – rkm 1807: Gönyü/KližskáNemá) with two sub-sections split at Devin/Morava confluence
Section Type 5: Hungarian Danube Bend	Hungarian Danube Bend (rkm 1807: Gönyü/ KližskáNemá – rkm 1497: Baja) with three sub-sections including the Danube bend breakthrough and stretches up and downstream
Section Type 6: Pannonian Plain Danube	Pannonian Plain Danube (rkm 1497: Baja – rkm 1075 : Bazias) with three sub-sections split at Drava and Sava confluences
Section Type 7: Iron Gate Danube	Iron Gate Danube (rkm 1075: Bazias – rkm 943: TurnuSeverin)
Section Type 8: Western Pontic Danube	Western Pontic Danube (rkm 943: TurnuSeverin – rkm 375.5: Chiciu/Silistra)
Section Type 9: Eastern Wallachian Danube	Eastern Wallachian Danube (rkm 375.5: Chiciu/Silistra – rkm 100: Isaccea)
Section Type 10: Danube Delta	Danube Delta (rkm 100: Isaccea – rkm 0 on Chilia arm, rkm 0 on Sulina arm and rkm 0 on Sf. Gheorghe arm)

For the hydromorphological assessment the Danube was subdivided into 241 segments of 10 rkm length following the current navigation map plus 18 segments for the additional Delta branches (Chilia (11) and St. Gheorghe branches (7) beginning from branch separation). Only the very first segment at Kelheim has only about 5 rkm and at the dam of Straubing the rkm was changed decades ago switching now from 2,330 rkm at the hydropower dam to 2,322.2 rkm downstream, which means nearly 8 km are missing. Therefore the segment from 2,320-2,330 is missing and the neighbouring segment calls 2,310-2,330 to keep a consistent counting in the database. Altogether 1,554 (269 x 6) sub-segments were evaluated for right and left floodplain, right and left banks, channel as well as the overall assessment. Those segments where dams fall not close to its lower ends (buffer up to 3 km to further downstream segment) were assessed as whole as having the dam inside.

The following table 4 shows the parameter groups morphology, hydrology and river continuity. For hydrology and river continuity only one parameter was used for each of these two parameter groups. For morphology eight parameters were used (see table 4), calculating the arithmetic mean. Each morphological parameter got the assessment classes 1-5: 1 (near natural), 2 (slightly modified),

3 (moderately modified), 4 (extensively modified) and 5 (severely modified). The parameters for hydrology and river continuity got only values 1, 3 or 5.

Table 4: Assessment scheme for WFD 3 digit continuous survey

	JDS Parameter	WFD parameters covered
Morphology	Planform (based on deviation from near to natural conditions for section types)	"Channel patterns"
	Substrates (Natural substrate mix or character altered) (based on deviation from near to natural conditions for section types)	"Substrate conditions"
	Erosion/deposition character (based on deviation from near to natural conditions for section types)	"River depth and width variation"
	Extent of reach affected by artificial bank material (% of bank length)	
	Land cover in riparian zone (top of banks and adjacent narrow strip) (% of bank length)	"Structure of the riparian zone"
	Land cover beyond the riparian zone (based on deviation from near to natural conditions for section types)	
	Degree of lateral connectivity of river and floodplain (Extent of floodplain not allowed to flood regularly due to engineering-based on hydromorphological surveys.) (based on deviation from near to natural conditions for section types)	
	Degree of lateral movement of river channel (% of length where lateral movement is artificially constraint)	
Hydrology	Changes of flow conditions due to artificial in-channel structures within the reach (impoundments, density of groynes and reflectors)	"Quantity and dynamics of water flow"
River continuity	Reach-based and local impacts of sluices and weirs on river continuity with regard to biological and sediment continuity	"The continuity of the river is not disturbed by anthropogenic activities, undisturbed migration of aquatic organisms and undisturbed sediment transport"

No residual water stretches were assessed (Bad Abbach, Szigetköz) with regard to parameter group hydrology. Hydropeaking and basin wide discharge regime couldn't be systematically assessed due to insufficient data or below level of significance as set by the countries.

The overall CEN assessment (Table 5) is based on individual parameters for channel, banks and floodplain and allows an assessment into five classes based on arithmetic mean values for each parameter group and the overall assessment. For channel, the parameter of "impacts of artificial in-channel structures" was assessed only in 1, 3 and 5.

Table 5: Assessment scheme for the continuous survey

	Parameter
Channel	Planform (based on deviation from near to natural conditions for section types)
	Substrates (Natural substrate mix or character altered), (based on deviation from near to natural conditions for section types)
	Erosion/deposition character (based on deviation from near to natural conditions for section types)
	Impacts of artificial in-channel structures within the reach (impoundments, groynes) (this single parameter was only assessed in 1, 3 and 5)
	Reach-based and local impacts of sluices and weirs on ability of biota (e.g. migratory fish) to travel through reach, and sediment to be transported naturally
Banks	Extent of reach affected by artificial bank material (% of bank length)
	Land cover in riparian zone (% of bank length)
Floodplain	Land cover beyond the riparian zone
	Degree of lateral connectivity of river and floodplain (Extent of floodplain not allowed to flood regularly due to engineering-based on hydromorphological surveys.) (based on deviation from near to natural conditions for section types)
	Degree of lateral movement of river channel (% of length where lateral movement is artificially constraint)

The overall assessment was applied to maintain the continuity with JDS2 assessments, while the 3-digit assessment was performed in order to address WFD requirements.

The results of the main assessment were visualised in form of a colour ribbon map and atlas showing the overall assessment as well as the individual assessments for channel, left/right banks and left/right floodplains and are available as digital annex on the supplementary CD attached to this report.

3.2.2 Methods of Site Survey – In situ Measurements

Hydrological, morphological and hydraulic parameters were selected to cover the main indicators of morphological alteration of the river channel in line with WFD (hydrology, continuity & morphology) considering time limit (4 hours/site) and technical equipment. The in-situ measurements included: discharge, velocity (flow pattern, surface velocity), cross sections, bed material sampling, suspended load sampling, water level fluctuation, and water level slope. Field measurements are accompanied by detailed visual observations, photos and sketches done for each survey site.

Purpose and methods of field measurements are described in Standard Operational Procedure (SOP, available for all core teams) but also briefly summarized in this report including modifications that had to be implemented due to specific site conditions. In-situ HYMO survey was prepared and performed by the team from VÚVH, Bratislava, Slovakia (4 experts – two of them always on board). Substantial part of the field survey at 67 sites of the Danube and main tributaries was done by two experts either from a small motor boat or from the river bank. Detailed site observation and documentation was done during the transport between sites.

Figure 1: Discharge & velocity measurements (ADCP)

Figure 2: Bed material sampling (bottom sampler)

Flow velocity (v) and discharge (Q) measurements: ADCP (Son Tek – River Surveyor, 0.7 m < H < 40 m) for 3D flow velocity measurements was used to provide spatial velocity distribution and cover the wide range of water depths and velocities in the Danube (Fig.1). ADCP measurements also provided data on river channel topography (cross sections). Measurements of surface velocity (SVR-Stalker, 0.2 m/s < v < 18 m/s) were performed mostly by the macrozoobenthos group. At the section between Kelheim (JDS2) and Budapest (JDS22) just one cross section was measured 5 times (two extreme values are excluded, resulting value is the average form remaining ones). Downstream of Budapest to Danube Delta five cross sections were measured once at the sections with constant discharge. This modification enabled to obtain more detailed topography data. Discharge & velocity were measured at 59 sites. The measurements from eight sites are missing due to weather conditions or too shallow water (tributaries). Accuracy of discharge measurement (ADCP) which is usually about 99% can be lower in case of strong impoundments (very slowly flowing water – velocity decrease bellow < 0.25 m/s, JDS43 rkm 1,073, JDS44 rkm 1,040, JDS45 rkm 956) due to specific flow conditions

Sediment sampling and analysis: bottom sampler – drag bucket type (Fig.2) was used to collect bed material samples. The sampler lowered to the river bottom was dragged along the bed to be filled with sediments. Minimum amount for each sample was about 20 kg. Collected sediments represent mixed composition of the river bed layer. Bed material was collected mostly in the middle part of the river channel on riffle sections. Only a few samples were taken on gravel bars. Each sample was

documented by a photograph. Sampling of the tributary confluences was skipped due to time and space constrains. Four sites could not be sampled mostly due to armouring on the river bed or weather conditions. Samples were transported to Hydraulic laboratory (VÚVH) and dried out. Sediment calibre was estimated using sieving method. Grain size distribution curves were compiled for all sites.

Suspended load sampling: depth-integrating sampler was used for measurements of suspended sediment load. The bottle with one litre volume was continually filled with water and sediments while it was slowly sank to the river bottom and lifted back. Suspended sediment sampling was performed in one vertical approximately in the middle of the river channel. Suspended sediment concentration was evaluated for 65 samples at VÚVH laboratories.

Water level slope: local water level slope was measured at sampling sites using the methods of classical geodesy (total station Leica TS06). Measurements were done from the river banks within the distance up to 1,000 m on the sites of the Upper and Middle Danube. Weather conditions particularly strong wind producing high waves in combination with decreasing value of river bed slope negatively influenced these measurements on the Lower Danube and in the Danube Delta.

Water level fluctuation – pressure probe located in sufficient water depth close to the river bank was used to record water level fluctuation. Observation was usually done during the whole available time (max. 4 hours) at 62 sites (missing sites: JDS23 rkm 1,560, JDS48 rkm 837, JDS56 Russenski Lom river, JDS57 rkm 488, JDS58 Arges river – technical reasons). Changes of water level were automatically recorded for adjusted time interval. Data were stored in the logger and downloaded to the laptop after observation. Changes of water level provided information on steady or unsteady flow conditions during the survey – relevant to HYMO measurements. Due to a relatively short time the range of hydropeaking could not be identified (usually hydropeaking occurs during morning/afternoon for peak energy demand (higher energy prices) and the fluctuation takes several hours).

Based on field measurements main hydrological, morphological and hydraulic parameters were estimated: Q_a – average discharge, v_a – mean velocity (+ flow pattern), cross sections, B_a – average channel width, H_{max} – max. depth, A- area of cross sections, D_{16}, D_{50}, D_{84} – characteristic grain size, S_{wl} – local water level slope, C_{ss} – suspended sediment concentration, ΔH_{max} – max. water level fluctuation. Field survey data including their evaluation are summarised in numerical (Hymo Summary Table – Annex 2.2.1) and graphical form (Hymo Survey Book – Annex 2.2.2) as a part of the Extended Report on the attached CD.

Methods for HYMOQ site assesment

Methods of "physical habitat assessment" (hydromorphological quality elements – HYMOQE) are one of the most common methods within the EU countries to characterise the hydromorphological conditions. These methods include general description of the site, characterisation and a visual assessment of physical in-stream and riparian habitats. There is a tendency to define high status/near to natural conditions only on the basis of presence and abundance of morphological features neglecting the river processes that generate and maintain the morphological units and the temporal context within which processes operate and river channels are adjusted. Therefore these methods are not comprehensive enough to adequately identify causes of hydromorphological alteration. There is an increasing need to improve the characterisation and analysis of the hydromorphological conditions of water bodies (Rinaldi, 2013). Methods which were used for hydromorphological site assessments in JDS3 are linked with these recommendations.

Hydrological regime relates to discharge variations in time including changes in flow dynamics and connection to groundwater. Morphological conditions include the physical characteristics of the river, mainly the width/depth variation, bed structure and substrate, river banks and riparian zone (floodplain should be included as well). River continuity refers to ability of water, sediments, and migratory species to pass freely upstream/downstream along the river. It should be pointed out that "fish migration aids" has no effect on river morphology.

Hydromorphological assessment neglecting the understanding of the river behaviour and physical processes in the context of human interventions may not provide sustainable solutions in the management and restoration strategies (RBMP) particularly on large rivers. Method of "physical

habitat assessment" can be improved by integration of key hydrological, morphological and hydraulic parameters (measurable/verifiable by monitoring), which reflect changes in the river processes thus can be used as indicators of hydromorphological alteration of the rivers. This approach was applied by VÚVH to develop the method for HYMOQ assessment (parameters partly harmonized by CEN standards) that was verified on many Slovak rivers within HYMO monitoring over a few years (as a part of ecological monitoring). As specific approach for site analysis only the main results of HYMOQ site assessment are briefly described in this report.

The HYMOQ assessment was done for JDS3 sites within 10 km stretches, which are consistent with 10 km segments of continuous longitudinal survey. The specific HYMO information collected during the survey along these stretches (sketches, photos, description, etc.) including visual monitoring of upstream and downstream sections are considered as well. This approach enhances reliability of the assessment as physical conditions result from processes and causes that occur at a wider scale.

Results of hydromorphological survey accomplished with site observations, technical information (river regulation, in-stream structures, infrastructures, channel maintenance, etc.), actual maps and aerial photos create the necessary background for hydromorphological quality assessment (HYMOQ). Historical maps document the near to natural conditions just before systematic river regulation was done (near to natural conditions). These maps indicate a degree of current morphological alteration and delineate important framework for sustainable river restoration to achieve ecological targets of WFD. Therefore historical maps for entire Danube were used in HYMOQ site assessment (Schwarz, 2013).

Eight indicators, which include several hydrological, morphological and hydraulic parameters are considered to estimate the final HYMOQ class: river planform, habitat diversity, flow regime & flow dynamics, sediment continuity (sediment, water, fish), local channel morphology, lateral connectivity, riparian zone and floodplain. Based on knowledge of hydromorphology, the main indicators are weighted as the impact of each differs. Final class is estimated as an average value ranging from class 1 to class 5 as follows:

near natural (1)	slightly modified (2)	moderately modified (3)	extensively modified (4)	severely modified (5)

Scoring results which are summarized in protocols for each site (including each indicator and all measured/estimated parameters) clearly show the most important hydromorphological deficits that can be used as a basis for proposal of effective restoration measures. This makes the process of HYMOQ assessment as transparent as possible.

3.3 Results

3.3.1 Continuous longitudinal survey in 10 rkm segments

The results will be shown for each content/parameter group for the whole Danube and then for the Upper (rkm 2,415 – rkm 1,880 at AT-SK border), Middle (down to Iron Gate at rkm 1,880 – rkm 943) and Lower Danube (rkm 943 – rkm 0). In the Danube delta only the Sulina branch is included in the analysis. The hydromorphological atlas is supplemented in the CD annex and shows the full resolution of assessment in map form. One segment has 10 km length, which allows a fast readability of results (e.g. 21 segments are 210 km of the Danube).

3.3.1.1 Entire assessed Danube from rkm 2,415 – rkm 0

The WFD-3digit analysis for the entire Danube indicates the general alteration (prevailing classes 3-5), in particular the best documented parameter group „Morphology", but also the „Hydrology". The longitudinal continuity is interrupted by 18 dams (segments). For two with functionning fish passes and partial sediment feeding (Wien-Freudenau and Melk) the value is „3" according to CEN standard.

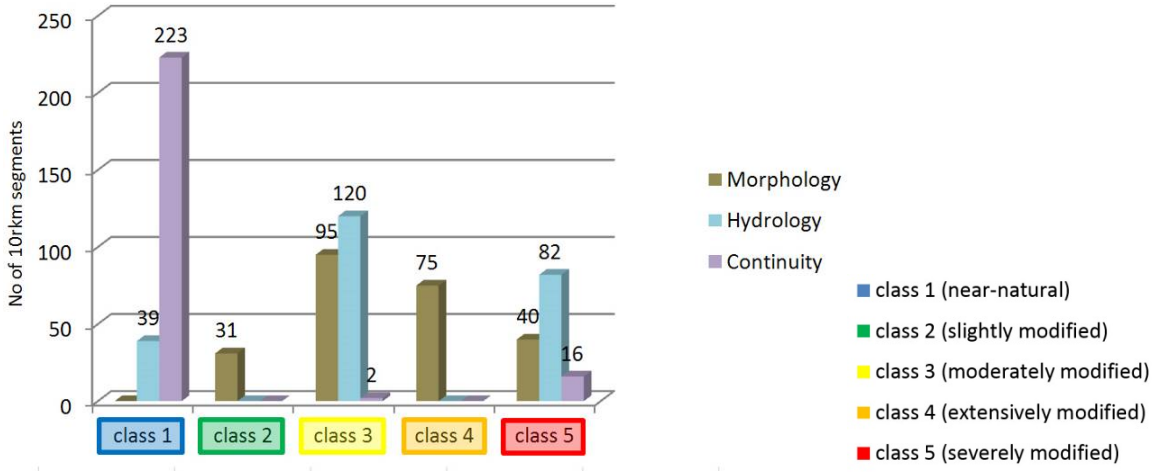
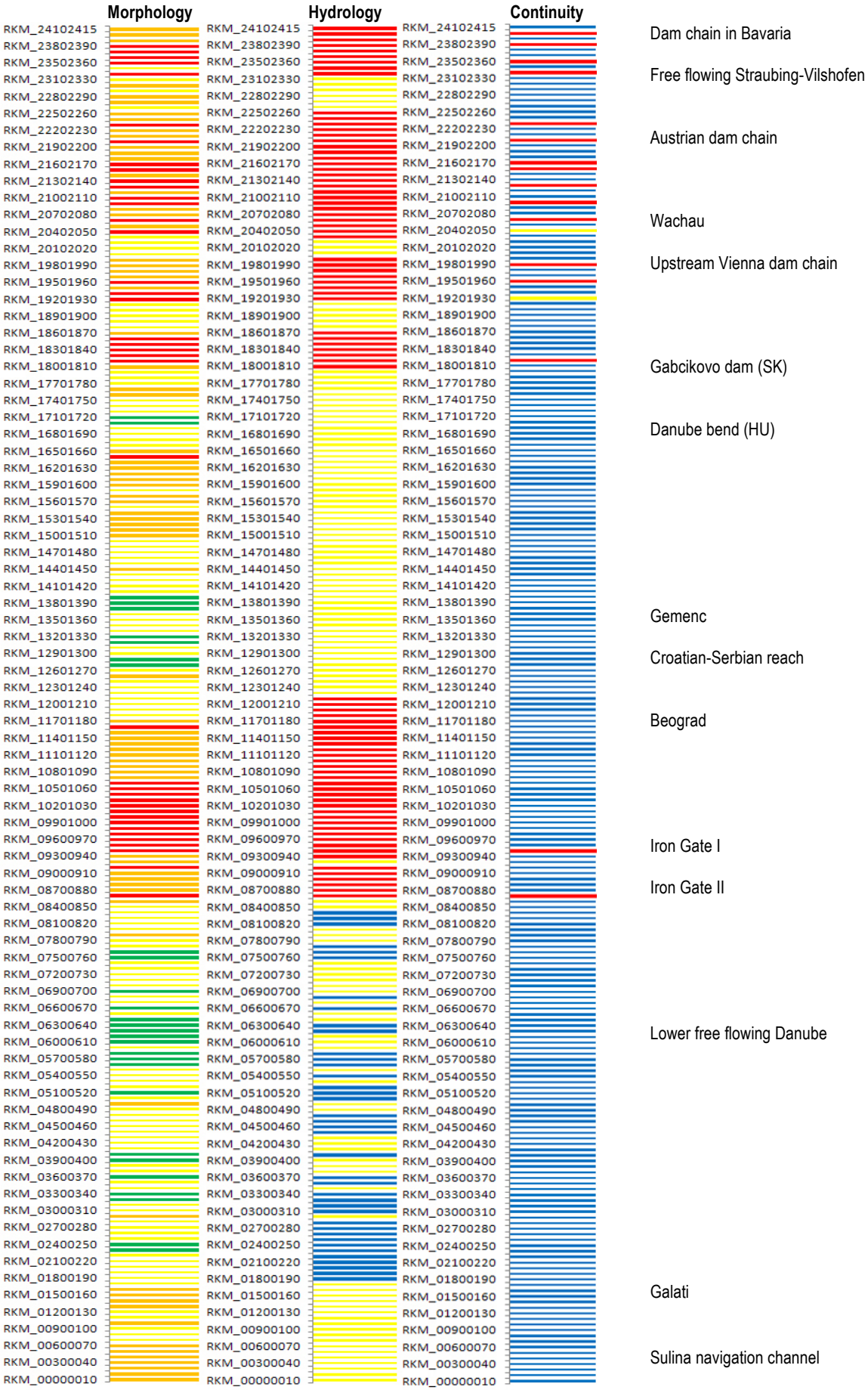


Figure 3: WFD-3Digit assessment[2]

Next page Figure 4: Longitudinal visualisation of the WFD-3Digit assessment (for coloured assessment classes compare with previous chart)

[2]For "Hydrology" and „Continuity" only the classes 1, 3 and 5 were evaluated


Morphology
Hydrology
Continuity
Dam chain in Bavaria
Free flowing Straubing-Vilshofen
Austrian dam chain
Wachau
Upstream Vienna dam chain
Gabcikovo dam (SK)
Danube bend (HU)
Gemenc
Croatian-Serbian reach
Beograd
Iron Gate I
Iron Gate II
Lower free flowing Danube
Galati
Sulina navigation channel

The longitudinal visualisation allows a comprehensive overview of impounded reaches with position of dams (middle and rigth coloumn) and the morphology on the left. The 10 rkm lables (text) can be not shown for each segment due to space reasons.

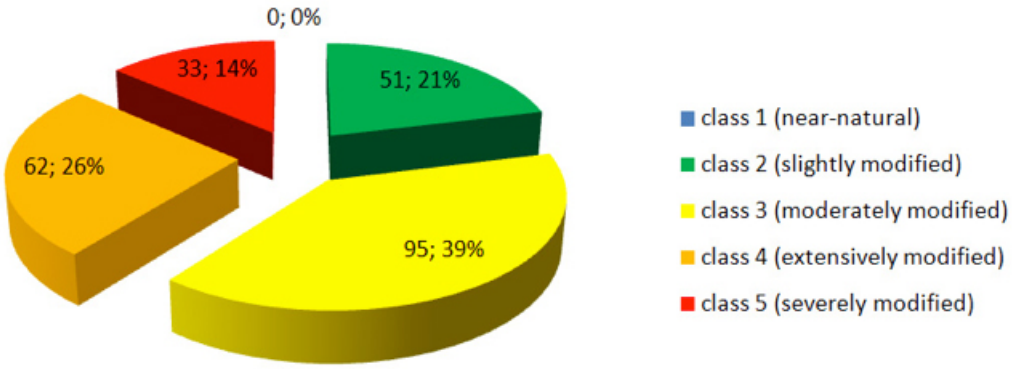


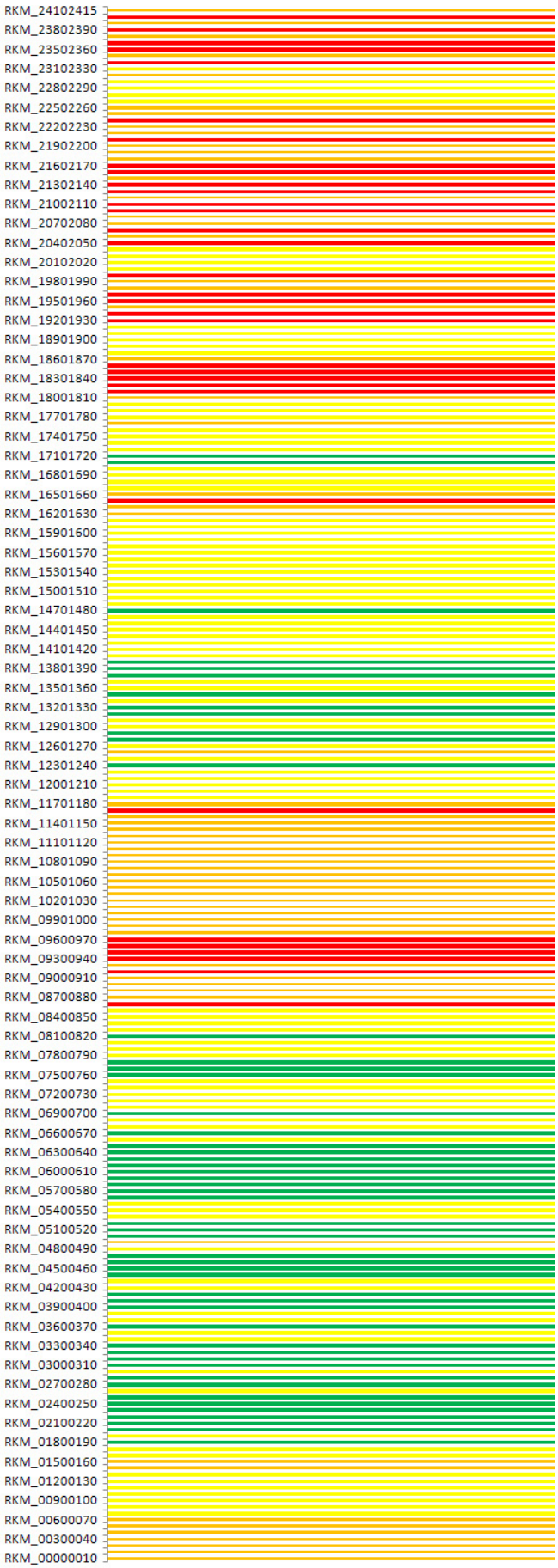
Figure 5: CEN-Overall assessment (with colour and assessment schema)

The class 2 (slightly modified) is represented by 21% of the analysed Danube reach (Fig. 5), followed by a significant portion of 39% in the "moderate" class (class 1 cannot be found at all). About 40% fall into the two worse classes 4 and 5. The overall picture is therefore split into a larger part with satisfactory conditions and a significant part of totally altered Danube reaches.

Figure 6 shows the whole longitudinal overview before comparing the three main sub-divisions of the Danube in detail and the single parameter groups in the next sub-chapters. The distribution of "good" and "poor" assessment in the upper and lower Danube is significant. The picture would be even more sharp taking the less modified two other delta branches (Chilia and St. Gheorghe) into consideration.

Regarding the direct comparison with JDS2 results from 2007 it is not possible due to changed methodology. Aside of the spatial increas of assessment stretches (from 66 with an individual length of up to 120 km to 10 rkm segemnts) allowing now to assess all impoundments and regulation works in much more detail, the qualitative improvement by the assessment of 10 parameters per segment instead of one global assessment for JDS2 lead to slightly shifitng assessments between neigbouting classes. However the overal picture having at least 60% in the classes two and three and up to 40% in four and five remains similar.

Figure 6 (next page): Longitudinal visualisation of the CEN-Overall assessment (for coloured assessment classes compare with previous chart)


RKM_24102415
RKM_23802390
RKM_23502360
RKM_23102330
RKM_22802290
RKM_22502260
RKM_22202230
RKM_21902200
RKM_21602170
RKM_21302140
RKM_21002110
RKM_20702080
RKM_20402050
RKM_20102020
RKM_19801990
RKM_19501960
RKM_19201930
RKM_18901900
RKM_18601870
RKM_18301840
RKM_18001810
RKM_17701780
RKM_17401750
RKM_17101720
RKM_16801690
RKM_16501660
RKM_16201630
RKM_15901600
RKM_15601570
RKM_15301540
RKM_15001510
RKM_14701480
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RKM_14101420
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RKM_03600370
RKM_03300340
RKM_03000310
RKM_02700280
RKM_02400250
RKM_02100220
RKM_01800190
RKM_01500160
RKM_01200130
RKM_00900100
RKM_00600070
RKM_00300040
RKM_00000010
Dam chain in Bavaria
Free flowing Straubing-Vilshofen
Austrian dam chain
Wachau
Upstream Vienna dam chain
Gabcikovo dam (SK)
Danube bend (HU)
Gemenc
Croatian-Serbian reach
Beograd
Iron Gate I
Iron Gate II
Lower free flowing Danube
Galati
Sulina navigation channel

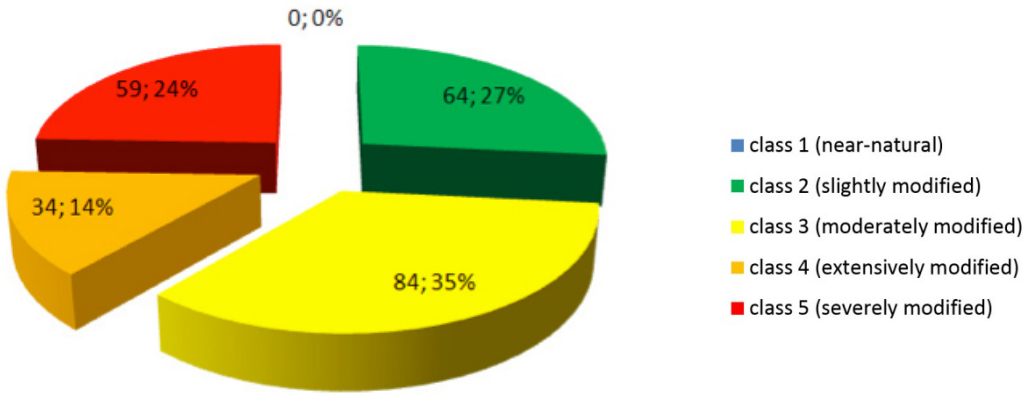


Figure 7: Assessment "channel"

The assessment of channel reflects very well the overall assessment. Significant amount of segments fall in the second and third class which is evident for the long free flowing stretches along the Middle (widely rectified channel, partially groynes) and in particular along the Lower Danube. About 590 km (out of 2,415 km) fall in the worst class (namely impoundments and severely altered stretches within dense settlements).

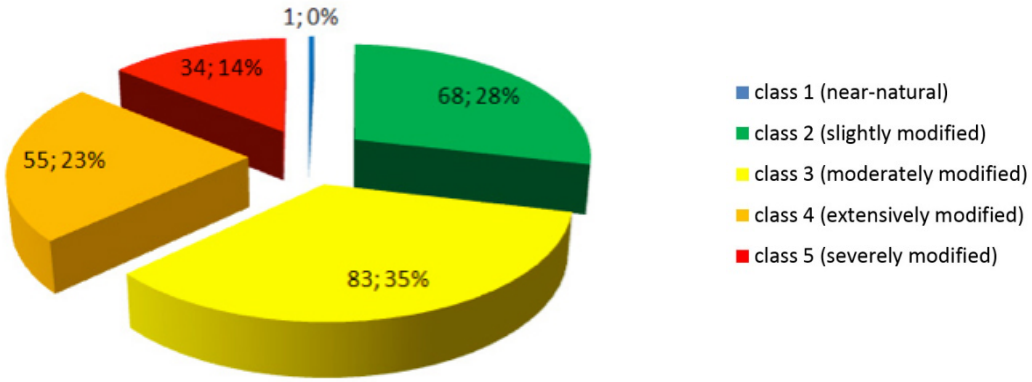


Figure 8: Assessment "banks" (integrating left and right bank assessments)

Over one quarter of the surveyed banks fall into the classes 1-2 which is mainly due in the Lower Danube. However the transition zone from banks to floodplains is covered often by increasing poplar plantations and neophyte stands. Along the middle Danube in Hungary, Croatia and Serbia long bank sections are not continuously fortified by riprap whereas these fortified banks – belonging to the categories 4 and 5 – can be find along the Upper Danube (in addition to the higher degree of urbanisation and hydropower usage along Upper Danube, significant slope and flow velocities in free flowing reaches causing lateral erosion and channel shift which is critical for navigation).

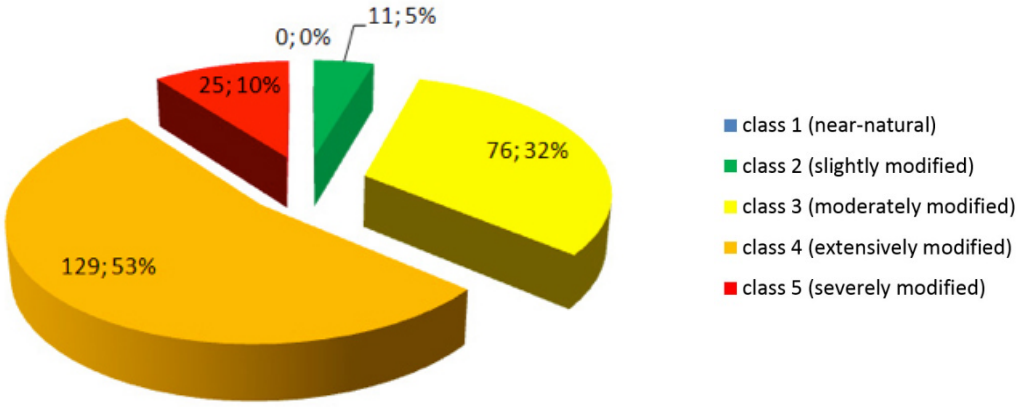
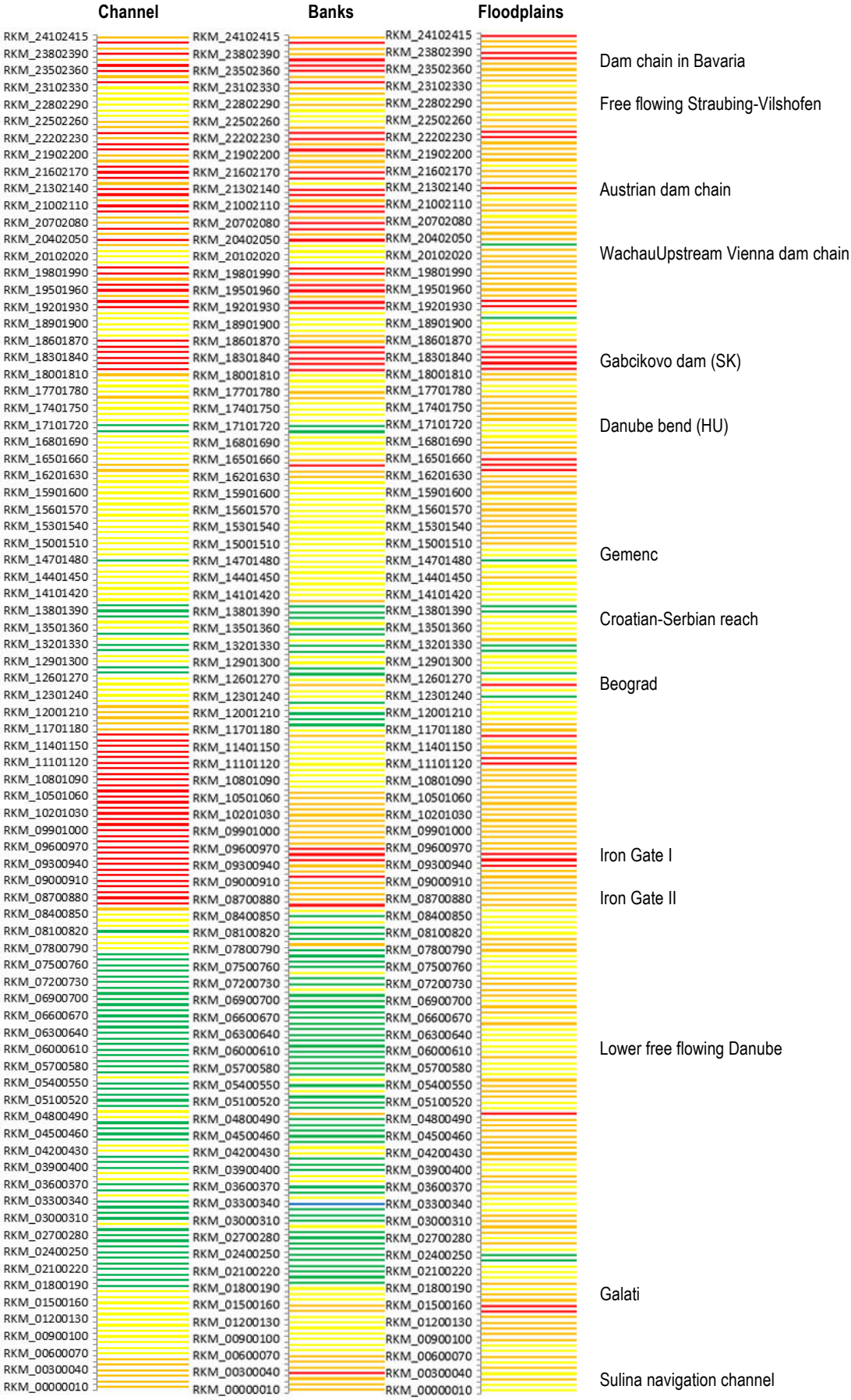


Figure 9: Assessment "floodplains" (integrating left and right floodplain assessments)

Only a very few stretches still host good conditions and stands of floodplains. The loss of floodplains can be assumed with at least 65-70% for the entire river represented by class 4 and 5 but partially also by class 3. Still remaining floodplains suffer in many cases by long lasting processes of channel incision (hydrological disconnection) and fine sediment aggradation caused by dams. Furthermore, poplar plantations substitute in many cases the natural floodplain vegetation.

Figure 10 (next page): Longitudinal visualisation for channel, banks and floodplains (for coloured assessment classes compare with previous chart)


Channel
Banks
Floodplains
Dam chain in Bavaria
Free flowing Straubing-Vilshofen
Austrian dam chain
WachauUpstream Vienna dam chain
Gabcikovo dam (SK)
Danube bend (HU)
Gemenc
Croatian-Serbian reach
Beograd
Iron Gate I
Iron Gate II
Lower free flowing Danube
Galati
Sulina navigation channel

Upper Danube (rkm 2,415 – rkm 1,880)

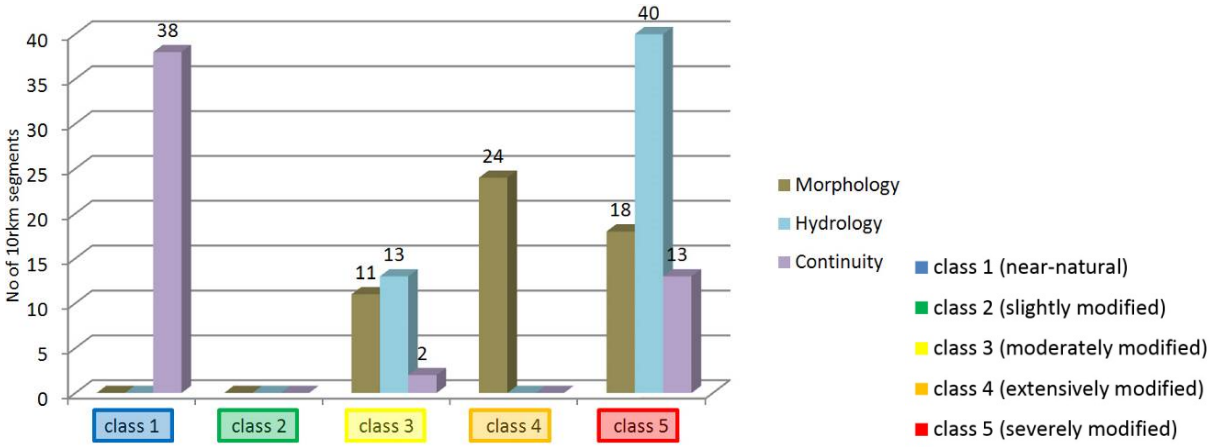


Figure 11: WFD-3Digit assessment[3]

The WFD-3digit analysis for the Upper Danube shows the rather high number of segments with continuum interruption (15 segments including two with fish passes). For "Morphology" class 4 and 5 prevail and the "Hydrology" clearly indicates the segments affected by impoundments and intensive river regulation works (Fig. 11).

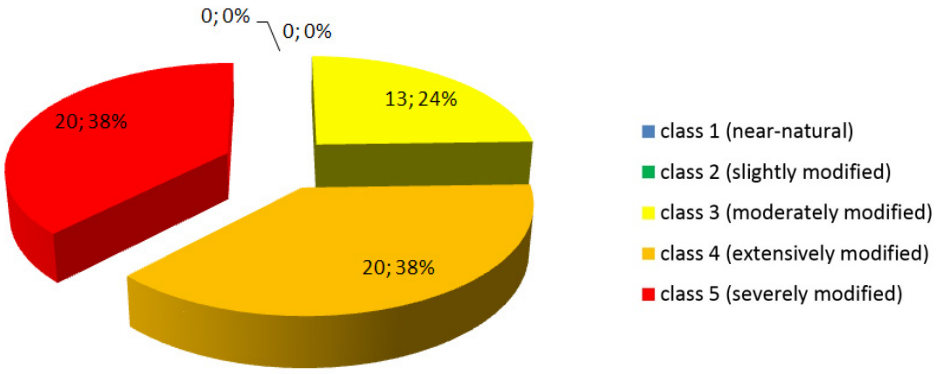


Figure 12: CEN-Overall assessment

Only the still free flowing reaches between Straubing-Vilsofen in Bavaria as well as Wachau and Vienna-Morava confluence fall into the „moderate" class (some segments come with an assessment value of 2.5 (arithmetic mean from individual parameter values) near to the second class). About one quarter is in class 3 „moderate" and the rest is intensively changed (Fig. 12).

[3]For "Hydrology" and „Continuity" only the classes 1, 3 and 5 were evaluated

Middle Danube (rkm 1,890 – rkm 934)

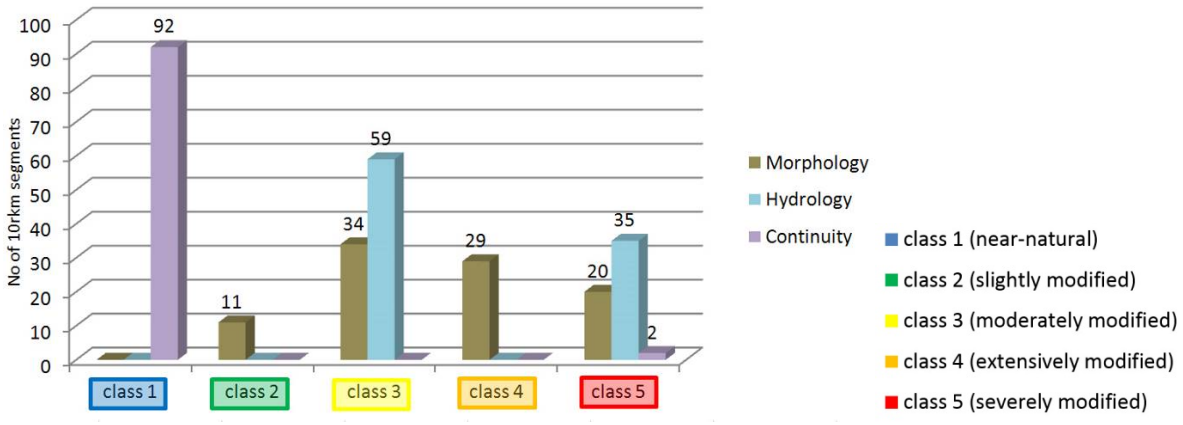


Figure 13: WFD-3Digit assessment[4]

The Middle Danube still hosts a couple of segments in the second class for "Morphology", but most of the segments fall into class 3 (Fig. 13). The significant number of segments for "Hydrology" in class 5 stands for the long impoundments of Gabčíkovo and in particular Iron Gate I dam. The river continuity is interrupted only in two segments (Gabčíkovo and Iron Gate I dams), but the effect of the two large dams comes along with long impoundments and sediment accumulation as well as deficits up and downstream of the dams.

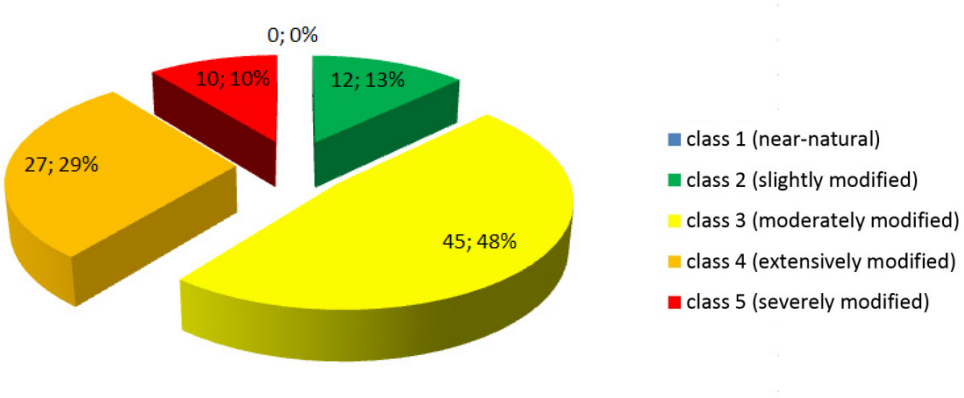


Figure 14: CEN-Overall assessment

At least 13% of the Middle Danube still has good hydromorphological conditions (Fig. 14), nearly the half falls in the moderate class. The rest can be found in the two reservoirs of Gabcikovo (not the Szigetköz fomer channel was assessed only the bypass canal) and Iron Gate as well as the city reaches of Budapest and Beograd.

[4]For "Hydrology" and „Continuity" only the classes 1, 3 and 5 were evaluated

Lower Danube (rkm 934 – 0 rkm)

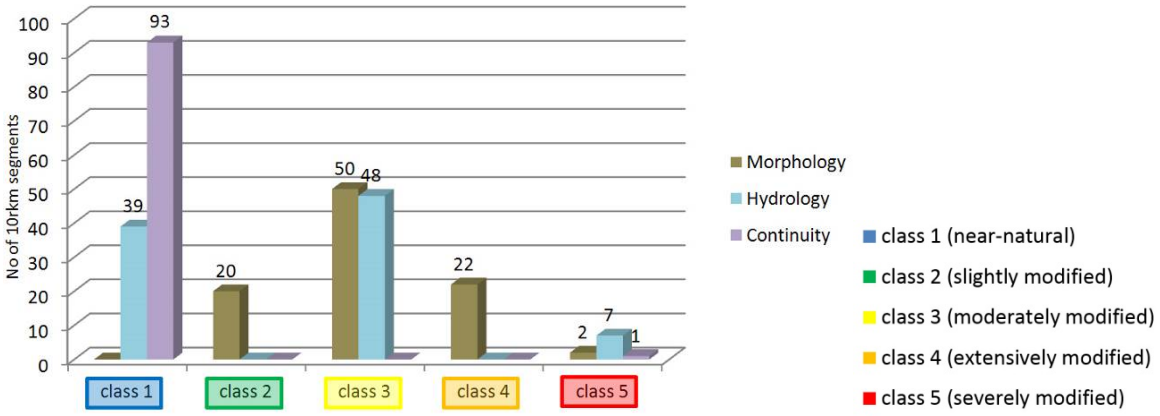


Figure 15: WFD-3Digit assessment[5]

Regarding the "Morphology" the Lower Danube still provides class 2 (slightly modified) stretches, but predominantly class 3 due to the limited lateral connectivity (floodplains). Class four and five fall mostly in the Iron Gate II reach. Regarding the continuity interruption only the Iron Gate falls in this reach, taking always into consideration that sediment and hydrological changes due to the two Iron Gate dams (and various dams on the Lower Danube tributaries) affect the Lower Danube in generally. With about 860 km the Lower Danube represents the longest free flowing stretch of the Danube at all, represented by "Hydrology" in first and third class (Fig. 15).

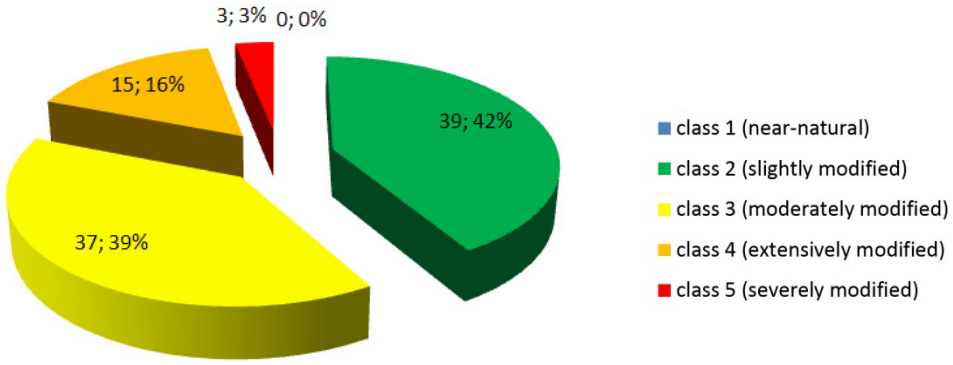


Figure 16: CEN-Overall assessment

Over 40% of the lower Danube stretch falls into the second class, which is remarkable in comparison with the upper Danube or e.g. Lower Rhine River. Moderate stretches fall into „town and harbour" stretches and free flowing stretches with moderate regulation works and/or cut floodplains, the rest is in Iron Gate II reach and canalised Sulina channel in the delta. However, the entire lower Danube is inter alia influenced by the Iron Gate dams (similar as Middle Danube is inter alia influenced by major hydraulic structures from the Upper Danube) and along major tributaries (Fig. 16).

[5]For "Hydrology" and „Continuity" only the classes 1, 3 and 5 were evaluated

3.3.2 Detailed JDS3 site analysis and assessment

Results provided by the detailed JDS site analysis and assessment consist of two substantial parts. The first part provides an overview of results and analyses of HYMO survey for the entire Danube. A more detailed interpretation is shown for the main morphological types defined on the Danube: Upper (rkm 2,412 – 1,880), Middle (rkm 1,880 – 943) and Lower Danube & Danube Delta (rkm 943 – 0). The second part of the results summarises the site assessment based on the results of hydromorphological survey using method VÚVH respecting WFD rules and CEN standards.

3.3.2.1 Results of hydromorphological survey – entire Danube

Relationship $Q_sD_{50} \sim QS$ represents proportionality between sediment discharge (Q_s), stream discharge (Q), particle size (D_{50}) and slope (S). A change in any of these variables sets up a series of mutual adjustments in the companion variables with a resulting direct change in the characteristics of the river (Lane, 1955). For example, changes in the bed load volume affect change in width, depth and river bed slope. Changes of the hydraulic and morphologic characteristics influence discharge capacity of the channel, which again affects river sediments. Except of sediment discharge the main variables controlling the river behaviour (Q, S_{wl}, S_{bed}, D_{50}) were measured or estimated during the HYMO survey. Interdependence of these variables (parameters) enables their exploitation as indicators sensitive to hydromorphological changes of the Danube river channel.

Variability of measured parameters clearly indicates the most significant changes in the river processes (erosion/deposition) that induce various degree of hydromorphological degradation along the Danube.

Flow conditions – interpreted by discharge, mean velocity and velocity pattern allow important insights to the hydrological and hydraulic situation during the survey. Unlike JDS2 when discharge downstream of Iron Gate significantly increased (data-gauging stations), relatively steady low flow conditions prevailed in the Danube during entire JDS3 (Fig.17). There was only one major discharge increase that occurred at short section between Vienna and Bratislava. Low flow conditions enabled better site description of the river morphology (in-stream forms, river banks, riparian zone). With exception of impounded sections there is highly variable flow dynamics along the Danube (Fig.19).

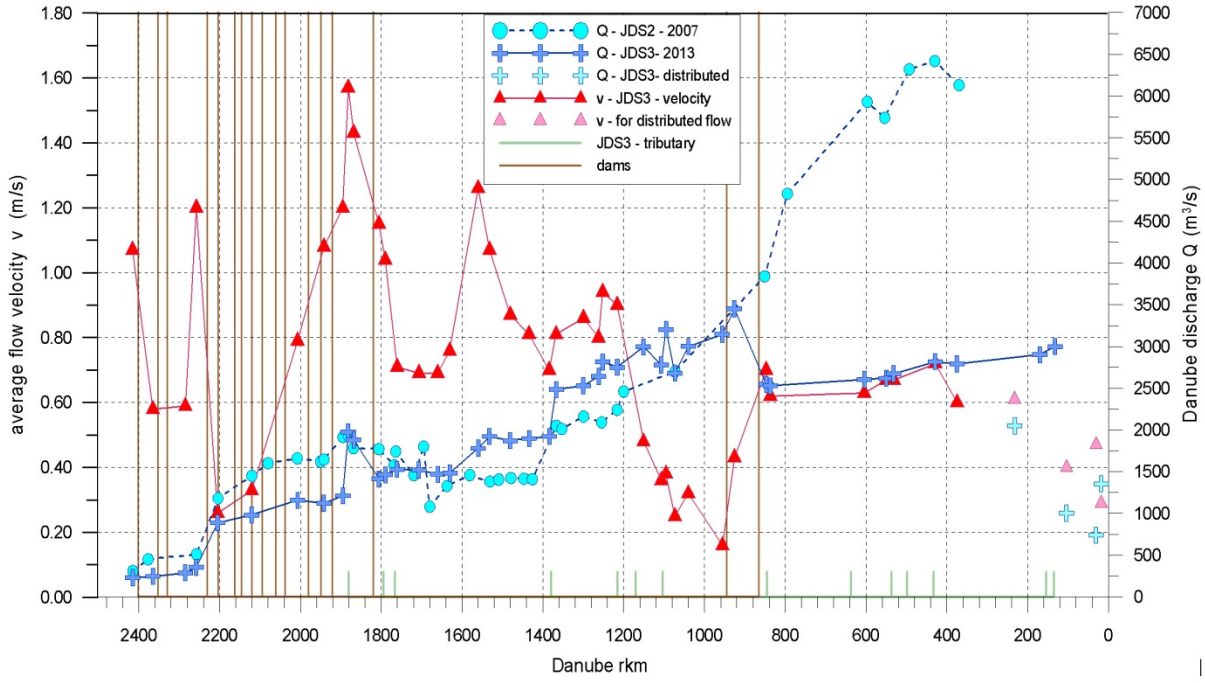


Figure 17: Flow conditions on JDS3 and JDS2

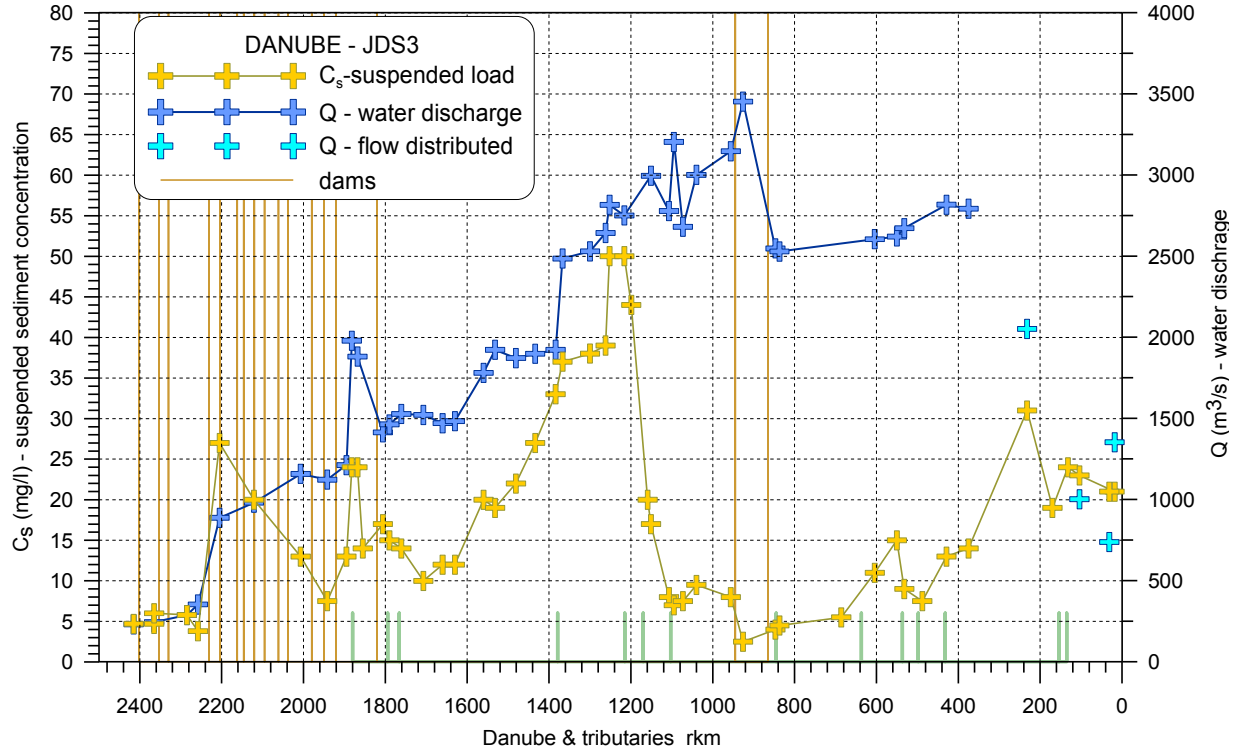


Figure 18: Suspended sediment concentrations & discharge

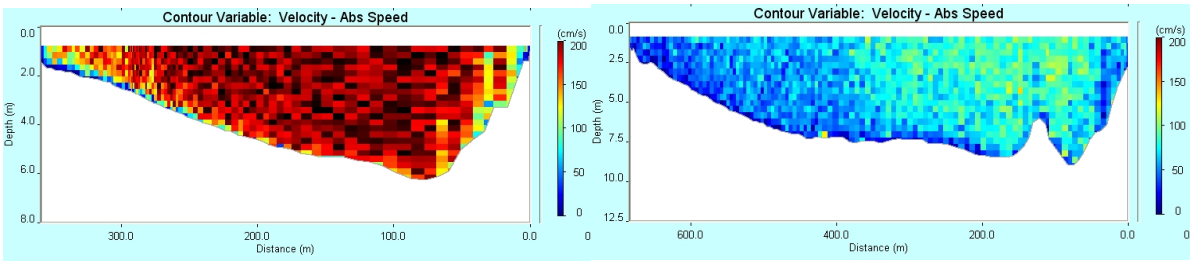


Figure 19: Flow pattern for the most dynamic flow (Upper Danube) and slowly flowing (Lower Danube)

Major changes of flow dynamics and sediment continuity along the Danube are caused by dams operated on the German – Austrian Danube (chain of dams), on the Slovak Danube (Gabčíkovo) and on the Serbian – Romanian Danube (Iron Gate). Danube dams create sections with flow deceleration (impoundment) or acceleration (just downstream of dams slope must be equalised if there is not immediately the backwater of next dam) where deposition/erosion prevail. These changes reflected in composition of the bed sediments (Fig.20), induce significant hydromorphological alteration at several longer stretches of the Danube. Changes of flow dynamics caused by groyne fields or other in-stream structures can have significant but mostly local effect on hydromorphology. There is an indication of flow regulation downstream of the Iron Gate where discharge decreased by 800 m^3s^{-1}. Flow regulation might cause certain effects on channel morphology downstream of the Iron Gate (discharge, sediments – see Fig. 17, 18).

Sediment continuity is documented by values of suspended sediment concentrations along entire Danube and tributaries (Fig.18) and implicitly by changes of flow dynamics and compositions of the bed sediments. Trapping effect of the Danube dams is documented by considerable decrease of suspended sediment concentration values along impounded sections. Disruption of sediment continuity generates not only deposition area upstream of the barrier but also lack of sediments in the downstream direction, usually related to erosion. Deficit of fine sediments downstream of the Iron Gate is obvious at long section of the Lower Danube & Delta (Fig.18). If fine sediment continuity (suspended load) is affected markedly then impact on coarser sediments (bedload) has to be even higher.

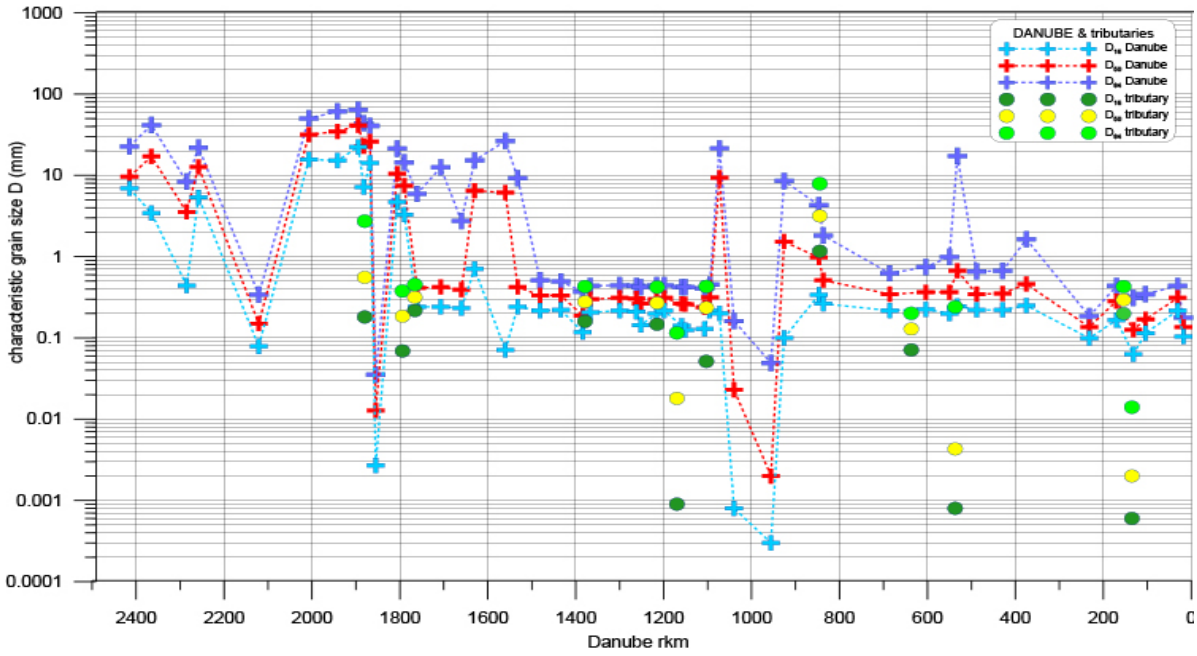


Figure 20: Downstream variation in bed material grain size on the entire Danube/tributaries

Bed material interpreted by grain size distribution curves represents an essential source of information to identify changes in channel morphology. Bed sediments vary in the downstream direction (Sternberg, 1875), the coarse sediments of headwaters giving way to progressively finer alluvium as bas-level is approached (Ried et al., 1997). Composition of the river bed sediments, rate of downstream fining (Fig.20) and sediment sorting provide important knowledge on river processes (erosion/deposition) so they can be used as diagnostic tools mainly in case no bedload data are available.

Natural composition and downstream fining of bed sediments for corresponding channel type and geomorphological environment have been changed dramatically along entire Danube mostly due to disruption of sediment continuity and other human interventions (dams, dredging, in-stream structures, etc.). Extent of these changes is proved by high variability of bed sediment size (D_{50}, fig.20). Except for strong impoundments where fine sediments are deposited (sand, silt & clay) there are localities mostly downstream of dams with highly sorted coarse sediments (missing fine fractions) that imply either bed erosion or some degree of artificial bed stabilization.

Variation in bed material grain size shows even downstream coarsening instead of fining at Upper Danube (Fig.20). Better situation can be seen on the Middle and Lower Danube where composition of bed sediments is less altered and the downstream fining is already indicated. Nevertheless, the impact of two big dams (Gabčíkovo, Iron Gate) and other interventions is still evident. Results of regression analysis for downstream fining underpin these findings (coefficients of determination ($D_{50,}$ distance) for Upper Danube $r^2 = 0{,}104$ Middle Danube $r^2 = 0{,}230$ and Lower Danube $r^2 = 0{,}473$).

Values of mean sediment size (D_{50}) indicate slightly coarser bed sediments at Lower Danube (without Delta) compared with the Middle Danube. This can be caused by lack of finer sediments trapped in Iron Gate and also by coarser sediments coming from tributaries. Only one sample taken form tributary mouth does not allow more comprehensive view on the tributaries function in changes of the Danube river bed.

Upper Danube (rkm 2,413 – rkm 1,880)

Flow dynamics at the Upper Danube has been influenced by operation of the chain of hydropower plants (HPP) that creates cascade of more or less impounded sections (in case of low water impoundments are nearly continuous). Only two free flowing reaches in Wachau valley and downstream of Vienna still remain. Changes in flow dynamics can be seen on Fig.21. There are

typical sections with slowing flow (just upstream of dams) or more dynamic flow (usually shorter section downstream of dams). More significant water level fluctuation (Δh > 50 cm) was not recorded on Upper Danube. The only increase in water discharge caused by more intensive precipitation occurred at short section downstream of Vienna.

Values of suspended sediment concentrations also show variability along impounded sections. (Fig.21). There are sites with evident decrease of values but also sites where suspended sediment concentration remains rather high (JDS6, JDS7) even if impounded (Fig.22). This indicates that suspended load can partly be transported through less impounded sections. Nevertheless, the chain of hydropower plants still creates a barrier for coarse sediment transport (bedload).

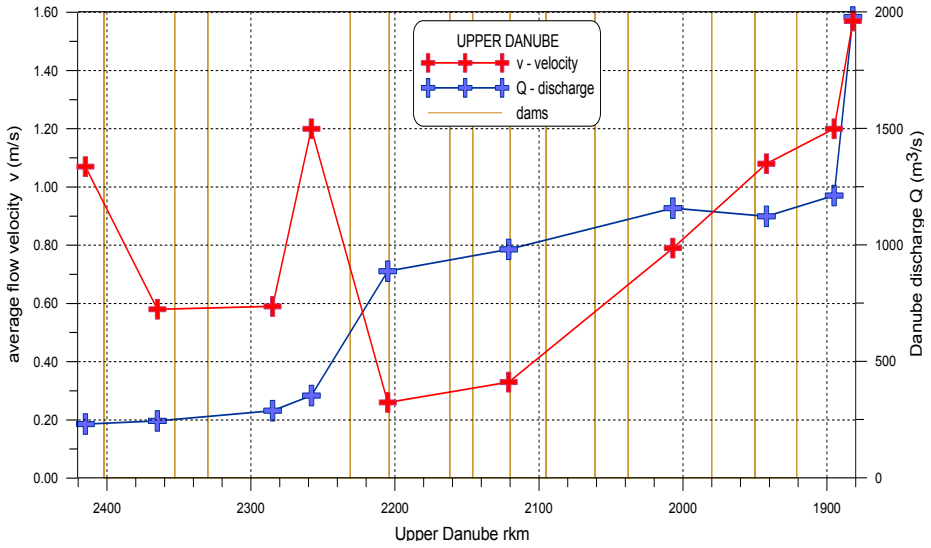


Figure 21: Mean velocity and discharge – Upper Danube

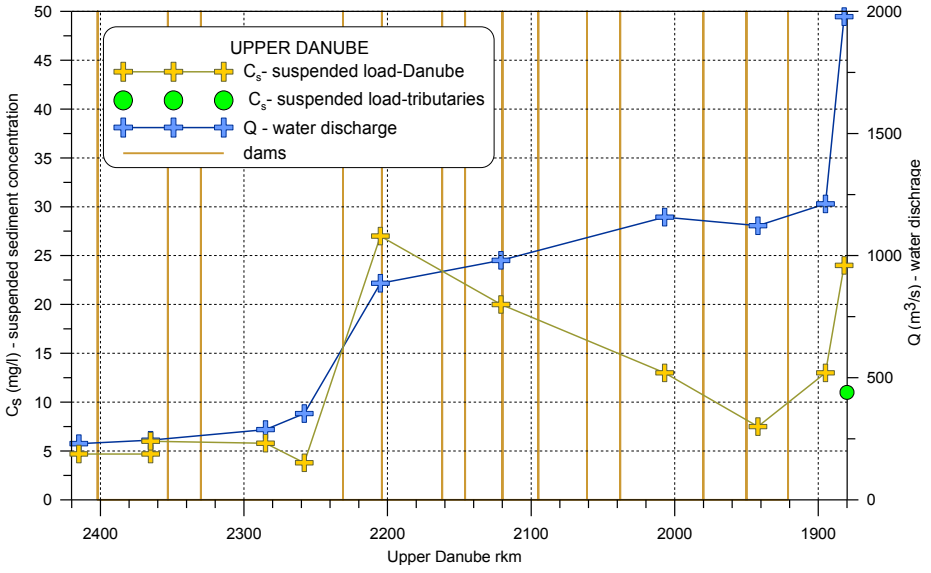
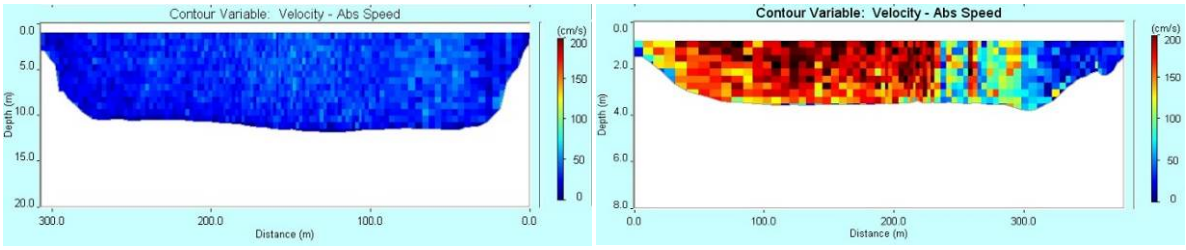


Figure 22: Suspended sediment concentrations and discharge



JDS7-Abwiden (rkm 2121) – impoundment JDS10 – Wildungsmauer (rkm 1895) – dstr. of HPP

Figure 23: Flow pattern for typical sites on the Upper Danube – just upstream and downstream of dam

Except for long impoundments the river bed consists of coarse and fine gravel with lower volume of cobbles. Characteristic composition of bed sediments and their variability can be seen on photos that document samples taken from the river bed at Upper Danube (Fig.24). Composition of the river considerably reflects flow conditions indicating river processes that prevail at particular site. Difference between two samples (JDS4 rkm 2,285, JDS7 rkm 2,121) is induced by impoundment (JDS4 – coarser sediments: fine gravel, coarse & fine sand) or impoundment (JDS7 – coarse & fine sand, silt).

These differences between particular sites can be seen on grain size distribution curves (Fig.25). There are some other samples (e.g. JDS8 rkm 2,007, JDS11 rkm 1,882) taken from the river bed just downstream of dams which demonstrate high degree of sediment sorting. Bed material consists of coarse gravel and cobbles. Fractions of fine sediments are almost completely missing (Fig.24). This indicates either erosion of the river bed or some kind of river bed stabilization downstream of dams.

JDS2 Kelheim JDS4 - Deggendorf JDS7-Abwinden JDS8-Oberloiben JDS10 Wildungsmauer

Figure 24: Bed material samples – Upper Danube

Impact of tributaries on sediment composition cannot be analysed because no tributary was included in JDS3 at this river section. Sediment continuity is highly altered at Upper Danube including two free flowing sections as due to lack of sediments from upper sections. This is proved by significant changes in river bed composition and also by high variability of sediment size along the river reach. Under these conditions downstream fining could not be identified – on the contrary, the coarsest sediments occurred at the lower edge of the river section (Fig.25, Fig.26).

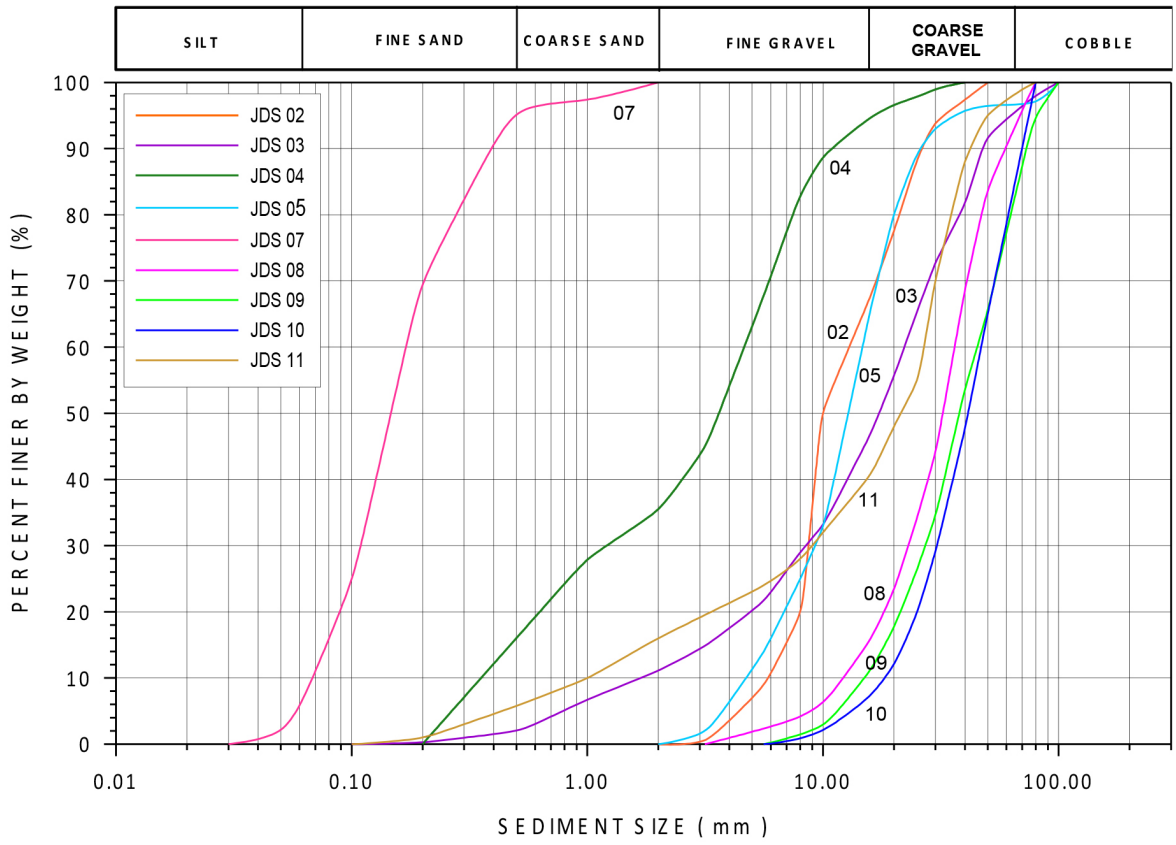


Figure 25: Grain size distribution curves- bed material

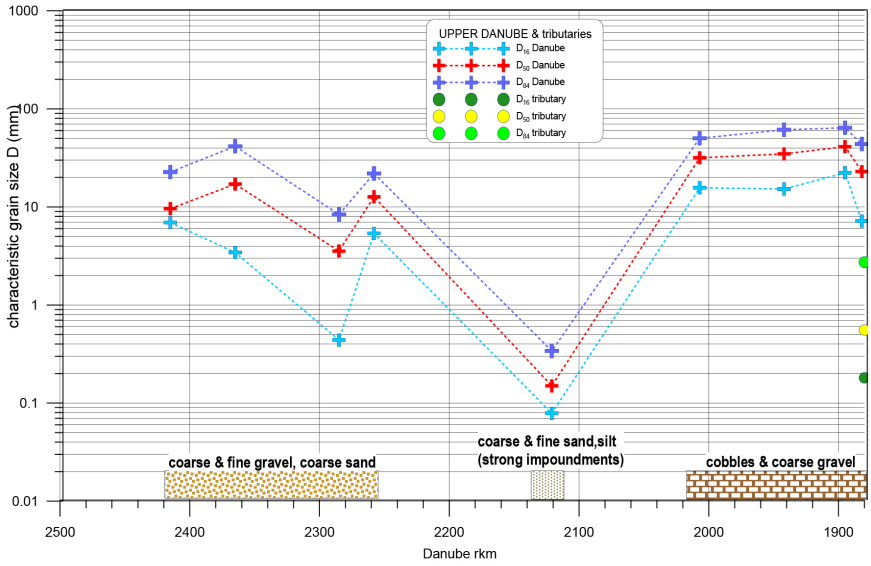


Figure 26: Downstream variation in grain size – Upper Danube

Changes in flow dynamics, sediment continuity and river morphology (regulated, uniform channel with stabilized river banks, in-channel structures e.g. groynes, deflective structures, etc.) induced high degree of hydromorphological alteration. That is the reason why the Danube sites are classified by 3, 4 and 5 in HMOQ site assessment for WFD. Nevertheless, there is still potential for improvement of the river hydromorphology as it can be seen upstream of Hainburg (area of the Danube National Park). This is the only green section (class 2) because of rather extensive ongoing restoration.

Middle Danube (rkm 1,880 – rkm 943)

Flow conditions at the Middle Danube have been influenced by operation of two hydropower plants (HPPs) at both edges: Gabčíkovo at the beginning and the Iron Gate at lower end (Fig.27). Flow dynamics in the section between is mostly influenced longitudinally by in-stream structures (e.g. groynes) and laterally by side arms closure. Effects of these interventions can be substantial but mostly local. Slowly flowing sections alternate more dynamic sections.

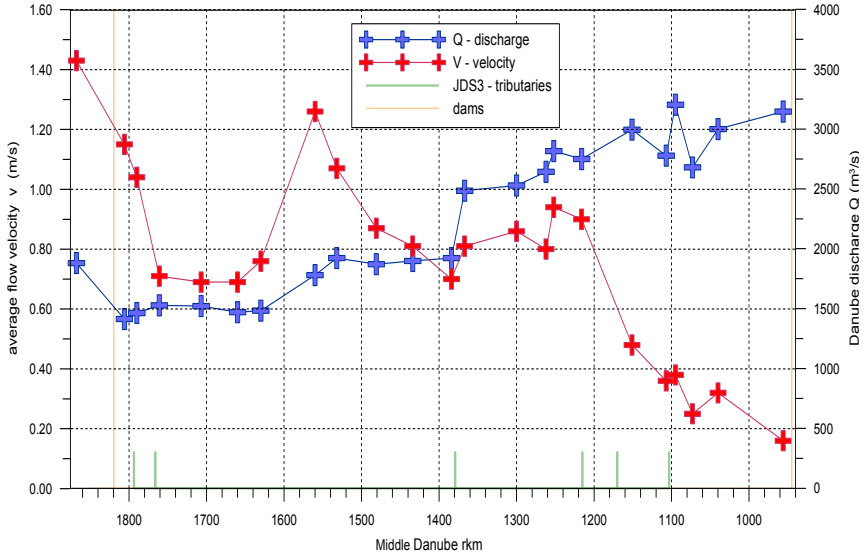


Figure 27: Mean velocity and discharge – Middle Danube

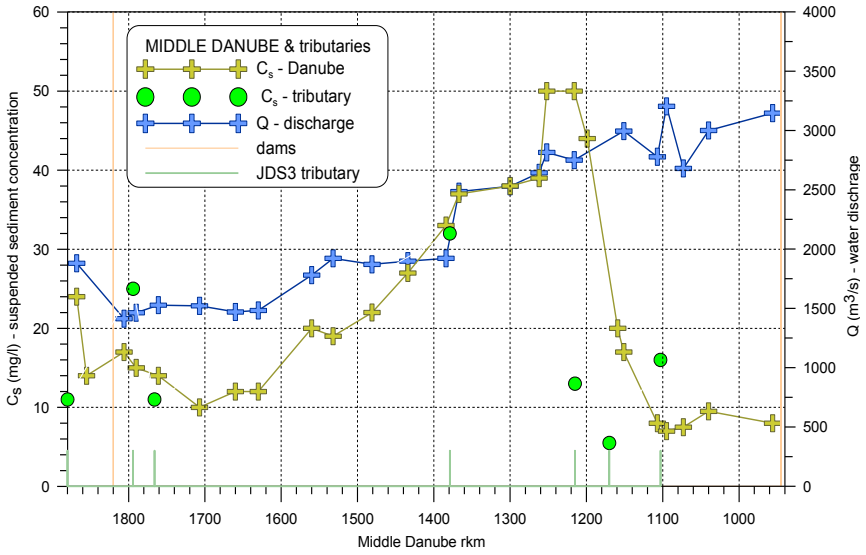
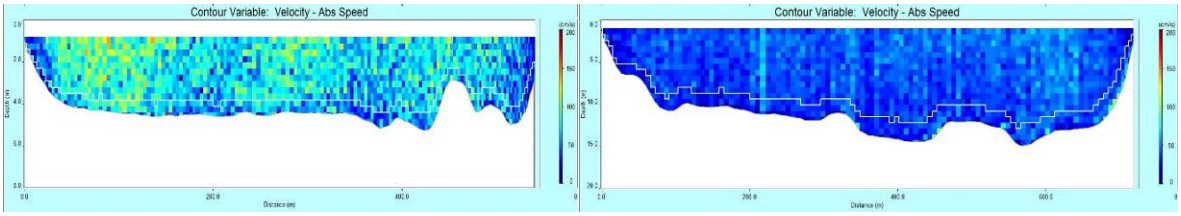


Figure 28: Suspended sediment concentrations & discharge

Gabcikovo HPP built on the bypass canal creates 40 km of abandoned channel of the Old Danube with strongly regulated flow but this part is not involved in JDS3. Impoundment reaches nearly 50 km upstream inducing significant decrease of flow dynamics. The effect of flow regulation in the Danube downstream of Sap is small (Fig.27). Slight indication of hydropeaking was recorded during the survey (JDS15 rkm 1,806 12cm/4 hours) but it had no effect on the sections downstream (JDS17 rkm 1,790, JDS20 rkm 1,707). Results of water level fluctuation for all sites can be found in the extended version of the report (CD).



JDS20 – Szob (rkm 1707) JDS42 – Downstream Velika Morava (rkm 1095)

Figure 29: Flow pattern for selected impounded (Iron Gate) and free flowing (Szob) Middle Danube

The Iron Gate I as the largest dam on the Danube has considerable effect on flow dynamics creating impoundment of around 300 km upstream. This is documented by flow pattern (Fig.29) and mean velocity distributed along the river section (Fig.27). As the Middle Danube ends just in the locality of Iron Gate dam the impact on flow regulation is included in the next chapter – Lower Danube. The trapping effect of the Iron Gate reservoir causes considerable decrease of suspended sediment concentration downstream of km 1,180 (Fig.28) and it is linked to velocity decrease (Fig.27).

JDS14–Gabčíkovo r. JDS15-Medvedov JDS19– Iza/Szony JDS26 – Baja JDS45-Irongate r.

Figure 30: Bed material samples – Middle Danube

Composition of the river bed that reflects flow conditions clearly shows the impact of impoundment at both ends of the Middle Danube (Fig.30). Except for smaller amount of fine sand larger volume consists of silt and clay as can be seen on grain size distribution curves (Fig.30). Similar composition can be seen in the section of strong impoundment from the Iron Gate upstream to km 1,040 (JDS44). Coarse grains in sample JDS43 (Fig.31) belong to sediment transported from tributary Velika Morava. The river bed has a rather uniform character at the next relatively long section up to km 1,252 (JDS33). Bed sediments mostly consist of fine sand (well sorted) as a result of a less strong impoundment.

Gabcikovo creates sections with deposition upstream (40 km) and erosion downstream. Due to trapping effect of HPP there is a lack of sediments at the section downstream resulting in the river bed incision. Process of the bed erosion continues at certain section while transport capacity is not fully restored. Nevertheless sediments trapped in the both reservoirs (impoundments) create big deficit that is missing at the downstream sections.

Section of the Middle Danube outside of strong effect of both HPPs shows much more natural composition of the river bed material (Fig. 31). Bed sediments largely consist of coarse & fine gravel and coarse and fine sand. Downstream fining is indicated but influenced by high scatter due to impediments ($r^2 = 0{,}230$).

Composition and arrangement of the river bed (bed structure) at this less effected section are influenced by in-stream structures that concentrate the flow into navigable channel creating deeper parts with coarser (main channel) and shallow parts with finer sediments (deposits between groynes).

This effect is mostly local fixed directly to the place where structures are situated. The river bed dredging has more significant negative effect as it causes sediment deficit inducing river bed incision that can initiate downstream and upstream river bed degradation.

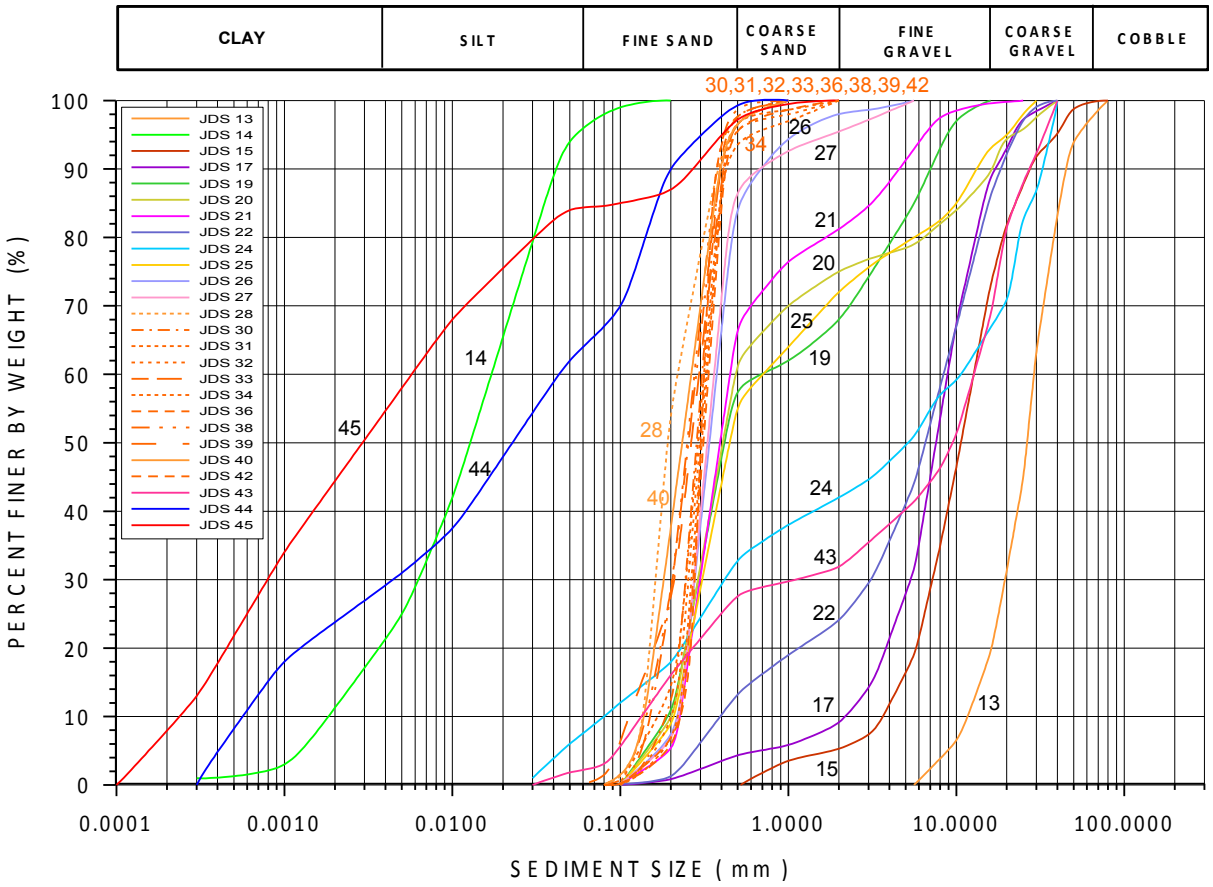


Figure 31: Grain size distribution curves-bed material

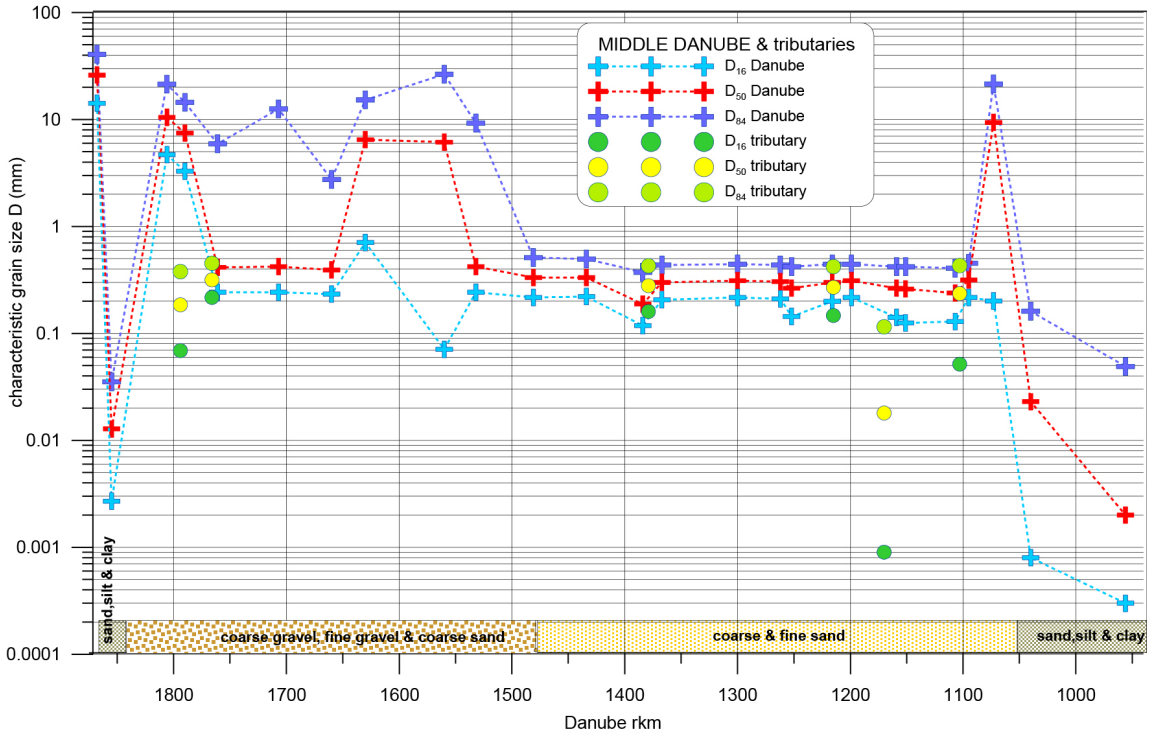


Figure 32: Downstream variation in grain size – Middle Danube

Hydromorphology of the Middle Danube is highly altered at sections of direct strong impact of both HPPs. At the section in between, there is mainly impact of river regulation (in-stream structures, dredging) but the character of the river indicates higher hydromorphological quality (e.g. higher channel variability B/H, in-stream habitats) compared with the upper sections. There are some parts with restored lateral connectivity (side arms, removal of bank stabilization, free banks). Except for strongly impacted sections which are classified by 4, 5 (extensively or severely modified), there are other sites classified mostly by 3 (moderately modified) and three sites by 2 (slightly modified). River section that is outside of strong effect of HPPs has relatively high potential for hydromorphological quality (HYMOQ) increase.

Lower Danube & Danube Delta (rkm 943 – rkm 0)

Flow conditions at the Lower Danube can be influenced by flow regulation on the Iron Gate I HPP as it is indicated by discharge changes (Fig.17, Fig.33) and already commented in the chapter 3.3.1. However, without more complex data on flow regulation or water level fluctuation it cannot be confirmed. Flow regime in the Danube Delta is influenced by the Black Sea but it is a natural situation. Except for some extent of flow regulation that can possibly influence the river morphology, flow dynamics is affected locally by in-stream structures.

The river at this section is slowly flowing but there are still more dynamic and less dynamic sections. Maximum velocity is not higher than 0,7 m/s and in downstream direction decreases to 0,4 m/s (Fig.33). Flow conditions can be seen on the flow pattern (Fig. 33 and 35). Except of indicated discharge regulation downstream of Iron Gate hydrological conditions were changed very slightly along the Lower Danube (Fig.33).

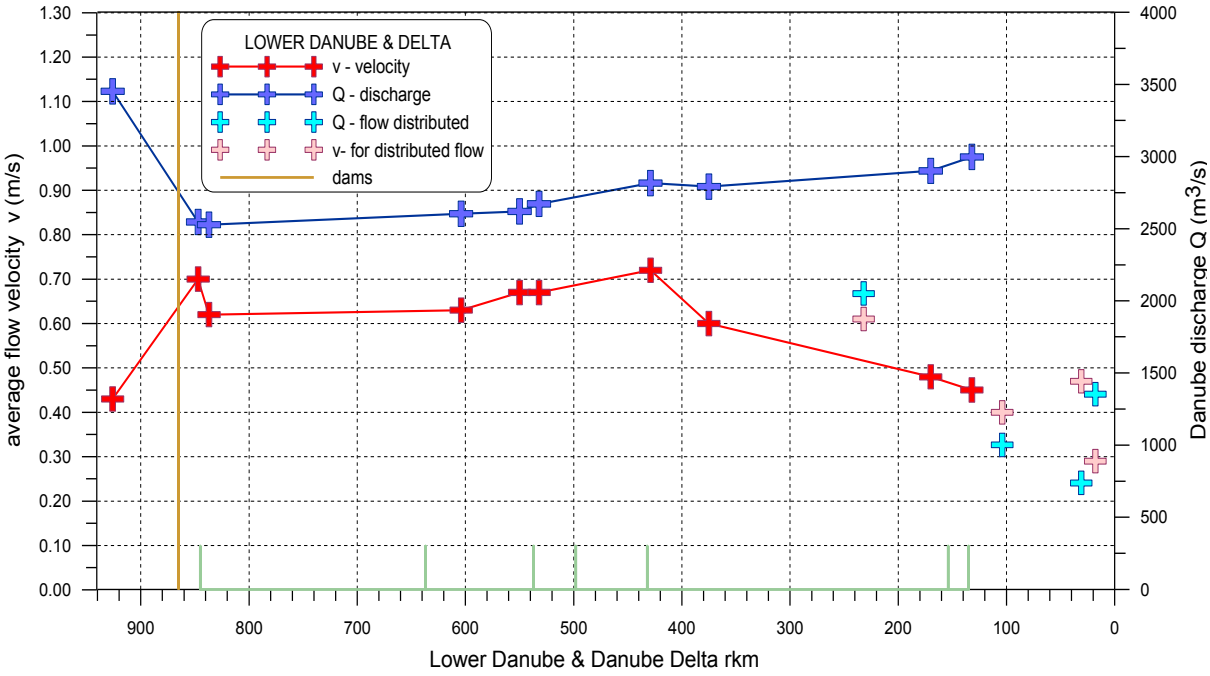


Figure 33: Mean velocity and discharge – Lower Danube

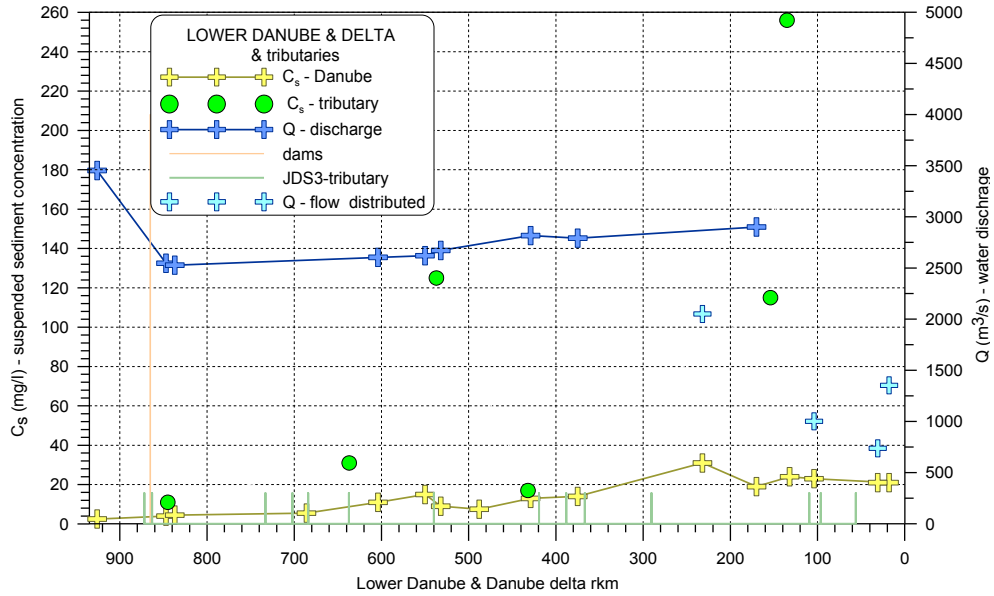
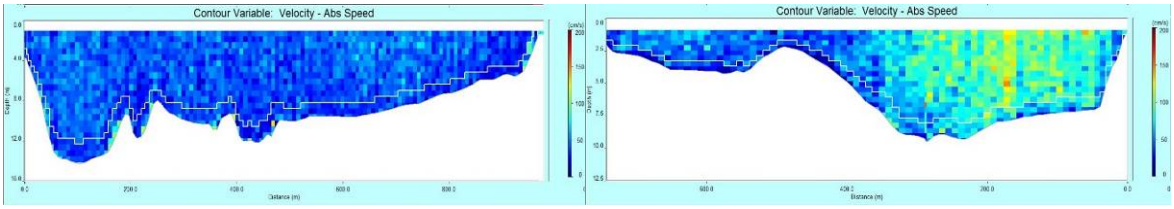


Figure 34: Suspended sediment concentrations & discharge



JDS46 – Vrbica/Simijan (rkm926) JDS55 – Downstream Jantra (rkm 532)

Figure 35: Flow pattern for selected impounded and free flowing Lower Danube

That was the reason why suspended sediment concentration increased on Jantra (125 mg/l) and Siret (154 mg/l). Extremely high value was measured on Prut (256 mg/l). Even though suspended load on these tributaries increased dramatically the effect on the Danube (Fig.33) was rather low (Fig.17).

Bed material on the Lower Danube consists of coarser gravel, fine gravel and coarse sand. Coarser sediments occur at the section just downstream of Iron Gate II – JDS47 which is influenced by more dynamic conditions. Finer sediments – mostly fine and coarse sand, comprise the river bed in the Danube Delta (Fig.36).

JDS47 Rudujevac/Gruia JDS52-Downstr.Olt JDS52 - Oltenia JDS65- Reni JDS68-Sf.Gheorghe

Figure 36: Samples of bed material – Lower Danube & Danube Delta

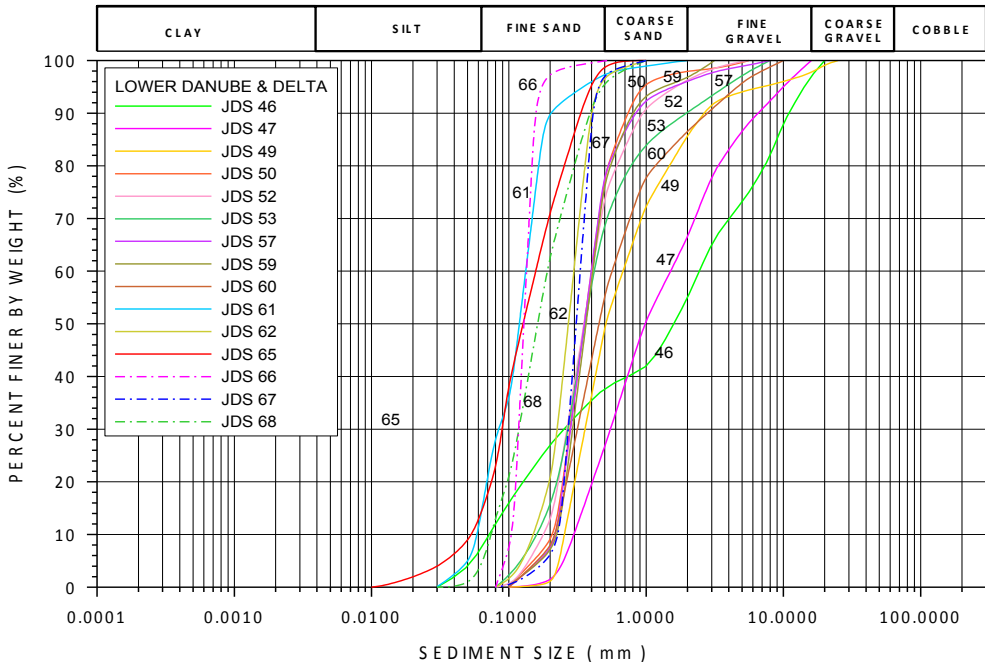


Figure 37: Grain size distribution curves – bed sediments

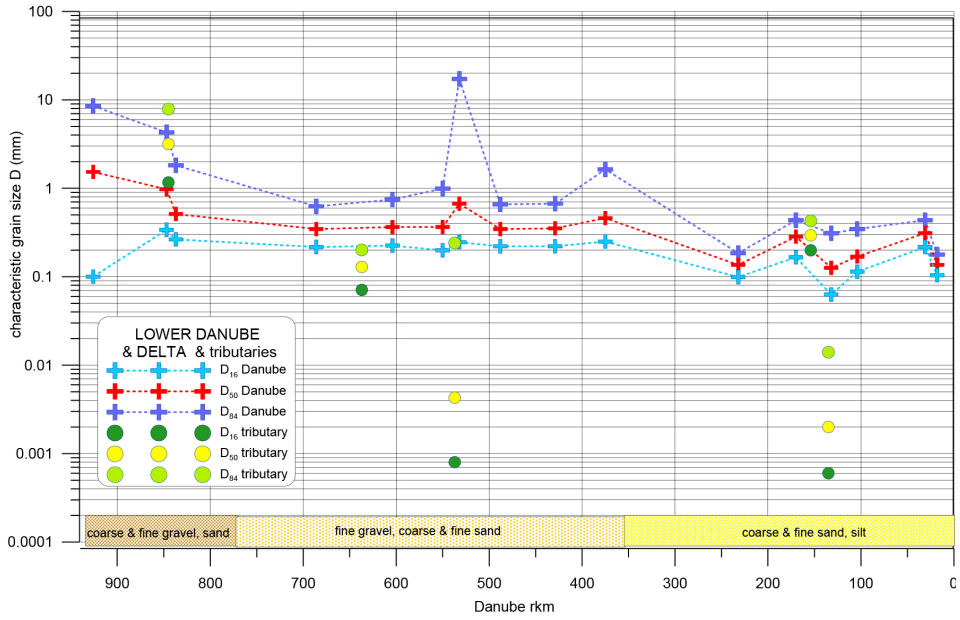


Figure 38: Downstream variation in grain size – bed sediments

Generally, proportion of smaller fractions nearly in all samples is very low and some fractions typical for river delta (silt and clay) are missing almost completely (Fig.37). This can be caused by the Iron Gate where large volumes of coarser and finer sediments are deposited. Significant deficit in sediment supply can be compensated by tributaries. Even though smaller fractions are mostly missing in the river bed. Downstream fining is identified with the highest value of coefficient of determination ($r^2 = 0{,}367$).

Lower Danube and the Danube Delta have a better hydromorphological quality compared to upstream sections. The river is negatively influenced by regulated discharges and a significant lack of sediments downstream of Iron Gate dams as well as the disconnection of floodplains by the construction of dikes, mainly in the 1970ties. However, the river channel shows a significant morphologically variability (width/depth) with sand bars and islands providing a diversity of habitats. There are some

localities more effected by regulation (mostly urban areas) but larger part of the Lower Danube including the Delta is classified by 3 (moderately modified) or 2 (slightly modified) – except for Sulina arm in the Delta (artificial, regulated arm).

3.3.2.2 Hydromorphological site assesment – JDS3 (VÚVH method)

Results of HYMOQ assessment indicate that the hydromorphological conditions of the Danube sites improve in the downstream direction. The highest degree of HYMO alteration has been assessed on the Upper Danube mostly due to the chain of HPPs and river regulation. Hydromorphology on the Middle Danube is still highly altered at long sections due to Gabcikovo and Iron Gate but in between the two huge dams the river channel indicates evident improvement towards moderate conditions. Although the Lower Danube and the Danube Delta is influenced by downstream effect of Iron Gate system (sediment regime) and also by other engineering measures the assessed HYMOQ quality is better compared with upper sections.

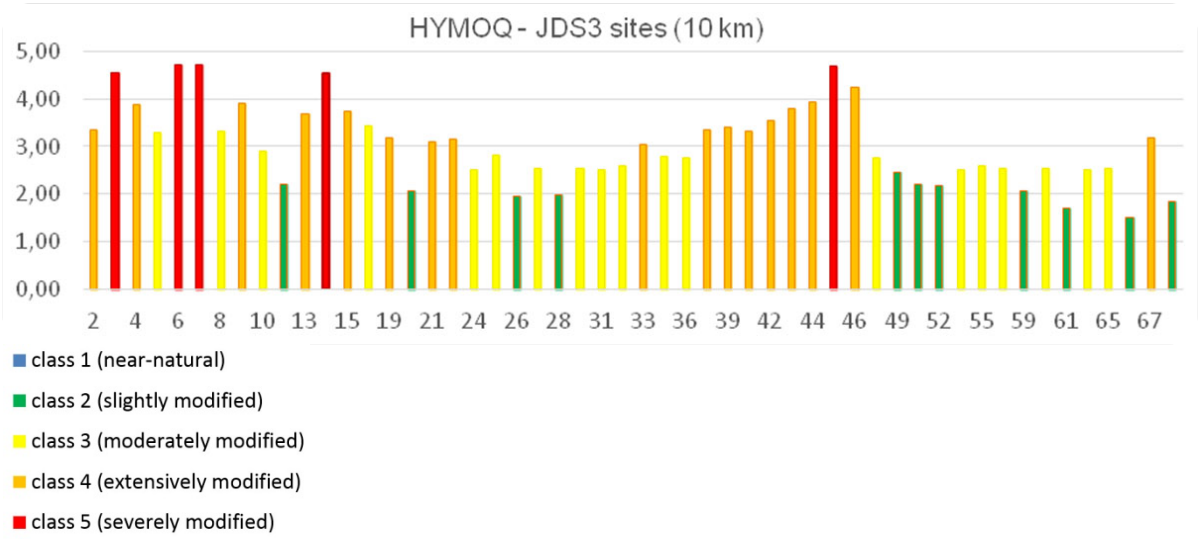


Figure 39: Results of HYMOQ site assessment for the 68 JDS3 sites

According to figure 39 (Lower Danube start with JDS site 46 in the Iron Gate II impoundment), 14 of the investigated sites belong to class 2 and 3 (each 7 sites), followed by two class 4 sites for Iron Gate II impoundment and Sulina branch in the delta.

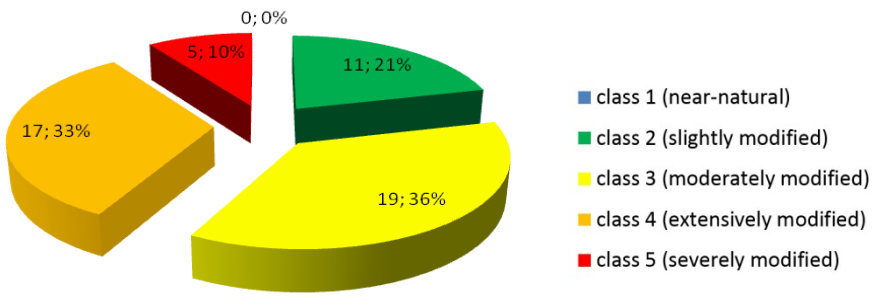


Figure 40: Proportionality of HYMOQ classes – JDS3 sites (VÚVH site method)

The most sections on the entire Danube belong to class 3 (19 sites) so they are moderately modified (36%). 17 sections and second largest group are in class 4 – extensively modified (33%). 11 river sections are only slightly modified (21%) class 2 – reflecting the best HYMOQ assessed on the Danube. Last small group – 5 sites indicate the highest degree of HYMO modification with the worst quality – class 5 severely modified (10%).

Assessment of hydromorphological alteration according WFD requires evaluation of three categories: hydrology, morphology, fish & sediment continuity. Each category has to be evaluated separately and rated by quality classes ranged from one to five. As the final score consists of three digits this approach is often referred to as the "3Digit method". This method of HYMO assessment was applied to data collected during JDS3. The main HYMOQ indicators (VUVH method) are be linked to three categories required by WFD method: hydrology, continuity and morphology. Results of this assessment applied on 10 km Danube sections, which include JDS3 sites are summarized in numerical and graphical form in the Extended Report (CD).

3.4 Conclusions

3.4.1 General conclusions

- Regarding the CEN WFD-3Digit assessment out of the 241 analysed 10 rkm segments 13% fall for morphology in class 2 (slightly modified), 39% in class 3 (moderately modified), 31% in class 4 (extensively modified) as well as 17% in class five (severely modified). For hydrology/flow regime and the continuity only the classes 1, 3 and 5 were assessed. For hydrology only 16% fall in the first class whereas class 3 with 50% and class 5 with 34% prevail. Regarding continuity, dams are located in 8% of segments (in total 18 dams, two dams with functioning fish passes and partial sediment management fall in class 3, the rest in class 5).
- The CEN overall hydromorphological analysis indicates that about 60% of the analysed Danube stretch falls below class 3 (21% in the second class „slightly modified" and 39% in the third class „moderately modified"), 40% fall in the two worse classes four (26%) and five (14%).
- Information on the hydromorphological conditions was significantly improved as in-situ measurements of hydrological, morphological and hydraulic characteristics were performed for the first time on the entire Danube and tributaries (JDS3-sites).
- Results of hydromorphological survey are used to identify present hydromorphological conditions of the Danube. These can be used further for the WFD compliant assessment of the HYMO alterations with regard to hydrology, morphology and river continuity having no ambition to replace the national assessment method.
- Ecological groups provided feedback that the HYMO survey provided valuable information for the interpretation of the biological data.
- Results of in-situ measurements used for hydromorphological assessment improved characterisation and analyses of the hydromorphological conditions (including consideration on physical processes) of the Danube, creating a basis for more reliable considerations on sustainable restoration actions.
- The hydromorphological database creates an excellent basis for further hydromorphological analyses.
- The assessment of defined 10 rkm segments improve spatial and thematically resolution of the survey and assessment based on a common methodology. It can serve as solid base for the management requirements and monitoring over the next decades.
- The assessment results confirm the main findings of JDS2 in 2007 (different situation along upper, middle and lower Danube), however the increased resolution allow a more precise assessment in particular of dams and their impacts but also regarding left/right banks.
- The importance and strong impact of existing dams in particularly regarding sediment balance up- and downstream, but also the hydrological changes (e.g. due to potential flow regulations) should be matter of further basin-wide investigations (sediment balance up- and in particular downstream of dams, detailed hydrological analysis downstream of dams).

3.4.2 Technical conclusions for next JDS

- JDS sites should be selected in close cooperation and discussion of all participated working groups (including hydromorphology group) to find out the most representative river sections.
- There is an increasing need to improve "descriptive" method of hydromorphological assessments in particular for large rivers as it should be more "physical process" based. Further the linkage of HYMO parameters and biological response as well as monitoring efficiency should be improved. The first steps in this direction were already done by performing in-situ measurements on JDS3.
- Based on field experience some technical improvements and optimization of hydrological and morphological measurements can be applied (in cooperation with other groups).
- To take fully into consideration the type-specific conditions according to WFD requirements.

3.4.3 Recommendations for measures:

- Taking into account the situation of the large European rivers which are severely altered to a large extent, it should be taken care that the remaining less altered water bodies along the Danube will be managed considering the environmental objectives.
- In addition to morphological restoration measures a management of the sediment balance is needed at Danube basin-wide scale.
- Prevention of fresh bank revetments and reinforcement to the absolute minimum.
- Continuation of restoration measures improving the hymo conditions to meet the good ecological status/potential along the entire Danube.
- Restoration of floodplains should be a long-lasting goal for ecological and flood mitigation planning.

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4 Riparian Bird Species (Little Ringed Plover, Sand Martin) as Indicators for River Dynamics and Morphology

Schmidt M., Bandacu D., Bogdea L., Bozhinova S., Costea G., Gáborik A., Grlica I.D., Hima V., Kiss G., Koev V., Kovarik A., Mešíková M., Milenkovic-Srbulovic M., Parrag T., Petrova V., Raluca A., Rožac V., Šakić R., Schneider T., Surovec P., Tatai S., Tóth B., Tucakov M., Vasić I., Frank G.

4.1 Introduction

River dynamics and natural morphological processes are key to the long-term preservation of rivers, and are necessary for forming a variety of important, highly valuable habitats with characteristic species communities. The forming of "new" habitats in early stages of succession, such as steep loam walls, gravel islands or large scale sand banks, is only possible through permanent relocation of sediments due to erosion and accumulation. Blocking river dynamics halts these initiating processes and, due to continuing succession, these characteristic habitats of natural rivers have become extremely rare. Consequently, the species communities associated with these dynamic habitats are highly threatened on a European scale. Furthermore, these habitats have also experienced the highest rate of species extinction of all habitat types along the Danube.

Two characteristic bird species for these habitats are the Little Ringed Plover *Charadrius dubius* and the Sand Martin *Riparia riparia.* In its primary habitat, the Little Ringed Plover inhabits large, bare, sparsely vegetated gravel or sand banks, laying its brilliantly camouflaged eggs on the blank sediment (Bauer et al. 2005a, Glutz v. Blotzheim 2001b). The Sand Martin needs steep natural river banks – the result of active lateral erosion of rivers – in which to burrow its nest (Bauer et al. 2005b, Glutz v. Blotzheim 2001b).

Figure 41 & 42: Little Ringed Plover *Charadrius dubius* and Sand Martin *Riparia riparia*: Indicator species for river dynamics and morphology (Pictures by M. Tiefenbach, C. Roland)

Both habitat types can only exist along rivers if there are enough dynamics to ensure natural morphological processes, although these processes can have on a short term negative effects (loss of broods, habitat availability during flooding periods) on the populations of the two species. Due to

various human activities (e.g. embanking and straightening of the Danube and its tributaries) many characteristic river habitats of high natural value vanished in the past centuries and decades, and have since become a high priority for nature conservation along rivers in Europe. As a result, the formerly widespread distributions of both species along the Danube are now reduced only to the remaining sections with sufficient river dynamics (Schmidt and Frank 2012; DANUBEPARKS 2012). Due to their adaption to artificial secondary habitats (e.g. sand and gravel pits) they are still relatively common in the Danube countries (BirdLife International 2004). Being adapted to dynamic habitats, the Little Ringed Plover and Sand Martin react directly and quickly to structural changes, and provide good and fast indication of positive (e.g. restoration actions) or negative impacts (e.g. river regulation) in their habitats. Important breeding sites of either species pinpoint valuable river sections in terms of dynamic river habitats, making both species excellent indicators for high natural value habitats.

DANUBEPARKS – The Danube River Network of Protected Areas (www.danubeparks.org) was established as a platform for continuous transnational cooperation of numerous Protected Area administrations from nearly all Danube countries, with the goal to preserve and restore the most valuable habitats of this international river and its tributaries. Besides the wide range of activities in the field of nature conservation, a monitoring of Little Ringed Plover and Sand Martin was implemented along the Danube – first in 2011 and again in 2013 – within the Joint Danube Survey 3, all funded by the EU Program for European Territorial Cooperation for South-East Europe (ETC-SEE).

According to the breeding season of both species, the survey was conducted from May to June (beginning of July), thus a few months earlier than other scientific groups of JDS3. Nevertheless, the integration of this monitoring in the Joint Danube Survey is important to analyse and discuss its results in an integrated way with other scientific disciplines and directions.

4.2 Methods

The large-scale approach of this monitoring was only possible due the intense cooperation of numerous experts from different parts of the Danube and its tributaries. The course of the Danube and its tributaries were split into sections, for each of which a local DANUBEPARKS partner implemented the monitoring. The shared responsibility ensured a flexible reaction to changing water conditions during the season and a synchronised implementation of the monitoring by all teams – highly important due to the limited time frame given by the breeding phenology of the two bird species.

All in all, 56 experts from 13 Danube Protected Areas representing the DANUBEPARKS Network were involved in the fieldwork.

4.2.1 Study Area

The study area covers the whole Danube from its source at the Black Forest to the Black Sea. The survey was mainly limited to the mainstream of the Danube, but includes the old Danube near Gabcikovo Slovakia, the Szendentre side arm in Hungary and the branches of the islands Balta Ialomiţei and Great Brăila Island in Romania. In 2013, the monitoring was extended to parts of the tributaries Drava River (rkm 240 – rkm 70; rkm 20 – rkm 0, Sava River (rkm 670 – rkm 467) and Prut River (rkm 120 – rkm 0) and the Mosoni-Duna (approx. 120 km). Overall, 4119 km were assessed in 2013.

4.2.2 Field Methodology

Faced with the large study area and permanent changes in water level, a simplified, flexible and efficient methodology was developed for this survey. The base methodology consisted of two surveys by small boats, one in May and one in June, along the whole Danube. All suitable habitats for both species were investigated, artificial areas like sand quarries were not considered. For each site, the number of birds and breeding pairs (bp) were assessed by the observers. Details of the habitat were

noted. For the Little Ringed Plover, habitat was classified according to one of six possible types (riverbank – gravel; riverbank – sand; gravel bank, sand bank, gravel island, sand island). For the Sand Martin, breeding habitat was classified as either loam wall or river bank. Each recorded bird was located in a map and the coordinates of the observation were noted. Details for the methodology are given in Schmidt and Frank (2012).

As a consequence of long periods of high water level (especially in 2013) or other obstructive conditions, in some sections it was not possible to accomplish two surveys per year. This fact was considered in the analysis.

4.2.3 Data Analysis

Considering the compacted methodology with only two surveys per year, the maximum numbers of territories per location and year were used for the analyses. Each data record was checked for plausibility, especially for location and type of habitat. Based on the comments of the observers, in few cases the number of territories and the classification of the habitats were harmonised.

For visualisation, the results were summed up for ten-kilometer sections. Mapping was done in Arcmap 10 and Quantum GIS 2.2. The base map was thankfully provided by NaturalEarth.

For presentation and comparison, the division of the Danube in Upper, Middle and Lower section according to Lászlóffy (1965) was applied. Additionally the Delta – representing a unique hydromorphological and ecological unit – was considered as a separate section.

To analyse the influence of the hydromorphological alteration of the Danube on the occurrence of Little Ringed Plover and Sand Martin, the results of the hydromorphological assessment (Chapter 3) were used. The analysis was only done for sections where data from both surveys – the Danubeparks Monitoring of Little Ringed Plover and Sand Martin and the hydromorphological assessment – were available (rkm 2415 to Delta, without any branches or side arms). The study area was subdivided into 232 x 10-kilometres segments. For each segment, the presence or absence of Little Ringed Plover and/or Sand Martin and the hydromorphological class were set. For the statistical analysis, a Generalized Linear Model (GLM; binominal) was calculated which describes the relationship between the presence of at least one of the two species and the hydromorphological situation. The analysis was done in the statistical software R (Version 3.1.0). For detailed description of Generalized Linear Models see for example McCullagh and Nelder (1989).

4.3 Results

4.3.1 Little Ringed Plover Charadrius dubius

4.3.1.1 Distribution and Population Density

In 2013, a total of 244 territories of Little Ringed Plover were recorded, out of which 182 breeding pairs occurred along the Danube itself. Mainly caused by the different water level conditions, these results differ strongly from the survey in 2011, where 369 territories of Little Ringed Plover had been surveyed along the Danube and its branches. The differences between the two years were more significant along the Upper and Middle Danube, whereas the Lower Danube was less affected by the extreme flooding of 2013, and the results are similar between the two years.

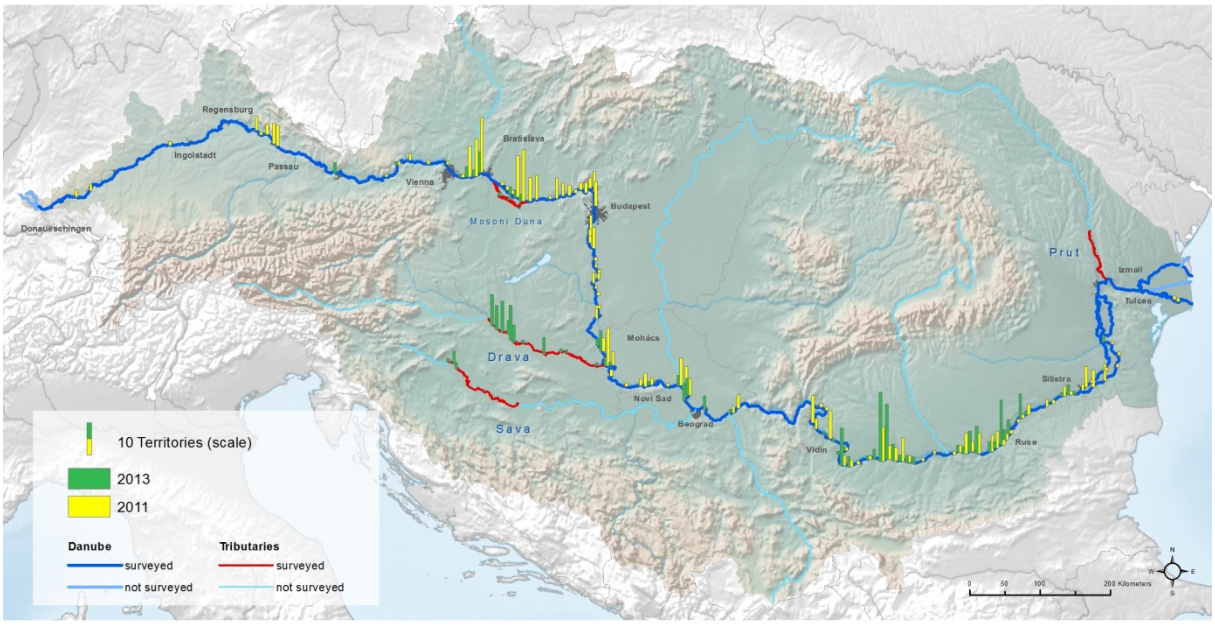

Figure 43: Distribution of territories of Little Ringed Plover along the Danube and selected branches and tributaries, presented for 10 km sections

The Upper Danube shows the lowest density of Little Ringed Plover along the Danube (mean abundance of 0.42 territories per 10 rkm). Both along the Middle and Lower Danube, much higher mean abundances were found (Middle Danube: 1.1 territories per 10 rkm; Lower Danube: 1.14 territories per 10 rkm). Along the middle section, there was a high fluctuation between the two years: In 2011, with more than 1.7 territories per 10 rkm, by far the highest number for the whole Danube could be recorded along the middle section, whereas in 2013 only about 0.4 territories per 10 rkm were recorded.

Compared with the tributaries, the results of both years from the Danube show a higher mean density than along the Mosoni-Duna, the Sava River and the Prut River. An extraordinarily high density of nearly 2.8 territories per 10-kilometres was recorded for the Drava River.

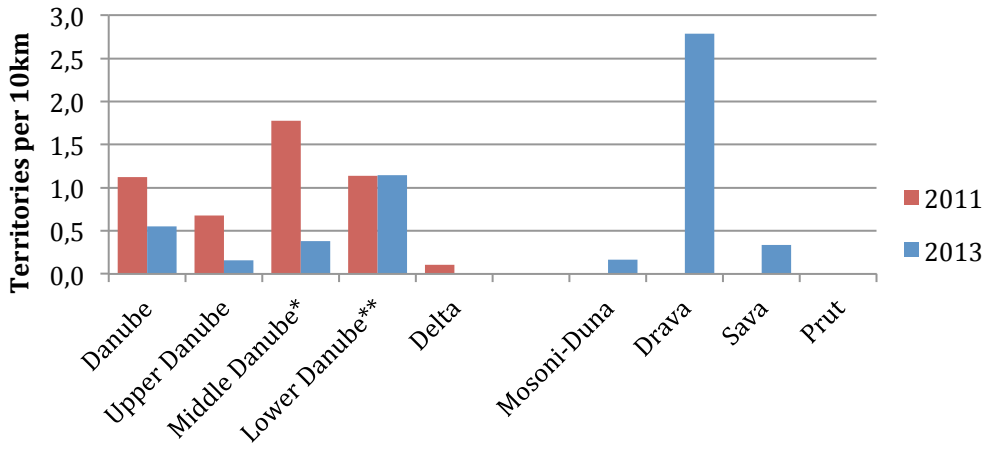

Figure 44: Mean population density of Little Ringed Plover along the Danube and selected parts of its tributaries

4.3.1.2 Habitat Selection of Little Ringed Plover

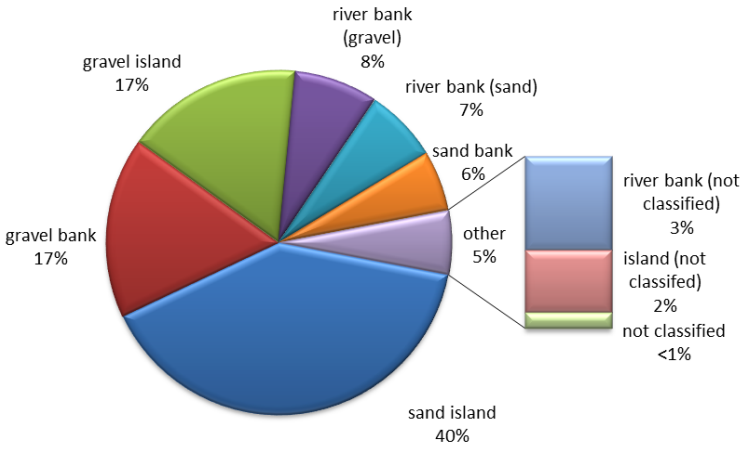

Figure 45: Types of breeding habits of Little Ringed Plover along the Danube

Nearly 60% of all recorded Little Ringed Plover territories were located on islands (sand or gravel). Sand islands in particular (40%), are of high relevance as breeding sites. Gravel banks and sand banks, very similar structures to islands with no connection to the bank, were used in 23% of the detected territories. This means that more than 81% of all Little Ringed Plover territories were found on river habitat structures without a connection to the bank (islands, sand or gravel banks in the river). These sites provide better protection against predators and can offer better feeding resources. Only 19% of territories were located in areas connected to the bank (river bank (sand) or river bank (gravel)).

4.3.2 Sand Martin Riparia riparia

In 2013, 103 colonies with a total of 10 453 breeding pairs of Sand Martin could be located along the Danube. Although these were more colonies than in the first survey (2011: 82 colonies), the number of breeding pairs was less than half of the 22 817 breeding pairs recorded in 2011. This discrepancy is a result of the different water levels, especially the extraordinary flooding along the Upper and Middle Danube in 2013, strongly influencing the number of birds per colony.

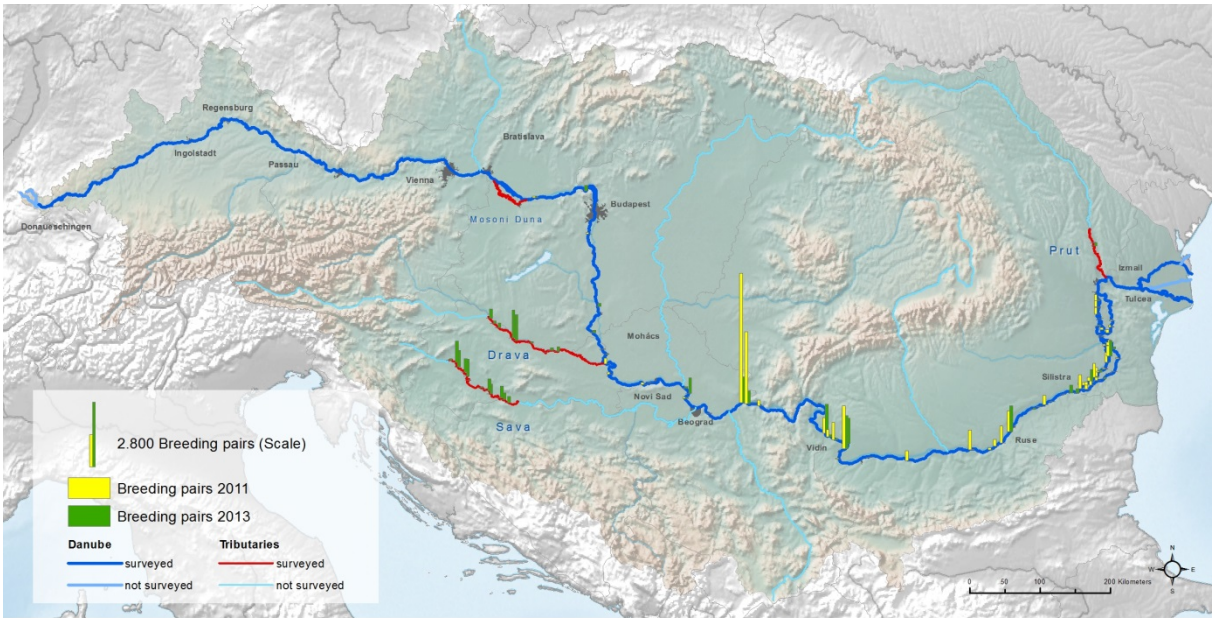

Figure 46: Distribution of breeding pairs of Sand Martin along the Danube and selected tributaries

In both years, no colonies could be recorded along the Upper Danube. Most colonies were found in the border area between Bulgaria and Romania. The largest colonies were located in "Deliblato sands" Special Nature Reserve in Serbia (2011: 5 580 bp).

The highest densities of Sand Martin were recorded along the tributaries Sava River (241 bp/rkm) and Drava River (179 bp/rkm). On Prut River, the densities match the numbers of the Lower Danube. No colonies of Sand Martin were found on the Mosoni Duna.

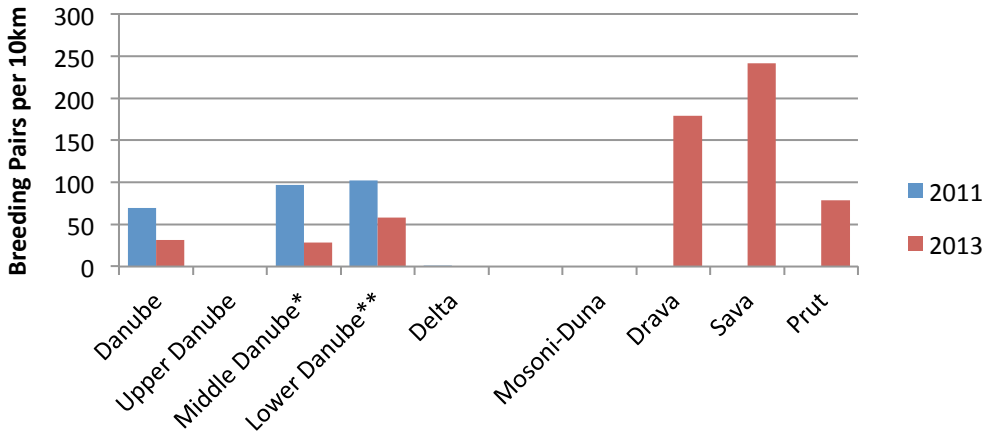

Figure 47: Mean population density of Sand Martin along the Danube and selected tributary rivers

4.3.3 Influence of Hydromorphological Alteration on Occurrence of Little Ringed Plover and Sand Martin

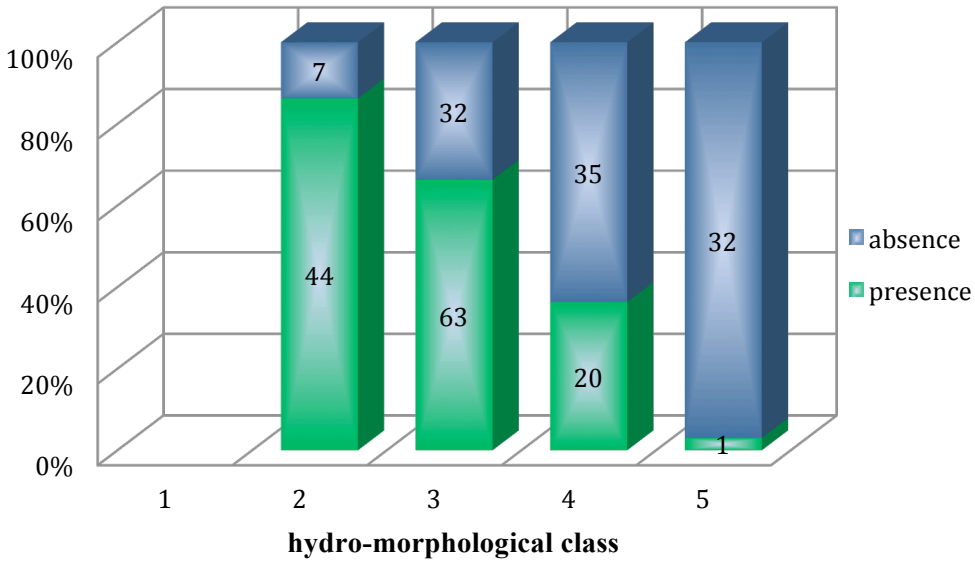

Figure 48: Presence and absence of Little Ringed Plover and/or Sand Martin in dependence of the hydromorphological alteration based on 10 km sections

Figure 48 shows the occurrence of Little Ringed Plover and/or Sand Martin per 10 km sections in relation to the hydromorphological classes assessed by the hydromorphological team of JDS3 (see chapter 3), proving the significant correlation between the extent of hydromorphological alteration and the presence and absence of these indicator species. The generalized linear model (Fig. 49) with binomial error structure shows a significant relationship between absence or presence of the species and hydromorphological class as the predictor (values 2-4, analysis of deviance (Type II tests),

Likelihood Ratio, Ch^2=75.794, Df =1, p<,001). The explained variance is almost 40% (Nagelkerkes R^2=0.37) and the model predicts in 83.6% of the cases the correct presence of Little Ringed Plover and/or Sand Martin (Predicted vs. Observed, cut-value =0.5).

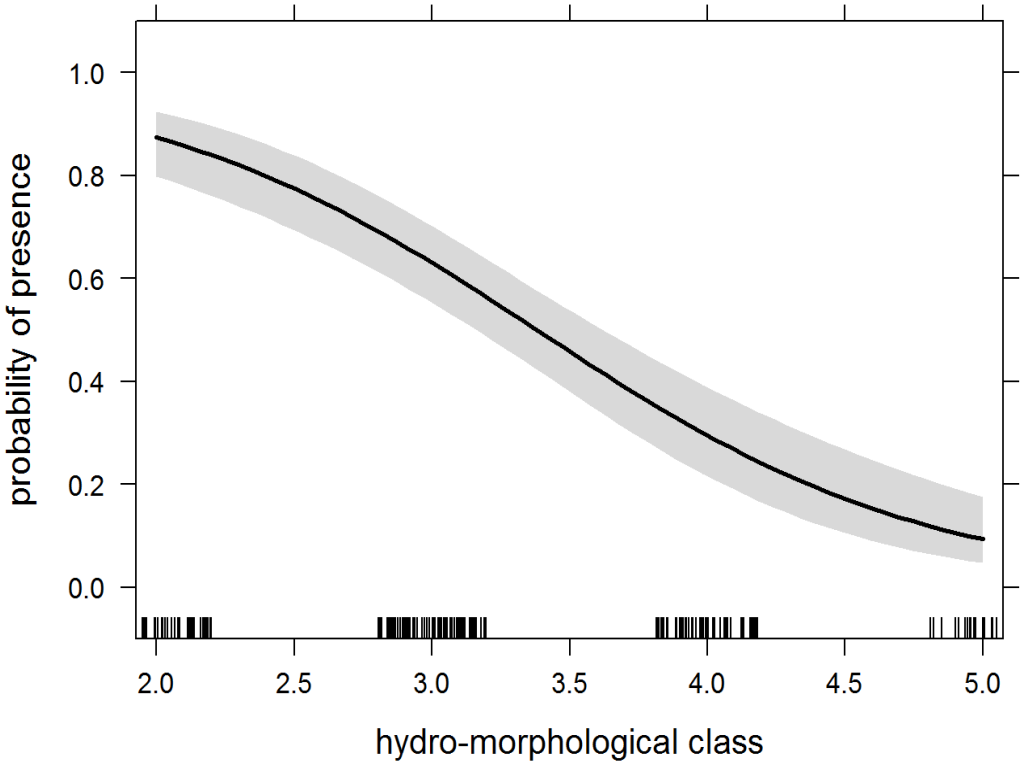

Figure 49: Probability of occurrence of Little Ringed Plover and Sand Martin according to the results of the hydromorphological assessment of the JDS3 (Parameter: "Total_2013")

The model shows that, in the hydromorphological class 2 (slightly modified), the probability of the occurrence of one of the two species is about 89%. According to the equation of the generalized linear model, in class I (near-natural – reference condition; extinct along the Danube) the indicator bird species could even be expected with a probability of about 97%. Stronger hydromorphological alterations greatly decrease the "biological potential" of a river for characteristic species, as shown for class 3 (moderately modified) by a limited probability of about 65%. In "extensively modified" sections (class 4) the probability of the occurrence declines to about 30%.

4.4 Conclusions

4.4.1 General Conclusions

- The results for both indicator bird species show the high natural value of the Middle Danube and the Lower Danube.
- The absence of Sand Martin and the low density of Little Ringed Plover stress the alteration of hydromorphological processes along the Upper Danube
- The high number of territories of Little Ringed Plover on the last remaining free flowing sections of the Upper Danube indicates the high potential of the Upper Danube and gives an imagination of the distribution and abundance of the species along a not anymore existing natural Danube (reference condition).This underlines the high relevance of river restoration projects along the Upper Danube.
- The assessment at selected sections at tributary rivers proves their high natural value and provides an idea of the natural potential of the main stream of the Danube.
- The clear preference of the Little Ringed Plover for island or island-like structures highlights the natural value of these habitats and the importance of an appropriate protection of these sites.
- The results of the monitoring of indicator bird species correlate significantly with the results of the hydromorphological assessment and show the added value of an interdisciplinary approach.
- The monitoring of indicator bird species stresses the high ecological value of river sections which are only slightly modified (class 2) or even in a better hydromorphological status. Stronger hydromorphological alterations reduce the ecological value: already in class 3 (moderately modified) the probability of occurrence of one of the two species is reduced to about 65%, and the probability is dramatically reduced to about 30% in class 4 (extensively modified).

4.4.2 Technical Conclusions

The water conditions in 2013 show how important it is to conduct a monitoring over several years. The results of 2013 give a good impression of the short-time effects of flooding on the populations of these bird species. Analyses concerning the hydromorphological aspect were only possible in combination with the results of 2011.

Further analyses concerning the hydromorphological situation on the side arms and tributaries would be valuable; harmonised data on hydro-morphology were not available upstream of rkm 2415, the inclusion of this section would increase the significance of the conclusions of this survey.

A follow-up survey for these bird indicator species is required to enable Danube-wide analyses based on population size. More detailed analyses on the correlation between biological indicators and hydromorphological alteration could be a step towards the formulation of biological thresh-holds for a good hydromorphological status on rivers.

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5 Macroinvertebrates

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5.1 Introduction

Benthic macroinvertebrates are one biological quality element used within the Framework of the European Water Framework Directive (EC, 2000/60; WFD) to assess the ecological water quality and were therefore monitored in all previously conducted Joint Danube Surveys (JDS). The methods applied were differing due to availability of devices, financial issues and the scientific focus. While in JDS1 grabs were used to investigate hard rocky substrates (Literathy et al., 2002), in JDS2 air-lift samples were taken to study the faunal composition of deep water habitats (Liška et al., 2008). During JDS3 a modified Multi-Habitat-Sampling (MHS) approach has been performed to highlight the importance of specific micro-habitats in terms of biodiversity and additionally as a sound basis for river restoration efforts and water management issues in general. The data gained from JDS3 can be seen as an important documentation of the current distribution of specific taxa and a completion regarding faunistics of earlier studies, (Russev, 1998; Slobodnik et al., 2005; Csányi & Paunovic, 2006) and of all previous JDS expeditions. The results will significantly contribute to the currently ongoing discussions regarding the WFD compliant assessment methods of large rivers either for field work as well as the analysing aspects.

5.2 Methods

5.2.1 Sampling

Sampling of benthic macroinvertebrates for JDS3 had three approaches carried out by three separate sampling groups:

Main approach:

- **Multi-Habitat-Sampling**, MHS: A standardised, WFD compliant method for the ecological (status) assessment (AQEM Consortium, 2002). Sampling of different habitats in the actual littoral zone was done with a Multi-Habitat-Sampling net (BOKU).

Additionally approaches:

- **Deep Water Sampling**, DWS: Cross-sectional survey by dredging in the deep water area (Laboratory of MTA (Hung. Acad. Sci.), Centre for Ecological Research, Danube Research Institute). This approach was decided for comparability reasons with the Airlift-data, a deep water sampling method which was applied during JDS2 in 2007.
- **Kick and Sweep Sampling**, K&S: Sampling with a hand net at the shore region (Siniša Stanković, University of Belgrade (IBISS)) in order to provide comparisons with the K&S data from JDS2.

The aim of the additional K&S sampling was to extend the investigated zone adding further mussel data to the results of the near-littoral MHS sampling program.

Sampling procedure and taxonomic resolution greatly influences the results of bioassessment (e.g. Birk et al., 2012; Hering et al., 2004). Therefore the standardised MHS approach was used for the ecological status assessment together with the DWS as well as to investigate habitat preferences of specific taxa. Samplings from the riparian zones are influenced by hydrological conditions. Therefore dredging (DWS) was used additionally to include deep water habitats of the Danube River. Until now only the Air Lift method provided systematic data on macroinvertebrates from the extended depths but the whole cross section of the river was not involved during former surveys (JDS1, AquaTerra, JDS2).

All three approaches are complementing each other, especially in terms of biodiversity and longitudinal distribution issues. Experiences of the JDS3 can therefore substantially contribute to the development of a comprehensive sampling methodology in large rivers.

5.2.1.1 Multi Habitat Sampling (MHS)

The habitat specific macroinvertebrate sampling at the littoral zone was done with a Multi-Habitat-Sampling (MHS) net with a frame of 25 x 25 cm. This semi-quantitative instrument provides a sampling area of 0.0625 m² per sampling unit and is positioned upstream in the riverbed whereas the sediment in front of the frame is stirred up so that the animals are drifting into the collecting net with a mesh size of 500 µm and minimum lengths of 1 m. This method can be applied in wadeable zones up to a maximum water depth of 1.5 m.

The original method focuses on a multi-habitat scheme designed for sampling major habitats in proportion to their presence within a sampling reach. A MHS-sample consists of 20 "sampling units" taken from all habitat types at the sampling site, each with a share of at least 5% coverage (AQEM-consortium, 2002).

During JDS3 at each sampling site all available habitats, regarding substrate type, such as lithal banks (of different grain sizes), rip-rap zones, macrophytes, woody debris (xylal), etc. were sampled and stored separately. The habitat types were selected by surveying shore-lines by motor boat. For each defined habitat five sampling units were taken for statistical reasons. Additionally water-depth and flow velocity were taken for each sampling unit. The sampling units of a habitat were pooled and stored separately. In case of homogeneous substrate diversity, the same substrate type was sampled under different hydraulic conditions. In total a minimum of 20 sampling units, representing at least four different habitats per sampling site were taken. All samples were fixed with formaldehyde (final concentration: 4%).

On the basis of this methodology, two approaches can be conducted:

- habitat preferences of different macroinvertebrate taxa can be ascertained and
- one WFD-compliant MHS, consisting of 20 sampling units, can be combined for standard analyses (e.g. Saprobity).

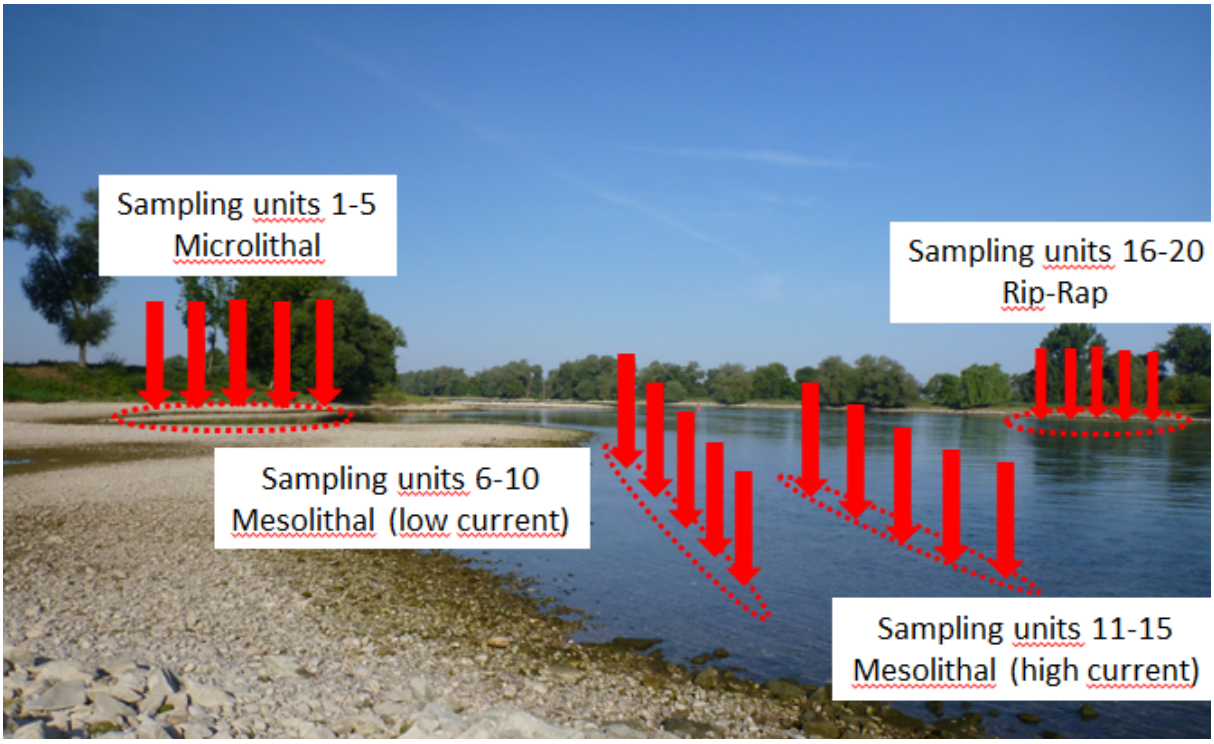

Figure 50: Habitat-specific sampling; example from JDS-site 5

The MHS methodology is based on the Rapid Bioassessment Protocols (Barbour et al., 1999), the procedures of the Environment Agency of England and Wales (Murray-Bligh, 1999), the Austrian Guidelines for the Assessment of the Saprobiological Water Quality of Rivers and Streams (Moog et al., 1999), ISO 7828, the AQEM sampling manual (2002), the AQEM & STAR site protocol (2002), the German methodology as described in www.fliessgewaesserbewertung.de, and the Austrian Standards M 6232 and M 6119-2.

5.2.1.2 Deep Water Sampling (DWS)

This dredging program provided rough information how the animal populations are distributed in the cross section the deep water space along the river bed.

Dredging was carried out with the help of the motor boat of the ARGUS. The iron-forked mouth of the triangle shaped dredge had a collecting net with 500 µm mesh size (Figure 51). Pulling the dredge was carried out with a rope downstream direction. The upstream-heading boat was driven backwards; so that the dredging was done from the frontal part of the boat. The dredging speed of the sampler on the bottom had to exceed the actual current velocity in order to avoid the washing out of the material from the net. The first 2 m of the pulling device was a heavy iron chain in order to keep the dredge horizontal on the bottom during dredging. We tried to keep the angle of the rope less than 25° during the procedure because this orientation made the dredge capable to dig in the bottom material efficiently.

Dredging locality was recorded with a GPS device, water depth was measured by hydro-acoustic equipment. The dredged material was filled into buckets marked with serial numbers I-V (Number I is near to right bank, II is far from right, III is in the middle, IV is far from left, V is near to left). Photos were taken to illustrate grain size distributions of the sample.

Usually 10 L of bed material was collected. Abundance data of dredging can theoretically be regarded as semi-quantitative: dredging 5 cm thick layer and 25 cm wide bed layer will provide this 10 l of volume if we pull the dredge roughly along a 80 cm long distance. This surface area (25x80 cm^2) represents 0.2 m^2. Thus the individual number of the sample multiplied by five roughly provides the individual number per square meter.

Figure 51: Bottom dredge with chain and rope for macroinvertebrate sampling

Deep water sampling was carried out in depths that are bigger than the wadeable, usually littoral (1.5 m) deep zone. The deepest part where the dredging was successfully applied was more than 20 m (Chilia arm).

5.2.1.3 Kick and Sweep Sampling (K&S)

Kick & Sweep (K&S) sampling (EN 27828:1994) carried out in a wet diving suit was used in the near-shore region. This way the sampling depth was bigger than 1.5 m in the littoral zone (up to 2.0 m) A hand net with 500µm mesh size was used. Free diving was also done in order to increase the sampling depth principally for collecting more data on freshwater mussels (up to 4 m water depth).

However, the results of the three sampling methods are complementing each other: MHS data are used for status assessment, DWS and K&S data provide more information characterizing biodiversity and analysing the spatial-temporal distribution of native and invasive taxa.

5.2.2 Sorting and Identification

In case of the habitat specific macroinvertebrate sampling at the littoral zone, the samples collected from a defined habitat were stored separately for further determination in the laboratory at the BOKU in Vienna. After a curing time of at least 2 weeks the material of each sample was sorted completely. The animals were counted, separated into their specific orders and determined by taxonomic experts to the best level possible. Additionally the crustacean order Amphipoda and the Bivalvia genus *Corbicula* were divided into size-classes for further investigation.

The following taxonomic experts were involved:

MHS – Ferdindand Sporka (Oligochaeta); Peter Borza (Crustacea); Wolfram Graf (Plecoptera, Trichoptera), Thomas Huber (Ephemeroptera); Patrick Leitner (Simuliidae); Berthold Janecek (Chironomidae/Odonata)

The samples collected by dredging (DWS) and K&S were partially processed in the field. Reduction of sample volume was done by rinsing (mesh size 500 µm) to separate organic from mineral fractions. The material was preserved with 4% formaldehyde.

Further sorting of material collected by dredging was performed in the Laboratory of MTA (Hung. Acad. Sci.), Centre for Ecological Research, Danube Research Institute, while the sorting of material collected by K&S was done in the Laboratory of the Institute for Biological Research "Siniša Stanković", University of Belgrade (IBISS).

The following taxonomic experts were involved:

DWS – Péter Borza (Crustacea); Béla Csányi (Mollusca, Hirudinea, Insecta); József Szekeres (Mollusca, Crustacea, Insecta); Ana Atanacković (Oligochaeta); Đurađ Milošević and Dubravka Čerba (Chironomidae)

K&S – Péter Borza (Crustacea); Ana Atanacković (Oligochaeta); Đurađ Milošević, Dubravka Čerba (Chironomidae); Jelena Tomović, Vanja Marković, Momir Paunović (Mollusca), Bojana Tubić, Momir Paunović (Insecta other than Chironomidae) and Stefan Anđus (Porifera).

5.2.3 Analyses

To ensure harmonised data storage the species-list per sampling unit including all measured parameters was filled into the Access-based software ECOPROF 4.0 (Moog et al., 2013), which is compatible with the ICPDR database. For the calculation of metrics and saprobic indices only WFD compliant (semi-)quantitative and area related approaches, represented by 20 combined sampling units (MHS-method) were used. Species list, diversity as well as cluster/NMS analyses for typological conclusions were based on all data collected during JDS3 including all habitat specific sampling units per site.

In the case of dredging and K&S method, data harmonization in respect to systematics was ensured using ASTERICS/PERLODES entering coding system. Coding system is principally harmonised with the ICPDR database and ECOPROF 4.0, which ensured comparability of the data.

5.2.3.1 Saprobic index and calculation of metrics

5.2.3.1.1 Saprobic Index

Saprobic indices based on the Fauna Aquatica Austriaca ed. by Moog (1995) were calculated based on available national methods using the software packages ECOPROF 4.0. and ASTERICS/PERLODES (www.fliessgewaesserbewertung.de). For calculations based on the Makovinska-catalogue (Sommerhäuser et al., 2003), a database has been created and linked with ECOPROF. For the calculation of saprobic indices based on German and Czech Standards, data have been exported to Excel and imported into the AQEM assessment software.

5.2.3.1.2 WFD-compliant criteria for assigning the ecological status

Much information has already been compiled with respect to hydrobiological (reference) conditions in the Danube basin (e.g. 'WFD Roof Report' ANNEX 3: Typology of the Danube River and its reference conditions [ICPDR, 2005]). Nevertheless, currently no WFD-compliant metrics for large rivers have been officially defined or agreed (Buijs, 2006), the intercalibration procedure is still in progress (Birk et al., 2013, Schöll et al., 2012).

5.2.3.1.3 Organic pollution

For monitoring the organic pollution the saprobic system has a long tradition – the WFD compliant implementation of this system is based on the deviation of the Saprobic Index from saprobic reference conditions (Stubauer & Moog, 2003; Ofenböck et al., 2010; Rolauffs et al., 2003). BMWP and ASPT are alternative indices that are widely used for assessment.

For the indication of water quality classes the threshold values of the Saprobic Index given in Table 6 were applied (Buijs, 2006). For the Upper Danube reach (from site 1 to site 8) the existing national classifications are used. In Germany the reference values are 1.80 for national type 9.2 and 1.85 for type 10 respectively (Rolauffs et al., 2003). In Austria the reference conditions are defined as 1.75 for ecoregion 9 (Stubauer & Moog, 2003) and 2.0 for ecoregion 11 which are changing between JDS site 8 and 9. Stubauer & Moog suggested in Sommerhäuser et al. (2003) a Saprobic Index of 2.0 as the highest threshold reference value for the Danube sections downstream. This value is consequently used as the saprobic basic condition for the Middle and Lower Danube reach. The same classification scheme was employed in the case of results obtained by the K&S sampling technique.

Table 6: Threshold values for the indication of water quality classes based on organic pollution.

	Saprobic reference condition (range of Saprobic Index)			
Ecological status class	Germany national type 9.2	Germany national type 10	Austria Saprobic basic condition 1.75	Austria Saprobic basic condition 2.0
I – High	1.65 – 1.80	1.75 – 1.85	≤ 1.75	≤ 2.00
II – Good	1.81 – 2.25	1.86 – 2.30	1.76 – 2.21	2.01 – 2.40
III – Moderate	2.26 – 2.85	2.31 – 2.90	2.22 – 2.68	2.41 – 2.80
IV – Poor	2.86 – 3.40	2.91 – 3.45	2.69 – 3.14	2.81 – 3.20
V – Bad	>3.40	>3.45	>3.14	>3.20

5.2.3.1.4 General Degradation

Due to the absence of commonly agreed metrics for the assessment of large rivers, up to now the river quality of large rivers was mainly assessed by organic pollution. To achieve the demands for an integrated biological assessment for macroinvertebrates and to assess the ecological status of a water body the taxonomic composition, abundance, ratio of disturbance sensitive taxa to insensitive taxa, and the diversity of biological indicators, have to be considered and compared to respective target values under reference conditions. The aim of JDS3 was to find valuable biotic scores that can be integrated into future assessment systems.

Hence, the recently developed Slovak method for large rivers (Nariadenie Vlady Slovenskej republiky, 2012; Sporka et al., 2009) of catchment sizes >1000 km² (separated into altitude classes between 200 and 500 m and <200 m respectively) was tested with the MHS-data, calculating the ecological status by means of this national method that combines Saprobity and selected (degradation-) metrics for each river type. This assessment method was chosen because it was already tested with prior Austrian Danube data (Leitner, 2013) providing reasonably results. The Slovenian multimetric index (Urbanović, 2012) is based on an analogue functional metric and was not tested therefore separately. Additionally Marković et al. (2012) developed a multi-metric index for the Middle Danube region which was not analysed further because of its type-specificity.

All relevant metrics for the Slovak method for each river type and benchmarks are listed in the Full report on the attached CD.

5.2.3.2 Multivariate analyses

For the following analyses the JDS-sites 11, 13, 28 & 32 were excluded from the calculation because of questionable results due to increasing water level or bad status and accordingly under-represented taxa numbers.

For the MHS-data the following statistical methods were applied by using PC-Ord Software Version 5.33 (McCune & Mefford, 2006).

- Cluster analysis – Distance measure: Sørensen (Bray & Curtis) coefficient (Sørensen, 1948); group linkage method: Flexible Beta (Beta = -0.25)
- Non-metric Multidimensional Scaling (NMS; Kruskal 1964) – Distance measure: Sørensen (Bray-Curtis)
- Indicator Species Analysis (ISA) – Dufrene and Legendre's (1997) method.

Data for sampling sites obtained by the K&S techniques were analysed using Correspondence analyses (CA) by employing Flora Software package (KARADŽIĆ, 2013). Basic variant ordination with Singular Value Decomposition (SVD) algorithm was used (KARADŽIĆ, 2013), as more precise method when compared to the Weighted Averaging.

5.3 Results and discussion

According to the selected main sampling method the following chapters are based mainly on the evaluation of the MHS data set. Due to spatial limitations the detailed discussion of DWS and K&S data is given in the CD supplement of this Report.

5.3.1 Overall taxa richness

During JDS3 a total of 460 macroinvertebrate taxa were identified by three applied sampling techniques. Insects, with 319 taxa, were the dominant component of the communities. Diptera were the richest insects order with 222 taxa, with 200 species belonging to the family Chironomidae. Other heterogeneous groups were: Oligochaeta (55 taxa), Mollusca (43 taxa – Bivalvia 23 and Gastropoda 20), Trichoptera (40 taxa), Ephemeroptera (32 taxa), Coleoptera (15 taxa), Amphipoda (15 taxa) and Odonata (13 taxa). Other taxagroups were less diversified.

5.3.2 Diversity and abundances

The following statistics provide the data of the **MHS-samples** (20 subsampling units per site) representing only the taxa of the proportional estimation of habitats for each single site. Additional samples of under-representative habitats (<5%) are not included to avoid deviations of means due to varying numbers of samples.

In total the combined MHS-samples comprised 345 invertebrate taxa; including the additional habitat-samples (of habitats which were additionally sampled but proportionately under-represented at a certain site, such as deadwood) an overall number of 393 taxa were documented.

The most heterogeneous groups were Diptera (162 taxa) and Oligochaeta (42 taxa) followed by Trichoptera (28 taxa), Ephemeroptera (24 taxa) and Molluscs (Gastropoda 17 taxa, Bivalvia 13 taxa, respectively). Coleoptera (11 taxa), Amphipoda (15 taxa) and Odonata (9 taxa) are as well noteworthy; other groups are important but less diverse. Along the three reaches of the Danube, Trichoptera and Ephemeroptera are decreasing in diversity, all other groups are quite constant or showing a peak at the middle reach (Figure 52).

Regarding Amphipoda a high number of invasive species (*Chelicorophium curvispinum, C. robustum, C. sowinskyi, D. bispinosus, D. haemobaphes, D. villosus, Echinogammarus ischnus, E. trichiatus* and *Obesogammarus obesus*) was documented.

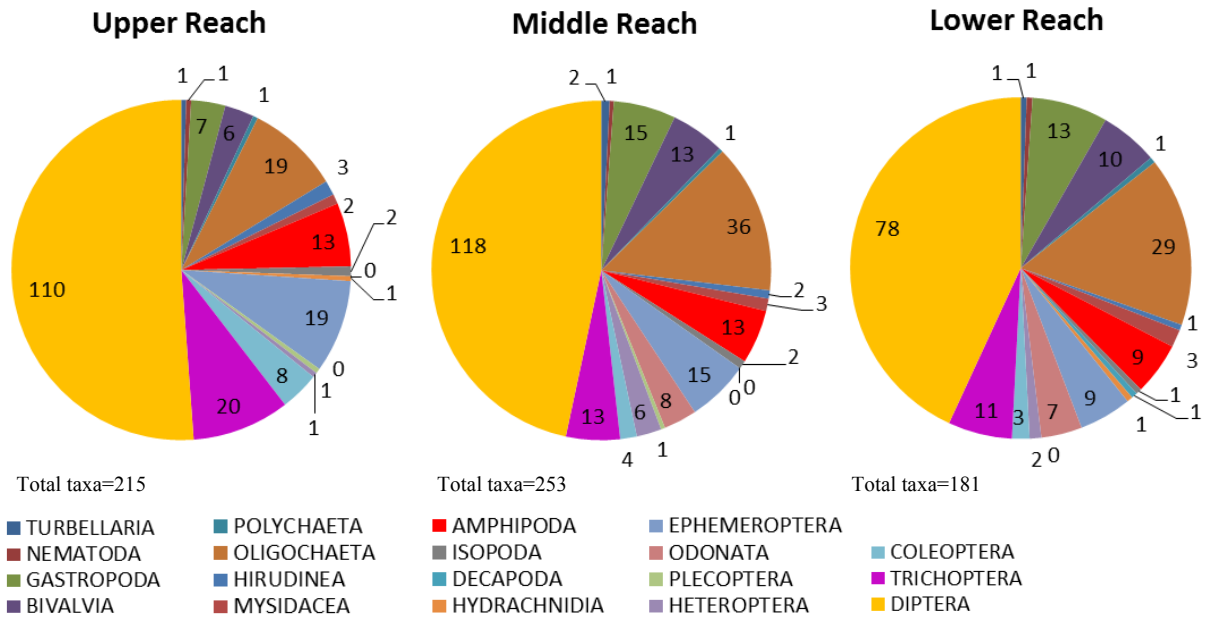

Figure 52: Number of taxa per taxa group along the different reaches of the Danube (MHS-Data)

Regarding abundance (ind./m²) Amphipoda are the dominant group in all Danube reaches and increase downstream (varying from 27 to 45%), while Diptera play an essential part in the Upper Reach (32%) and decrease downstream (17%). Oligochaeta and Mollusca were found in increasing numbers in the Middle and Lower Reach. Higher abundances of EPT-Taxa (Ephemeroptera, Plecoptera and Trichoptera) were only documented for the upper stretch, whereas Trichoptera showed highest abundances within this group. Regarding aquatic insects, only Chironomidae play a major role along the whole Danube stretch (Figure 53).

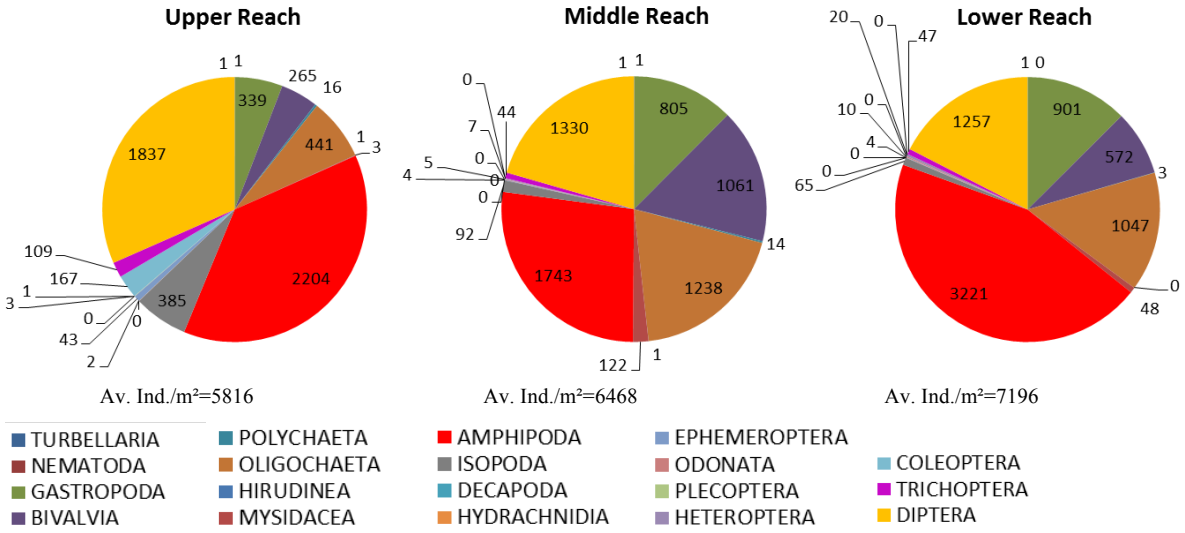

Figure 53: Average density (individuals/m²) per taxa group along the different reaches of the Danube (MHS-Data)

On the basis of the **DWS method** altogether 172 taxa were detected in 53 different cross sections (5dredges/site). The most abundant groups are Insecta (82 taxa, Chironomidae with 54 taxa) and Mollusca (15 Gastropoda- and 20 Bivalvia-taxa). The Annelida group contains 22 Oligochaeta, 7 Hirudinea taxa and one Polychaeta taxon. The 23 Crustacea-taxa are characterised by 8 Amphipoda, 7 Mysididae, 4 Coropiidae, 2 Decapoda and 1-1 Isopoda and Cumacea.

14 of these taxa are considered as invasive. Most of these species are of Ponto-Caspian origin. Their presence on the Lower Danube should be regarded as natural (native species for that reach). Only two taxa are relatively new in the Danubian Fauna: *Theodoxus fluviatilis* was firstly reported from the Budapest section of the Danube not long ago (Frank et al. 1990). Similarly, *Corbicula fluminea* was found at first in the lower Hungarian Danube in 1998 (Csányi 1998-1999) as a new species for Hungaria.

Based on the **K&S sampling** procedure, all together 282 macroinvertebrate species were identified. Aquatic insects were found to be the dominant component of the communities, with 160 taxa recorded.

The number of taxa per sampling site ranged from 13 (JDS32, Upstream Novi Sad) to 63 (JDS14, Gabčikovo Reservoir).

The number of taxa of the main taxonomic groups per sampling method is given comparatively in Figure 57.

5.3.2.1 Habitat specific assessment

The focus of the habitat-specific sampling was to investigate the habitat preferences of taxa as a basis for river restoration and management in general. For the following analysis all samples (also from proportionally under-represented habitats) taken by the MHS method were integrated.

The NMS scatterplot in Figure 54 (left) shows a distinct faunal gradient from fine (pelal to akal) to coarse substrates (gravel to boulders), rip-rap and woody debris (xylal). Other organic habitats as macrophytes and roots are widely spread over the scatterplot.

This indicates a clear correlation between taxa composition and habitat type along the whole Danube stretch having a higher explanatory value regarding biological composition than the longitudinal distribution along the 3 reaches of the Danube (Figure 54, right) as especially the samples of Middle and Lower Danube reach show no distinct separation. This implies a relatively homogenized fauna (except in the Upper Danube reach) and the occurrence of specific taxa is predominantly habitat-determined.

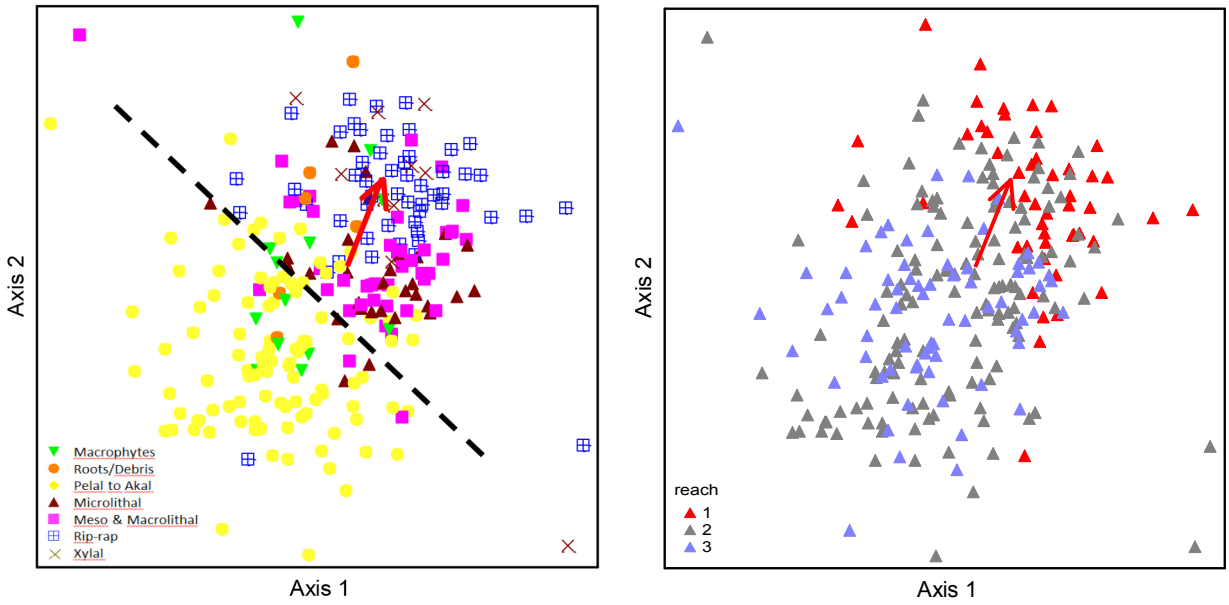

Figure 54: NMS scatterplot, based on taxa assemblages per sample (each point represents a pooled habitat sample of 5 single units); overlay: substrate types, partly combined (left), Danube reaches (1=Upper, 2=Middle, 3= Lower Danube reach), (right); final stress for 3-d solution: 16.7, final instability: 0.00338, iterations: 250; red vector: correlation between substrate type, Danube reach and the number of invasive Crustacea (cutoff value r^2=0.30)

The number of significant indicator taxa per taxonomic group for the defined substrate types are presented in Figure 56.

Organic habitats provide the highest numbers of indicator taxa, whereas Diptera, as the most frequent taxa group along the Danube, are dominating. The highest diversity of indicators was found in samples of roots/woody debris representing 19 taxa. Coarse lithal substrates like meso- and macrolithal as well as rip-rap comprise 4 indicators in total only, whereas rip-rap is preferred only by two taxa groups. Indicators of the sensitive group of EPT-Taxa were allocated to roots/woody debris and meso-/macrolithal.

In a nutshell, organic habitats share a highly diverse indicator fauna compared to lithal habitats, especially artificial substrates as rip-rap which presence is correlated with the number of invasive Crustacea (see Figure 55).

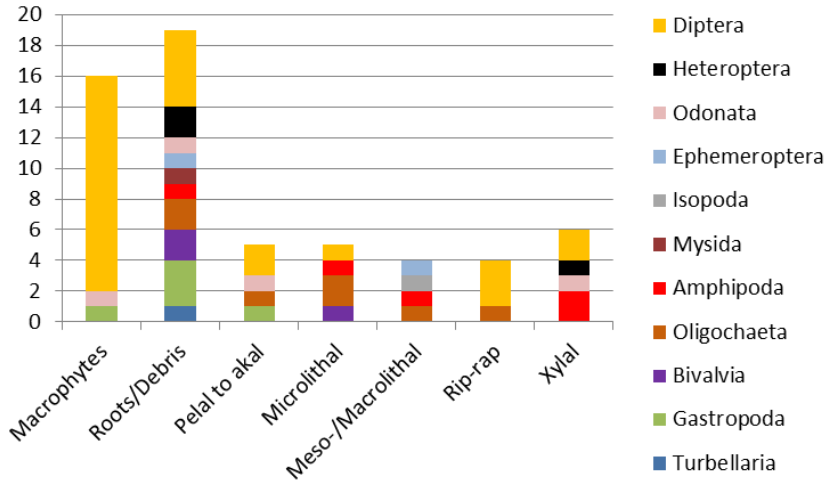

Figure 55: Significant indicator species per substrate type

Neozoa taxa reach highest average densities on hard substrates (mostly due to the mud shrimp *Chelicorophium* sp.) like meso- and macrolithal, rip-rap and xylal; highest species numbers are found in organic habitats like macrophytes and roots/woody debris (Figure 56).

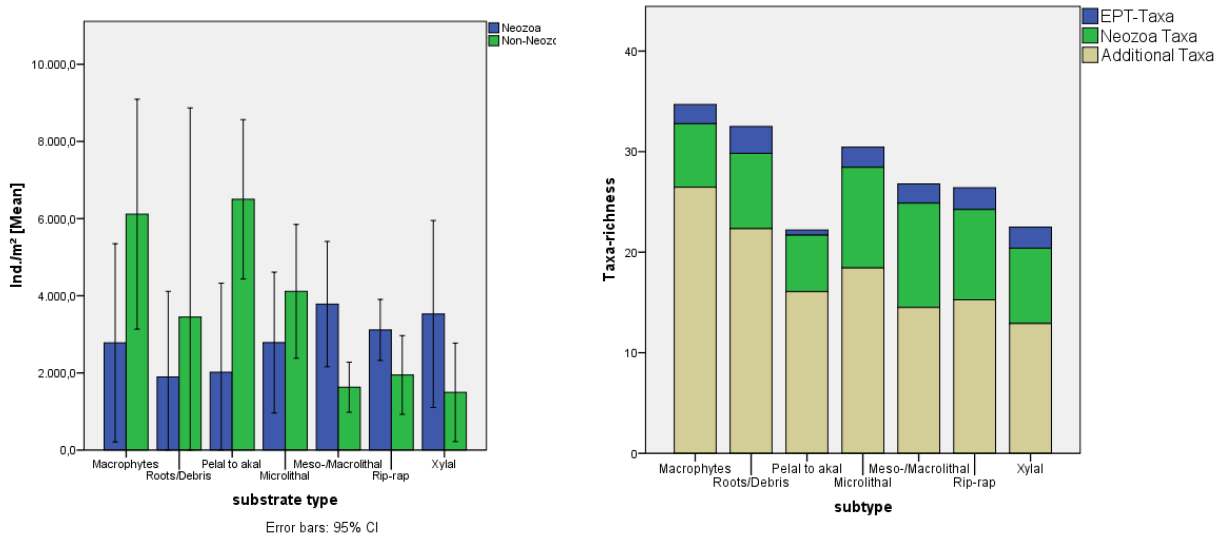

Figure 56: Average density of neozoa and indigenous taxa on different substrate types (left); Taxa richness and substrate type (right)

A more detailed analysis per section type and reach with a comprehensive splitting into all substrate types with detailed information about the indicator taxa is given in Full Report on macrozoobenthos on the attached CD.

5.3.3 Comparative analysis of the different applied methods

Large rivers consist of two distinct habitats: a lentic riparian zone and a much wider, non wadeable deep water area with higher water current. While margin habitats reveal more local conditions, the lotic environment tends to be shaped by the whole catchment. MHS and K&S were performed in the wadeable zones, DWS focused on the deeper, lotic habitats.

A comparison of the three sampling methods of JDS3 is given in Figure 57.

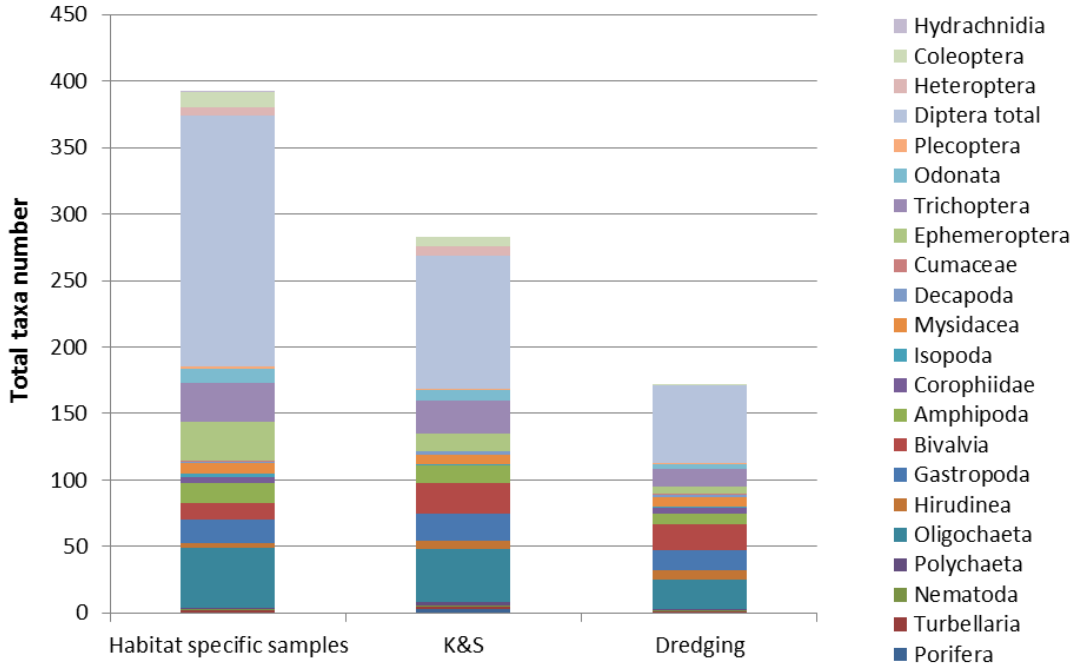

Figure 57: Number of taxa per taxonomic group recorded by habitat specific sampling method, K&S and Dredging

Less taxa were detected in the lotic deep water region (DWS) than either by MHS or K&S sampling in the littoral wadeable zone. This can be explained by the fact that deep water sections of large rivers are generally less densely and diversely colonized mostly caused by instable sediment conditions (Moog et al., 2000; CSÁNYI et al. 2012).

These results are confirmed by comparing MHS data from JDS3 with the Airlift data from JDS2 Figure 58 (left). The number of taxa shared by both methods is 220 only, which is quite low compared to the total taxa number. It indicates that each method provides a unique fauna – a deep-water fauna and a riparian related fauna. The allocation of the samples into the 3 main Danube reaches shows comparable accuracy; faunas from both methods indicate a similar gradient regarding longitudinal zonation (Figure 58, right).

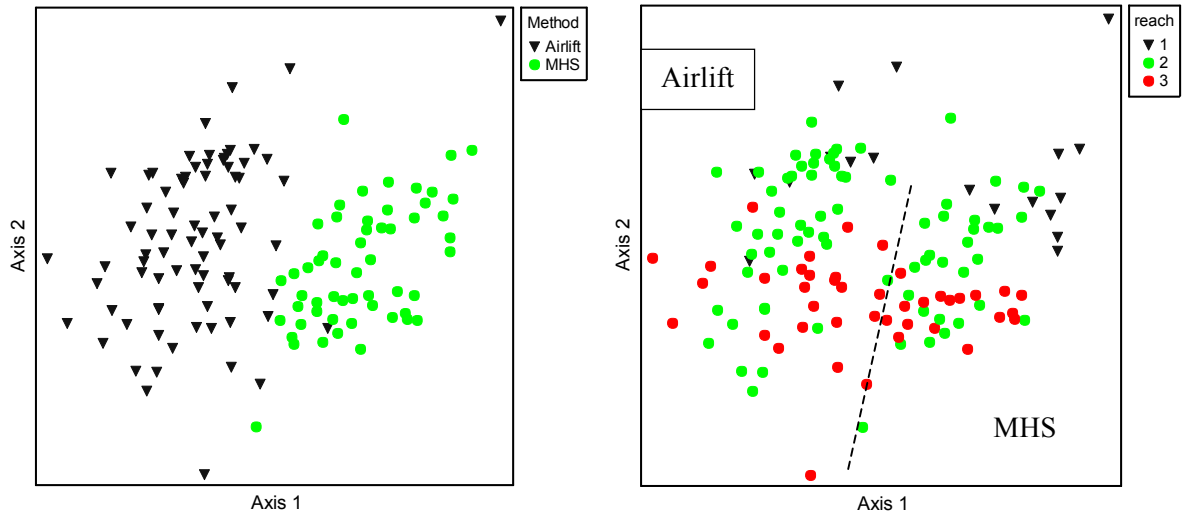

Figure 58: NMS scatterplot based on taxa assemblages of the Airlift method (JDS2) compared to MHS data (JDS3); overlay: sampling method (left), Danube reaches (right); final stress for 3-d solution: 14.56, final instability: 0.000, iterations: 194

Neale et al. (2006) compare the effectiveness and suitability (regarding the assessment system of Great Britain) of available techniques for sampling invertebrates in deep rivers (airlift, dredge, margin

samples and long-handled pond net). They recommend the air-lift as the most suitable method but explicitly state: "to permit the effective assessment of river quality at deep water sites, sampling activity should target deep water habitats and margin habitats".

This is underlined by findings of JDS3. The combination of all habitat-specific approaches provides a more comprehensive insight in the faunal composition of a specific site for large lowland rivers. As JDS3 focuses equally on issues like ecological status, biodiversity and documentation of invasive species the precise study objectives are prerequisite for methodological recommendations.

Further discussion on all three spatial aspects of the macroinvertebrate community collected by the different sampling methods is provided in the Full Report on macrozoobenthos on the attached CD.

5.3.4 WFD-compliant criteria for assigning the ecological status

The lack of appropriate methods to assess the ecological status in large rivers like the Danube is a fundamental obstacle in implementing the WFD compliant monitoring (Birk, 2003). In the past the river quality was mainly evaluated by assessing organic pollution. To achieve the demands of the WFD for an integrated biological assessment of macroinvertebrates and to assess the ecological status of a water body, further attributes of the species assemblage have to be considered and evaluated.

As already applied and proved in several EU member states a modular assessment system is recommended (Ofenböck et al., 2010; Hering et al., 2004; Birk et al., 2012) for the biological quality indicator 'benthic invertebrates' based on:

1. the assessment of **organic pollution** (saprobic condition) and
2. the assessment of the **general degradation** (hydromorphological and hydrological impact like damming, impoundment etc.) e.g. using multimetric indices (MMI) or predictive models.

5.3.4.1 Organic pollution

For monitoring the organic pollution the saprobic system has a long tradition – the WFD compliant implementation of this system is based on the deviation of the Saprobic Index from saprobic reference conditions (Stubauer & Moog, 2003; Ofenböck et al., 2010; Rolauffs et al., 2003). It has to be clearly pointed out that a WFD compliant assessment of the ecological status based exclusively on saprobic indices can provide only a rough indication of the status as several other pressures are not revealed by assessment tools based on saprobic systems.

The data gathered by MHS method (JDS3) were analysed using all available national systems of saprobic indices and transferred to water quality classes and are given for each single site investigated during both surveys in comparison with Airlift from JDS2 (Table 7). During JDS3 all saprobic classes from high to bad status were assessed. Serious organic pollution was detected upstream Novi-Sad (indicating bad status). Saprobically "poor status" was indicated upstream Drava, downstream Velika Morava and at Vrbica/Simjan in the Irongate reservoir.

In some cases questionable results – underlined by a statistically under-represented number of total taxa – were obtained due to rising water level (Table 7, indicated by italics).

A proportion of 73% (=40 sites) of all 55 sampling sites can be classified as "indication of good ecological status", nine sites (16%) as "indication of moderate ecological status" and two sites (4%) actually as "high ecological status" according to the WFD.

During JDS2, the highest values of Saprobic Indices indicating serious organic pollution (poor status) were detected downstream Pancevo and at Giurgeni. Regarding organic pollution 74% (=58 sites) of all 78 sampled Danube sites were classified as "indication of good ecological status" according to the WFD. For eight sites the SI showed an "indication of moderate ecological status", for three sites "poor ecological status" and for nine a "high ecological status" was indicated.

Compared to the JDS2 data, the proportions of sites per status class are generally comparable, although a change of the quality class is detected at certain sites. About 60% of the shared sampling sites at both surveys indicate the same status; at 12% of the sites a better ecological status is indicated and at 28% of the sites a worse status. This must not be interpreted as an aggravation of organic

pollution; it is a result of the applied methodologies: Airlift samples are usually taken at higher depths in lotic parts of the river which are colonised by a different fauna than riparian zones. Saprobic Indices of both faunas (riparian and lotic) show a similar range but abundances of saprobic indicators are different regarding the two methods (Figure 59) leading to deviations of the overall ecological status. In a case study at the Austrian Danube Moog et al. (2000) found similar results comparing Saprobic Indices from cross-sectional samples.

As mentioned earlier riparian habitats provide information on more local conditions, deep water areas reveal the overall characteristics. Both habitats are essential for ecological processes and the functioning of the ecosystem. We therefore propose a worst-case approach to overcome this dilemma and to include indications in a holistic way.

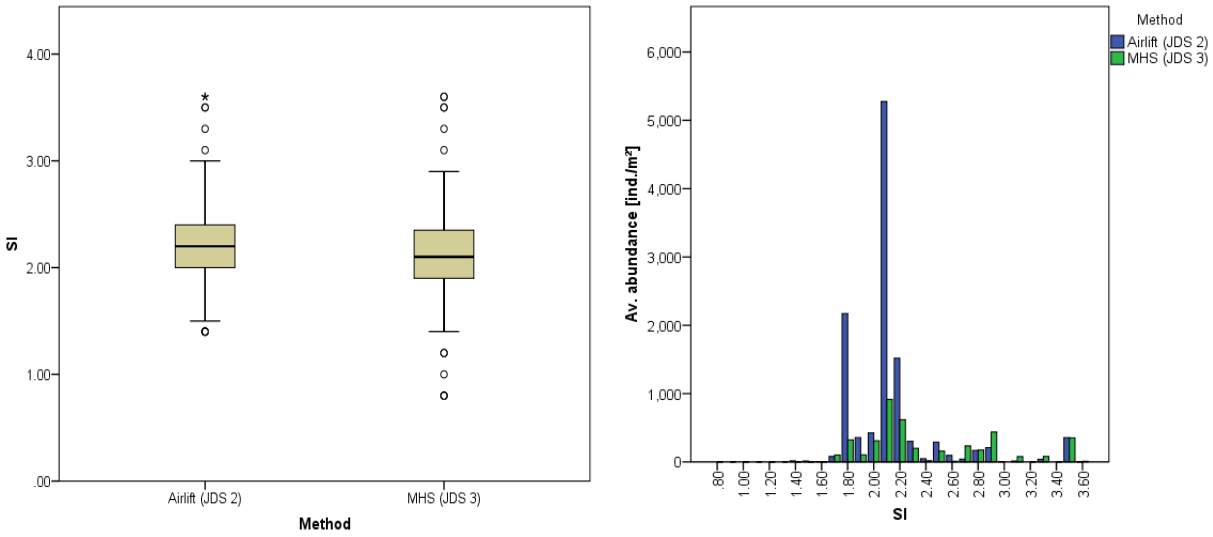

Figure 59: Boxplots of Saprobic Indices of all classified taxa found during JDS2 by Airlift method and JDS3 by MHS method (left); average abundances [ind./m²] of taxa per Saprobic Index class of all samples per method (right)

5.3.4.2 General Degradation

The results of the Slovak method for large rivers applied for the JDS3 MHS-data (Table 7) indicate quite balanced ecological classes of good (26 sites) and moderate (27 sites) status. Only Klosterneuburg indicates class 1 (high status) and site 32 upstream Novi-Sad class 4 (poor status). The results are thoroughly comprehensible as the sampling site Klosterneuburg provided a high variation of different substrate types and current velocity classes and therefore a diverse fauna sharing a comparatively high number of (EPT-) taxa. At Novi-Sad the Saprobic Index already indicated an alteration compared to other sites.

On the basis of this method the morphological high degraded sites (channelized or impounded, with rip-rap dominating at the shore zones) in the Upper Danube reach indicate moderate status, while sites with less morphological impact, providing adequate gravel banks, indicate generally good status. The parameter saprobity only indicates quite constantly a good status in the Upper reach not capturing hydromorphological degradation. The results implicate that the general degradation of large rivers can be largely covered by this assessment method. A compatibility of the Slovak method in the Lower Danube reach has to be further tested and possible adaptations of boundary values have to be critically revised due to the fact that the environmental conditions show a distinct change along the Danube stretch and deviate considerably from reference conditions used by the Slovak method.

Marković et al. (2012) report on moderate ecological status at 7 sampling sites along the Iron Gate reservoir (rkm 849-1,077) by using 7 selected metrics. This partly deviates from the JDS3 results which are ranging between good and poor status (MHS) in this certain stretch.

More details are given in the Full Report, whereas this information could be used to implement a multimetric index in a national assessment method or within the Danube intercalibration process.

Table 7: Saprobic indices (SI) and indication of water quality classes for all Danube sites; results from JDS2 (Airlift) in grey, results from JDS3 (MHS, DWS and the multimetric Slovak method for large rivers (SK)) in black; Country specific Saprobic Indices were applied for the German, Austrian and Slovakian stretch; for all other countries the Romanian SI was calculated; values and indications of water quality based on under-represented (less than 10 taxa for DWS and JDS2 data; less than 27 taxa for Upper Danube reach and less than 20 for Middle and Lower Danube reach following standardised residuals for MHS data) indicator taxa are scientifically questionable and written in italic.

JDS3JDSrkm [JDS2/JDS3]	Site no. [JDS2/JDS3]	Sampling Site	Saprobic basic condiciton	JDS2 Airlift		JDS3 MHS		JDS3 DWS		SK
				SI	Class	SI	Class	SI	Class	Class
2599.8 /	1 /	Donaurieden	1.65	1.94	II					
/ 2581	/ 1	Böfinger Halde	1.75			2.08	II			2
2412.4 / 2415	2 / 2	Kelheim – gauging station	1.75	2.23	II	2.14	II			2
/ 2365	/ 3	Geisling power plant (upstream)	1.75			1.94	II	2,19	II	3
2353.5 /	3 / 3A	Geisling power plant (downstream)	1.75	2.2	II	1.88	II	2,15	II	3
2287 / 2285	4 / 4	Deggendorf	1.75	2.18	II	1.93	II	2,14	II	3
2278 /	5 /	Niederalteich	1.75	2.16	II					
/ 2258	/ 5	Mühlau	1.75			1.90	II	2,10	II	2*
2203,5 / 2205	7 / 6	Jochenstein	1.75	2.31	III	2,33	III	2,95	*IV*	4
2120,5 / 2121	8 / 7	Upstream dam Abwinden-Asten	1.75	2.12	II	2.18	*II*	2,11	*II*	*3*
2062 /	9 /	up. KW Ybbs/Persenbeug	1.75	2.2	II					
2007.5 / 2007	10 / 8	Oberloiben	1.75	1.87	II	2.00	II	2,02	II	3
1950.6 /	11 /	Greifenstein	2.00	2.54	III					
1942 / 1942	12 / 9	Klostemeuburg	2.00	1.84	I	2.06	II	2,19	II	1
1895 / 1895	13 / 10	Wildungsmauer	2.00	1.83	I	2.03	II	2,12	II	2
1881.9 / 1882	14 / 11	Upstream Morava (Hainburg)	2.00	1.95	I	2.02	*II*	2,16	II	2
/ 1868	/ 13	Bratislava	2.00			2.20	*II*	2,25	*II*	2
1865 / 1865	16 / 13A	Bratislava (downstream)	2.00	2.27	II	2.30	II	2,23	II	2
1851.5 / 1855	17 / 14	Gabcikovo resevoir	2.00	2.3	II	2.27	II	2,25	II	2
1806 / 1806	18 / 15	Medvedov/Medve	2.00	2.09	II	2.03	II	2,20	II	2
1794 /	19 /	Mosoni Danube	2.00	2.84	IV					
/ 1790	/ 17	Klizska Nema	2.00			2.05	II	2,24	II	2
1768 /	20 /	Komarno	2.00	2.11	II					
1761 / 1761	22 / 19	Iza/Szony	2.00	2.09	II	2.13	II	2,08	II	2*
1719 /	23 /	Esztergom	2.00	2.12	II					
1707 / 1707	26 / 20	Szob	2.00	2.11	II	2.12	II	2,02	II	2
1692 /	27 /	Szetendre Island	2.00	2.11	II					
1692 /	28 /	Szetendre Island arm	2.00	2.15	II					
1659 / 1660	29 / 21	Budapest upstream – Megyeri Bridge	2.00	2.07	II	2.16	II	2,05	II	3
1658 /	30 /	Budapest up. Sidearm	2.00	2.09	II					
1632 /	31 /	Rockere-Sorokser Sidearm	2.00	2.31	II					
1632 / 1630	32 / 22	Budapest downstream – M0 bridge	2.00	1.94	I	2.44	III	2,08	II	3
1598 /	33 /	Adony/Lorev	2.00	2.12	II					
1586 /	34 /	Rockere-Sorokser Arm end	2.00	2.28	II					
1560 / 1560	35 / 24	Dunafoldvar	2.00	2.06	II	2.13	II	2,38	II	2
1533 / 1532	36 / 25	Paks	2.00	2.26	II	2.24	II	2,11	II	2
1481 / 1481	38 / 26	Baja	2.00	2.35	II	2.06	II	2,01	*II*	2*
1434 / 1434	39 / 27	Hercegszanto	2.00	2.23	II	2.17	*II*	2,05	*II*	*3*
1424 /	40 /	Batina	2.00	2.13	II					
1384 / 1384	41 / 28	Upstream Drava	2.00	2.2	II	3.05	*IV*	2,03	*II*	*3*
1367 / 1367	43 / 30	Downstream Drava (Erdut/Bogojevo)	2.00	2.17	II	2.51	III	2,16	*II*	3
1355.3 /	44 /	Dalj	2.00	2.2	II					
1300 / 1300	45 / 31	Ilok/Backa Palanka	2.00	2.13	II	2.27	II	2,14	II	3
1262 / 1262	46 / 32	Upstream Novi-Sad	2.00	2.25	II	3.32	V	2,00	II	4
1252 / 1252	47 / 33	Downstream Novi-Sad	2.00	2.15	II	2.33	II	2,01	II	3
1216 / 1216	48 / 34	Upstream Tisa (Stari Slankamen)	2.00	2.16	II	2.41	*III*	2,10	II	*3*
1200 / 1199	50 / 36	Downstream Tisa/Upstream Sava	2.00	2.11	II	2.03	*II*	2,01	II	*2*
/ 1159	52 / 38	Upstream Pancevo/Downstream Sava	2.00	2.22	II	2.12	II	2,13	*II*	3
/ 1151	53 / 39	Downstream Pancevo	2.00	3.09	IV	2.41	III	2,10	II	2
/	54 /	Grocka	2.00	2.29	II					
/ 1107	55 / 40	Upstream Velika Morava	2.00	2.26	II	2.62	III	2,48	III	2
/ 1095	57 / 42	Downstream Velika Morava	2.00	2.27	II	2.86	IV	2,00	II	3
/	58 /	Starapalankaram	2.00	2.43	III					

JDS3JDSrkm [JDS2/JDS3]	Site no. [JDS2/JDS3]	Sampling Site	Saprobic basic condiciton	JDS2 Airlift SI	JDS2 Airlift Class	JDS3 MHS SI	JDS3 MHS Class	JDS3 DWS SI	JDS3 DWS Class	JDS3 SK Class
/ 1073	59 / 43	Banatska Palanka/Bazias	2.00	2.15	II	2.36	II	2,00	II	2
/ 1040	60 / 44	Irongate reservoir (Golubac/Koronin)	2.00	2.58	III	2.35	II	2,00	II	2
/	61 /	Donij Milanovac	2.00	2.69	III					
/ 956	62 / 45	Irongate reservoir (Tekija/Orsova)	2.00	2.44	///	2.67	III	2,44	III	3
/ 926	63 / 46	Vrbica/Simijan	2.00	2.47	III	3.02	IV	2,16	II	3
/	64 /	Irongate II	2.00	2.13	II					
/ 847	65 / 47	Upstream Timok (Rudujevac/Gruia)	2.00	2.21	II	2.39	II	2,26	II	3
/ 837	67 / 49	Pristol/Novo Selo Harbour	2.00	2.13	//	2.08	II	2,05	II	2
/	68 /	Calafat	2.00	2.26	II					
/ 686	69 / 50	Downstream Kozloduy	2.00	2.29	II	2.02	II	2,01	//	2
/	70 /	up. Iskar	2.00	2.06	//					
/	72 /	ds. Iskar	2.00	1.78	/					
/	73 /	up. Olt	2.00	2.14	//					
/ 604	75 / 52	Downstream Olt	2.00	1.9	/	2.36	II	2,09	II	2
/	76 /	ds. Turnu Magurele	2.00	1.93	/					
/ 550	77 / 53	Downstream Zimnicea/Svishtov	2.00	2.38	//	2.27	II	2,01	II	3
/ 532	79 / 55	Downstream Jantra	2.00	2.32	II	2.00	I	2,01	II	2
/	80 /	up. Ruse	2.00	2.18	//					
/ 488	82 / 57	Downstream Ruse/Giurgiu	2.00	1.48	/	2.00	/	2,03	//	3
/	83 /	up. Arges	2.00	2.1	//					
/ 429	85 / 59	Downstream Arges. Oltenita	2.00	1.81	/	2.12	II	2,03	II	2
/ 375	86 / 60	Chiciu/Silistra	2.00	2.76	III	2.04	II	2,00	II	3
/	87 /	ds. Crnavoda	2.00	2.16	II					
/ 232	88 / 61	Giurgeni	2.00	3.15	IV	2.49	III	2,02	//	3
/ 170	89 / 62	Braila	2.00	2.23	//	2.12	II	2,34	II	3
/ 132	92 / 65	Reni	2.00	2.16	//	2.19	//	2,00	II	3
/ 18	93 / 66	Vilkova – Chilia arm/Kilia arm	2.00	2.24	II	2.72	III	2,01	II	3
/	94 /	Bystroye Canal	2.00	2.15	//					
/ 31	95 / 67	Sulina – Sulina arm	2.00	2.16	II	2.01	//	2,05	II	3
/ 104	96 / 68	Sf.Gheorghe – Sf.Gheorghe arm	2.00	2.11	//	2.08	II	2,00	//	2*

* EQR values close to thresholds (≤ 0.01 points) are rounded up to the next best status class

5.4 Conclusions

During JDS3 samples were taken at wadeable and riparian areas (MHS and K&S), as well as in deeper parts (DWS) of the river at 55 sites along the Danube stretch. According to the different sampling methods the following main conclusions are stated:

General characteristics of the Danubian Fauna

- Altogether 460 macroinvertebrate taxa were identified by means of all used sampling techniques.
- Insects, with 319 taxa, were the dominant component of the communities. Diptera were the richest insects order with 222 taxa, with 200 species belonging to the family Chironomidae. In terms of abundance, Diptera play an essential part in the Upper Reach and decrease downstream.
- Amphipoda (mostly invasive Corophiidae) are the dominant group in all Danube reaches and increase downstream, while
- Oligochaeta and Mollusca were found in increasing numbers in the Middle and Lower Reach, whereas the Asian clam *Corbicula fluminea* occurs in high densities.
- Higher abundances of EPT- Taxa (Ephemeroptera, Plecoptera and Trichoptera) are restricted to the upper stretch, whereas Trichoptera show the highest abundances within these sensitive groups. Regarding aquatic insects Chironomidae play a major role along the entire Danube stretch.

- Highest taxa-richness was recorded with the MHS-approach. Some species were detected only in the middle region of the river bed on the lowest part of the Danube by dredging: *Paramysis ullskyi*, *Schizoramphus scabriusculus*, *Niphargoides spinicaudatus.*

Methodology

- The MHS method is especially applicable for ecological status assessment of large rivers at low water period: it is standardized, stressor-specific and habitat-oriented.
- K&S and diving method can provide additional information particularly on mussel populations inhabiting deeper zones next to the bank.
- DWS is not affected by water level and discharge so much and is appropriate for data collection from all of deep parts and habitats of a large river. Carefully operation of the dredge can provide semi-quantitative data.
- Regarding detailed surveys of Mollusca a detailed habitat monitoring in the field is necessary.

Saprobiological assessment

- The different methodological approaches produce clearly different datasets leading to different assessment results. While Saprobic Indices from riparian habitats (obtained K&S and MHS) are largely comparable, DWS collates more lotic faunas associated with lower Saprobic Indices resulting in a better ecological status. To overcome this phenomenon a worst-case approach of deep water and riparian sampling is applied.
- Saprobic Indices and based on that, water quality status class per site, are comparable to the JDS2 data.
- Regarding Saprobity in total 73% of 55 sampled sites in 2013 can be classified as “indication of good ecological status”, 15% of the sites as “indication of moderate ecological status” and 4% actually as “high ecological status” according to the WFD. This proportion is similar to the JDS2 results.
- Serious organic pollution was identified upstream Novi-Sad (bad status). Saprobically “poor status” was indicated in Jochenstein, upstream Drava, downstream Velika Morava and at Vrbica/Simjan in the Irongate reservoir.

General degradation

- On the basis of the Slovak assessment method for large rivers, the morphologically high degraded sites (channelized or impounded, with rip-rap dominating at the shore zones) in the Upper Danube reach indicate moderate status, while more natural sites at the Upper and Middle Danube reach indicate generally good status.
- These results implicate that the general degradation of the main channel of large mountainous rivers can be roughly covered by this assessment method.
- Compatibility of this method in the Lower Danube reach has to be further tested as substrate composition differs considerably from the Middle Danube.
- Additionally the inclusion of WFD- compliant assessment methods based on biological quality elements of associated floodplains of large rivers, is needed in respect of a holistic aquatic ecosystem approach.

Habitat preferences of indicators with implications on management actions

- As habitat degradation is one main stressor of large rivers the preferences of taxa were one main focus of JDS3. Organic habitats provide the highest numbers of indicator taxa. The highest diversity of indicators was found in samples of roots/woody debris.
- Coarse lithal substrates like meso- and macrolithal as well as rip-rap comprise only four indicator taxa in total, whereas rip-rap is preferred by only two taxa groups.
- Indicators of the sensitive group of EPT-Taxa (Ephemeroptera, Plecoptera, Trichoptera) were allocated to roots/woody debris and meso-/macrolithal.
- Invasive crustaceans show high affinities to stabile substrates, especially rip-rap.

The following topics are discussed in the Full report: on macrozoobenthos on the attached CD:

- Longitudinal, sectional and cross sectional change of the main taxonomic groups based on comparative analysis of results gained by different sampling methods
- Comments and conclusions about the Danube typology
- Analyses of the indicative power of selected taxa groups regarding organic pollution and habitat preferences
- Analyses of the distribution of Crustacea

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6 Phytobenthos

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6.1 Introduction

Benthic algae (periphyton or phytobenthos) are the most successful primary producers in aquatic habitats. They are widely considered to be the main source of energy for higher trophic levels in many, if not most, unshaded temperate region streams (e.g., Minshall, 1978, Lamberti, 1996). In large rivers, the leading role in primary production is governed by phytoplankton (Vannote et al., 1981). The specific conditions in such river types favour phytoplankton development and the algal biofilms are often restricted to the littoral zone because of limited light availability and high turbidity of the flow. Therefore, studies on phytobenthos from large rivers naturally refer to the river-bank area respectively visible and suitable for collecting samples. Nevertheless, phytoplankton as bioindicator mirrors environmental conditions in flows in short term, whilst attached benthic algae that are exposed to fluctuations of environmental factors and water chemistry within a period of time reflect a long-term status of aquatic health.

Phytobenthos together with macrophytes are identified as Biological Quality Element under the European Water Framework Directive (2000/60/EC), and as such need to be monitored to identify anthropogenic influences on aquatic ecosystems. Especially in the rivers, phytobenthos is considered to be a suitable parameter to determine the impact of nutrient pollution. Organisms are generally sessile and therefore reflect to the nutrients enrichment as well as to other pollution.

In the Danube, nutrients have been identified as an important anthropogenic pressure threatening the quality of the river water (ICPDR, 2009). In such conditions, benthic algae are an essential component of all bio assessment studies.

6.2 Methods

6.2.1 Sampling and sample processing

For phytobenthos sampling, a river segment (usually up to 50 m long) with a suitable substrate (preferably cobbles) was chosen at each sampling site. Diatom sampling followed instruction of the CEN 13946 (2003), non-diatoms sampling was carried out according to CSN EN 15708 (2009).In principle, at least five stones occurring in the current (if possible) and euphotic zone down to 1m of depth (preferably cobbles with a diameter between 64 to 256 mm) were used for sampling and chl-*a* measurements. Where hard substrata were absent, epiphyton was sampled following the CEN 13946 (2003), CSN EN 15708(2009) and Slovak Standard STN 757715. On the stones selected, first chlorophyll-a concentration was measured in situ (see below for details). After the measurements, a minimum area of 10 cm^2 was brushed thoroughly from each stone (as much concentrated as possible) into two containers (for diatoms and non-diatoms analyses) and labelled. Samples for benthic diatoms analyses were preserved by formaldehyde (final solution of 1 – 4%). Samples for non-diatoms analyses were refrigerated and analysed alive on-board. If any macroscopic algae were observed at site (e.g. *Cladophora, Hydrodiction*), a separate subsample was taken for easier determination. Diatom samples were further treated following the European standards CEN 13946 (2003) and CEN 14407 (2004). The diatom samples were treated by hot

hydrogen peroxide method to obtain clean frustule suspensions. Finally, the oxidised samples were rinsed with deionised water by decantation of the suspension several times, and permanent slides were mounted with Naphrax®.

6.2.1.1 Biomass measurements

Quantification of phytobenthos biomass has been done in situ on natural substrate by fluorescence fingerprint measurements using the BenthoTorch® (bbe Moldaenke) provided by Benten Water Solution (The Netherlands). On each of five or more stones (cobbles) five sub-areas were measured to obtain sufficient data of chlorophyll-*a*. Three main algal groups were distinguished: diatoms, green algae and cyanobacteria. For each of these groups and for total benthic algal biomass, the chlorophyll-a level was determined in µg/cm^2.

6.2.1.2 Microscopic analyses

After sampling the microscopic analysis of non-diatom community has been performed using light microscopy at 400 x – 1000 x magnification. All taxa were identified to the lowest taxonomical level possible. The taxa identified were quantified on the scale 1 – 5 (1: rare, 5: dominant). Diatom samples were further treated following the European standards CEN 13946 (2003) and CEN 14407(2004). Diatoms were analysed from permanent slides. On average, 400 valves were counted on each slide in random transects with a Zeiss scope A1 (Axio) microscope with 100x oil immersion objective. A list of taxa data was made from each slide and the counts were used to calculate species relative abundance (in%).

6.2.2 Data treatment

The diatom species data were processed with the OMNIDIA ver. 5.3 (Lecointe et al 1993, 1999, http://clci.club.fr/index.htm) to calculate all the 18 diatom indices included in the software. The community structure was further explored by calculating the proportion of species belonging to three ecological guilds (low profile, high profile and motile) adopted from Passy (2007) and Berthon et al. (2011) and to two life forms (planktonic, benthic).

6.2.3 Statistical methods

Results of the chlorophyll-*a* measurements and analyses of species composition of non-diatoms and diatoms were treated separately. Environmental variables were standardized and log-transformed before the statistical analysis. For species-based statistical analysis of non-diatoms, only 43 taxa were taken into account. For diatoms, only diatom taxa reaching a relative abundance of more than 3% in at least one sample were included in the statistics (86 taxa in total). Diatom species data were arcs in square root transformed prior to any statistical analysis, non-diatoms were not transformed. Appropriate tests for normality were conducted using STATISTICA 10 (StatSoft Inc., 2011) on all environmental and biological data. In total, 21 explanatory variables were treated for statistical analysis and comprised data on water chemistry (conductivity, temperature-t, pH, dissolved oxygen-O_2, total nitrogen-TN, total phosphorus-TP, nitrates- N-NO_3, phosphates-P-PO_4, potassium-K, calcium-Ca, sodium Na and dissolved organic carbon-DOC) and hydromorphological variables (discharge-Q, slope, mean velocity, suspended solids) and general descriptors such as river kilometre (rkm) and 10 Danubian types (Moog et al. 2004) as follows Type 1: 2581 rkm, type 2: 2415 – 2258 rkm, type 3: 2204 – 2008 rkm, type 4: 1942 – 1790 rkm, type 5: 1761 – 1533 rkm, type 6: 1481 – 1097 rkm, type 7: 1071 – 954 rkm, type 8: 926 – 378 rkm, type 9: 235 – 130 rkm, type 10: 107 – 26 rkm).

The chlorophyll-a content was correlated with environmental variables (water chemistry, river kilometres and hydromorphology) in order to identify the relationships between the algal biomass and environmental factors. Spearman correlations were applied using STATISTICA 10 (StatSoft Inc., 2011).

The variance in diatom and non-diatom community regardless the environmental variables was explored by Detrended Correspondence Analysis (DCA, Hill and Gauch, 1980) in order to test the respond of species composition on the environmental gradients. The DCA was made using PC-ORD v. 6 (McCune and Mefford, 1999), rare taxa were down weighted and the randomization test was

performed with 999 runs. The DCA gave a gradient lengths of 3,382 SD (Axis 1 of non-diatoms) and of 2,199 for Axis 1 and of 2,475 for Axis 2 of diatoms. A consequent Canonical Correspondence Analysis was performed on both algal sets (non-diatoms and diatoms) to describe the relationships between and among the diatom species composition at sites and environmental variables. The CCA was run with manual forward selection, Monte Carlo permutation tests (full model, n=999) and Bonferroni correction of the significance levels to determine the factor significantly contributing to the model. Hill's scaling was chosen with focus on inter-sample distances. Manual selection and Monte Carlo permutation test (999 runs) were used to reduce the environmental variables to those correlated significantly with the derived axes, at a cut-off point of P=0.05. Hill's scaling was selected with inter-sample distances.

Relationships between the diatom metrics (diatom indices, diatom guilds and life forms) and all environmental variables (general, physico-chemical and hydromorphological) were assessed with the non-parametric Spearman correlations using STATISTICA 10 (StatSoft Inc., 2011). We further used a non-parametric Kruskal-Wallis one-way analysis of variance by ranks to evaluate whether the diatom indices differed significantly between the different Danubian types (N=108). The Kruskal-Wallis ANOVA by Ranks was performed using STATISTICA 10 (StatSoft Inc., 2011).

6.3 Results

6.3.1 Phytobenthos biomass

A total of 108 samples was evaluated for chlorophyll-*a* concentration on the hard substrate. The values of the total chlorophyll-a measured in situ varied between 0.06-7.19µg/cm^2 (Figure 60). The highest values were detected in the Upper Danube down to the station JDS10 (rkm 1895) and started to increase again at JDS40 (rkm 1107).

The phytobenthos structure evaluated via chlorophyll-*a* content was mainly formed by cyanobacteria and diatoms, green algae created only a minor part of the biofilm (Figure 60). The cyanobacteria reached more than 50% of proportion in 52 samples, whilst diatoms prevailed in 37 samples. In general, diatoms prevailed in the upper Danube (down to JDS10 – 1895 rkm).

With regard to the relationships of the chlorophyll-a concentration with other environmental variables, the Spearman correlations showed that it is most significantly related to the concentrations of suspended solids (Tab. 8). The negative correlation coefficient indicates that higher concentrations of suspended solids impede the phytobenthos development. This caused the low values of chlorophyll-*a* concentrations at sites in the type 6, which were proved to contain significant amounts of suspended solids. Furthermore, the chlorophyll-a was significantly positively correlating with phosphates and dissolved organic carbon (Tab. 8).

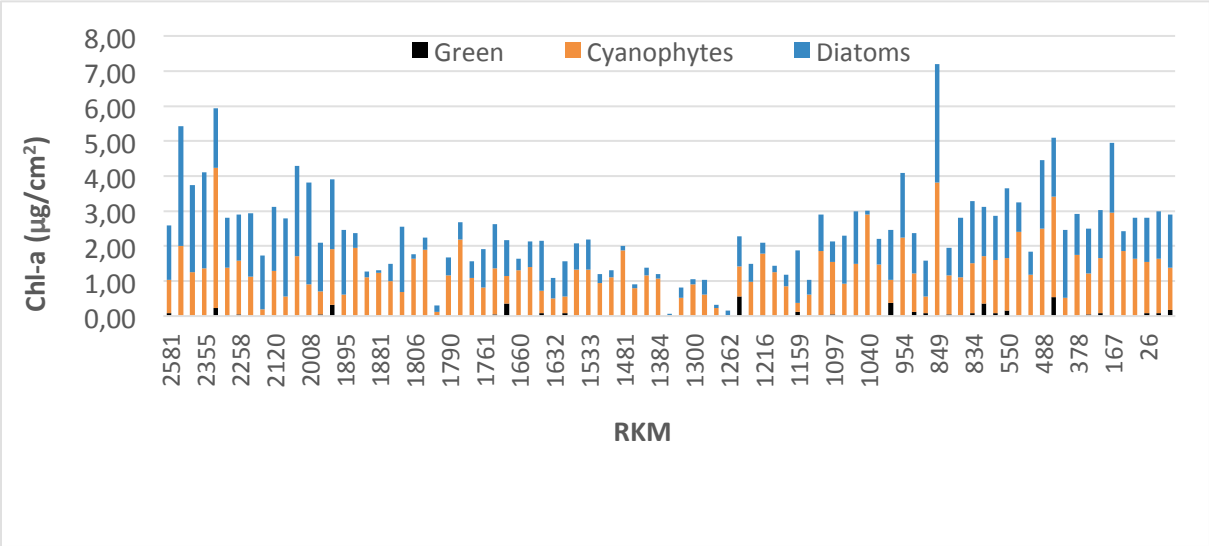

Figure 60: The total biomass of chlorophyl-*a* (µg/cm²) and distribution of different algal classes (green algae, cyanobacteria, diatoms) among the sites investigated. River kilometres refer to the sites investigated. Data from tributaries are not involved

6.3.2 Algal species composition

6.3.2.1 Non-diatoms

In total 68 taxa were identified in 108 non-diatom samples of non-diatom community. Non-diatom species diversity was mainly created by species of cyanobacteria (Cyanophyta), green algae (Chlorophyta) and red algae (Rhodophyta). Together 40 taxa of **cyanobacteria** were found in the samples from the Danube and the tributaries, represented by mainly **filamentous** genera such as *Calothrix* Agardh ex Bornet et Flahault, *Heteroleibleinia* (Geitler) L. Hoffmann, *Homeothrix* (Thuret ex Bornet et Flahault) Kirchner, *Leptolyngbya* Anagnostidis et Komárek, *Lyngbya* C. Agardh ex Gomont, *Oscillatoria* Vaucher ex Gomont, *Phormidium* Kützing ex Gomont, *Stigonema* Agardh ex Bornet et Flahault. **Coccal cyanobacteria** were observed as well, mainly *Chroococcus* Nägeli, *Chamaesiphon* Braun, *Geitlerinema* (Anagnostidis et Komárek) Anagnostidis, *Geitleribactron* Komárek, *Pleurocapsa* Thuret in Hauck, *Stanieria* Komárek et Anagnostidis were present. Among **green algae**, a total of 24 taxa occurred at individual sampling stations. The most abundant filamentous species was *Cladophora glomerata* (Linnaeus) Kützing that was usually accompanying water macrophytes. *Hydrodictyon reticulatum* (Linnaeus) Bory de Saint-Vincent, *Oedogonium* Link ex Hirn sp. and *Spirogyra* sp. Link were abundant in the shallow poles of the Danube River. Downstream of Novi Sad *Pseudendoclonium basiliense* Vischer was found quite often down to the Danube delta together with coccal cyanobacteria. There were three taxa of **red algae** (Rhodophyta) found, *Bangia artropurpurea* (Roth) Aghard, *Hildebrandia rivularis* (Liebmann) Aghard and *Thorea hispida* (Thore) Desvaux.

6.3.2.2 Diatoms

A total of 318 diatom taxa belonging to 62 genera were detected in 108 samples. Among them, only 148 taxa reached a relative abundance of at least 1% at minimum of one site, 86 taxa with a relative abundance over 3% and only 61 species a relative abundance of at least 5%. With regard of the species frequency, only 28 species occurred at more than 50% of sites. The most frequent species detected in more than 75% of samples (more than 81) were *Amphora pediculus* (Kützing) Grunow, *Cocconeis placentula* Ehrenberg, *Cyclotella meneghiniana* Kützing, *Navicula cryptotenella* Lange-Bertalot, *Navicula recens* (Lange-Bertalot) Lange-Bertalot, *Nitzschia dissipata* (Kützing) Rabenhorst, *Nitzschia fonticola* Grunow in Van Heurck, *Nitzschia palea* (Kützing) W. Smith var. *debilis* (Kützing) Grunow in Cleve & Grunow and *Nitzschia palea* (Kützing) W. Smith.

6.3.3 Relationships of algal biofilms with environmental variables

The Canonical Correspondence Analysis on both diatoms and non-diatoms showed that the species composition differed between the Danube types (Figure 61). The different Danube types appeared gradually arranged along the axis 1, which correlated significantly with natural changes in the longitudinal profile (e.g. velocity, slope, oxygen, river kilometres) on one hand and pollutants (e.g. phosphates, DOC, potassium) on the other. Second axis correlated significantly with suspended solids and allowed separation of the type 6 in both cases (Figure 61).

In particular, distribution of non-diatom taxa in the Danube showed to change mainly with river kilometres, velocity, pH, suspended solids, nitrates, phosphates, potassium and DOC (Figure 61). However, the environmental variables tested explained only 21% of the total variance in the non - diatoms data. The two first axes accounted for 57% of the explained variance. The first axis clearly separated sites from the types 1-5 from that reflected the higher velocity and oxygen content from the sites in the types 7-10 in the direction of the gradient of increasing phosphates and potassium.

Similarly, based on diatoms, sites from the upper Danube (types 1-4) were clearly separated along the first axis in the direction of the gradient of river kilometres, slope, oxygen, nitrates and calcium from the types 7-10 that arranged mainly along the gradient of phosphates and potassium. The first axis accounted for 12% of total variance, the second axis explained 7,6% of the data variance. All canonical axes accounted for a total of 37% of the variance in the diatom species data. Second axis correlated with suspended solids and dissolved organic carbon (DOC) and showed to separate the diatom communities of the type 6. These assemblages were distributed in the ordination space mainly along the gradient of suspended solids, which are positively correlated with the proportion of centric diatoms in the samples. The proportion of centrics in samples from the type 6 reached an average relative abundance of 52% with a maximum of 83%, which is the highest proportion in the dataset. These results confirm that the benthic algal communities at sites belonging to the type 6 are significantly influenced by higher rates of suspended solids that greatly increase the proportion of planktonic diatoms in the biofilms.

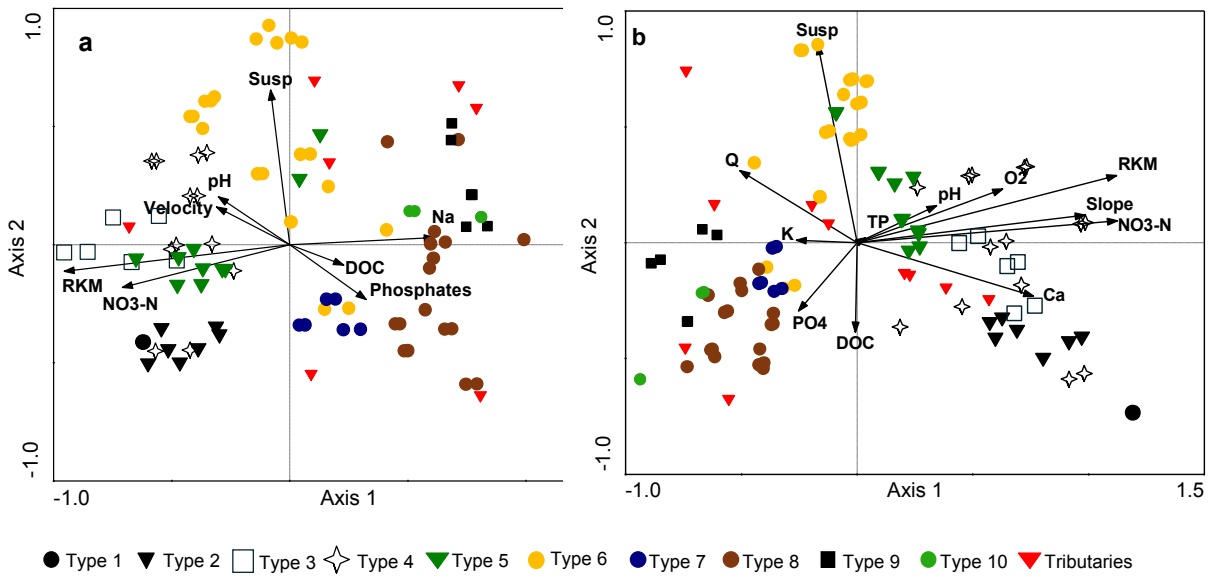

Figure 61: The distribution of samples in the ordination space of a Canonical Correspondence Analysis based on non-diatoms (a) and diatoms (b). The different Danube types and tributaries are differentiated

Table 8: Spearman correlation coefficients between the environmental variables (water chemistry and hydromorphology) and algal descriptors (chl-*a* concentration, diatom indices, diatom guilds and lifeforms). Correlations significant at p>0.05 (*) and p>0.001 (**) are shown. Best performing diatom indices are highlighted. RKM: river kilometre, TN: total nitrogen, TP: total phosphorus, DOC: dissolved organic carbon. Q: discharge, Susp: suspended solids, Diatom guilds: motile, low, high.

Variable	chl-*a* Greens	Chl-*a*Cyano	Chl-*a*Diato	Chl-*a* Total	GENRE	IPS	TID	Motile	Low	High	Planktonic	Benthic
RKM	-0,27*	ns	ns	ns	0,77**	0,64**	0,75**	-0,62**	0,56**	0,64**	ns	ns
Cond.	0,27*	ns	ns	ns	-0,27*	ns	ns	ns	ns	ns	ns	ns
O2	-0,28*	ns	ns	ns	0,46**	0,41*	0,53**	-0,35*	0,34*	ns	ns	ns
pH	ns	ns	ns	ns	0,41*	0,52**	0,41*	ns	ns	ns	ns	ns
T	ns	ns	ns	-0,24*	ns	ns	ns	ns	ns	ns	ns	ns
TN	ns	ns	ns	ns	0,64**	0,35*	0,67**	-0,6**	0,56**	0,45*	ns	ns
TP	ns	-0,32*	-0,25*	-0,36*	0,35*	0,36*	ns	ns	ns	0,33*	ns	-0,29*
Ca	ns	ns	ns	ns	0,38*	0,3*	0,35*	-0,28*	ns	0,28*	ns	ns
Mg	ns	0,23*	ns	0,28*	-0,31*	ns	ns	ns	ns	ns	ns	ns
K	ns	ns	ns	-0,22*	ns	ns	ns	ns	ns	ns	ns	ns
Na	0,33*	ns	ns	0,24*	-0,69**	-0,51**	-0,6**	0,56**	-0,5**	-0,55**	-0,26*	0,33*
NO3-N	-0,27*	ns	ns	ns	0,63**	0,42*	0,78**	-0,56**	0,51**	0,53**	ns	ns
PO4-P	0,24*	ns	ns	0,23*	-0,26*	ns	-0,25*	0,28*	-0,3*	ns	ns	ns
DOC	ns	ns	0,22*	0,25*	-0,35*	ns	-0,34*	0,34*	-0,29*	-0,42*	ns	ns
Q	ns	ns	ns	ns	-0,44*	-0,56**	-0,4*	ns	ns	-0,36*	ns	ns
Velocity	-0,24*	ns	-0,28*	ns	0,49**	ns	0,42*	-0,49**	0,38*	0,43*	ns	ns
Susp	ns	-0,32*	-0,53**	-0,55**	0,26*	ns	ns	-0,35*	0,34*	ns	0,5**	-0,49**
Slope	ns	ns	-0,27*	ns	0,52**	0,37*	0,51**	-0,36*	ns	0,61**	ns	ns

6.3.4 Diatom indices

Among all environmental variables evaluated, the strongest correlations in the dataset were calculated for river kilometres indicating that all diatom indices decrease longitudinally from the Upper Danube down to the mouth. The highest correlation coefficients were calculated for the indices GENRE (Rumeau & Coste 1988, Coste & Ayphassorho 1991), TID (Rott et al. 1999), SID (Rott et al. 1997) and IPS (Coste in Cemagref 1982) (Tab. 8). With regard to the water chemistry, the indices showed to be most significantly related to oxygen, pH, total nitrogen, total phosphorus, Ca, sodium and nitrates. The strongest correlations were detected between indices and total nitrogen and nitrates. However, these correlations are most likely an artefact as the coefficients obtained were positive and not negative as expected from an indicator whose value decreases with an increase of a pollutant. On one hand, this might be due to the fact that diatoms as long term indicators might not reflect single values of chemical parameters gathered concurrently with diatom sampling as they are adapted to a scale of values within a certain period. However, the correlations with water chemistry and hydro-morphological variables were incomparably lower than with river kilometres. Therefore we assume that values of diatom indices were in this case reflecting a complex of overall changes in the longitudinal profile (rather than a particular pressure), related to both natural longitudinal variability and human-induced degradation of water environment. The best performing indices (GENRE, IPS, TID), and the diatom guilds and life-forms were further correlated with hydromorphological variables. The three diatom indices (GENRE, IPS and TID) correlated significantly with most of the hydromorphological variables, the strongest correlations were detected for discharge (negative

correlations) and slope (positive correlations) (Tab. 8). All these variables change naturally and gradually in the longitudinal profile.

Moreover, all diatom indices proved to differ between the 10 Danubian types at p<0,001 except for LOBO and IDP that differed at p<0,05 (N=108). The indices decreased significantly along the entire Danube from the stream down to the river mouth. Most significant gradual decrease was detected between the types 1-5, from the first station at Böfinger Halde (JDS1: 2581 rkm) down to Budapest (JDS24: 1632 rkm). Diatom indices downstream Budapest (types 6-10)remained systematically low (see Figure 62 for example) with a less significant decreasing trend (Average IPS for types 6-10: 9.5, Standard deviation: 1.2). Interestingly, indices of the type 2 (JDS2: 2415 – JDS5: 2258 rkm) were lower than those of the type 3 and 4 indicating an intensive degradation of the aquatic environment in the type 2.

6.3.4.1 Diatom guilds and life-forms

Similarly to diatom indices, the ecological guilds structure showed to change significantly in the longitudinal profile. The high guild reached relatively higher proportion in the higher Danube, whilst the motile guild proportion increased significantly at sites in the lower Danube. Furthermore, there were strong positive correlations detected between all the three guilds with river kilometres, the high profile guild performed the best. Also, the guilds correlated significantly with oxygen, calcium, sodium, total nitrogen, nitrates, phosphates and DOC. With regard of their relation to hydromorphological parameters, the high profile guild was related to all variables tested except for suspended solids. These results indicate that the diatom guilds composition reflect both chemical and hydromorphological variables. In general, the indication power of the three ecological guilds showed to be similar to the best performing diatom indices.

The two life-forms tested showed to strongly correlate with suspended solids (Tab. 8). There were high positive correlation coefficients calculated between the suspended solids and the proportion of planktonic diatoms in phytobenthos. This is most probably caused by the fact that planktonic diatoms, which do not move actively, can be pulled down onto the river substrate by the sedimenting solids. Therefore higher rates of suspended solids, which imply higher rates of their sedimentation especially near the river banks, might have increased the percentage of planktonic species in phytobentos by purely accelerating their sedimentation. This might explain the high proportions of centrics reached in the biofilm at several sites mostly belonging to the type 6.

6.3.5 Indication of ecological status assessment

Among the diatom metrics tested, the IPS complies the conditions of being used by most of the member states on national standardized level (see Kelly et al., 2009 and Kelly, 2013).It is regularly being updated in OMNIDIA software and was applied in the intercalibration exercise of phytobenthos-based assessment of ecological status of rivers in Central Baltic Geographical group (Kelly et al., 2009) and large rivers (Birk et al., 2012). The IPS was also previously applied to JDS2 results (Makovinská et al., 2008) and based on the results presented above it turned to be among the most appropriate indices for ecological status assessment of the JDS3 data. In order to confirm with the results of the intercalibration exercise, the ecological status was evaluated using the two intercalibrated boundaries between high/good and good/moderate status. For this purpose, the intercalibrated values of the IPS from the Slovak assessment methods were used (High ecological status: IPS>15.5, Good ecological status IPS>13.1). The entire Danube was assessed using the same classification scheme.

Based on this assessment approach the ecological status of most of the sites in the upper Danube down to Gabčíkovo reservoir in Slovakia (1852 rkm) in the types 1-4 appeared in the high-good band (Figure 62) except Geisling power plant (JDS3L, 2355 rkm) and Deggendorf (JDS4R, 2285 rkm) in Germany. The sites from Gabčíkovo down to Budapest (1632 rkm) varied between good and moderate status and all sites downstream Budapest (downstream the 1852 rkm) appeared consistently bellow the good/moderate boundary reaching a moderate or worse ecological status. Nevertheless, the assessment method and the single classification scheme applied do not take into account the particular differences between Danubian types and as such was also intercalibrated for the whole Danube.

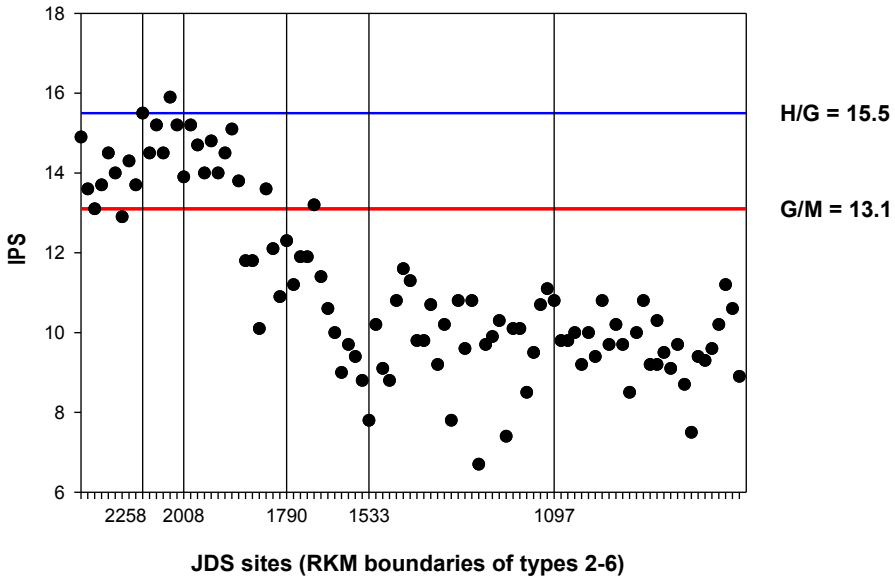

Figure 62: Indication of ecological status assessment based on IPS index using the two intercalibrated boundaries of the Slovak classification system: high/good (H/G) and good/moderate (G/M). Type 2: 2415 – 2258 rkm, type 3: 2204 – 2008 rkm, type 4: 1942 – 1790 rkm, type 5: 1761 – 1533 rkm, type 6: 1481 – 1097 rkm

6.4 Conclusions

Both diatoms and non-diatoms in the Danube indicated that there is a strong environmental longitudinal gradient in the Danube profile related to natural changes in the river typology as well as to increasing anthropogenic disturbance.

The Danube phytobenthos was mainly composed of diatoms and cyanobacteria, with the former prevailing in the upper Danube. The algal biomass showed to increase in the upper and lower Danube and was most significantly influenced by phosphates and suspended solids.

Both species composition of diatoms and non-diatoms as well as the diatom metrics changed gradually downstream. The structure of diatom assemblages in the Danube showed to be closely related to natural longitudinal changes and clearly differed between Danubian types. On the other hand, the distinct longitudinal decrease of diatom indices indicated that besides natural variability, there is also a significant gradient of water environment degradation increasing downstream.

The algal assemblages in the upper reaches (types 1-5) were significantly influenced by velocity, slope oxygen content, pH and nitrates. The assemblages in the middle and lower Danube (types 6-10) reacted mainly on phosphates, potassium, sodium, DOC and suspended solids. Suspended solids showed to greatly influence the community structure by increasing the proportion of planktonic diatom species and decreasing the overall biomass of algal biofilms.

All diatom indices tested decreased gradually from the stream down to Budapest in Hungary (1632 rkm) indicating a longitudinal increase of disturbance and pollution between types 1-5, but also reflecting the natural longitudinal differences. In lower reaches, between Hungary and the river mouth in Romania (types 5-10), all diatom indices remained systematically low with a less distinct descending trend downstream. The increase of general degradation in the longitudinal profile of the Danube River was confirmed by high correlations of diatom metrics with river kilometres as well as with water chemistry. In summary, structure of phytobenthos and diatom indices in the upper Danube were driven by both natural longitudinal changes and pollution, whilst in lower reaches the influence of human disturbance prevailed. Among the diatom indices available, the GENRE, IPS and TID appeared to be the most appropriate for further application in the ecological status assessment of the Danube. The indication power of the three ecological guilds showed to be similar to the best performing diatom indices. The composition of the three diatom guilds as well as the life forms showed to be closely related to hydromorphology as well as water chemistry. However, the particular pressure or pollutant causing the distinct longitudinal gradient identified could not be extracted from

the data available. The results obtained indicate that diatoms reflected a combined effect of general pollution (mainly differences in oxygen, pH, phosphorus, sodium and DOC) and natural hydromorphological differences and alterations (differences in slope, discharge, velocity in upper sections and banks degradation, increased suspended solids concentrations and shear stress and sediments loads in middle and lower sections).

The IPS-based indication of ecological status assessment of the Danube showed that the ecological status of the Upper Danube (sites down to Gabčíkovo reservoir at 1852 rkm) varied between high to good except too moderate sites in Germany (Geisling power plant at 2355 rkm and Deggendorf at 2285 rkm). Sites downstream Budapest (after the 1852 rkm) appeared consistently bellow the good/moderate boundary indicating that the ecological status of the middle and lower Danube might be moderate and worse. Nevertheless, the assessment method applied (even though intercalibrated) does not fully take into account the Danubian typology and the results should be therefore considered as indicative.

These results confirm that despite the methodological limitations related to phytobenthos surveys in large rivers, benthic algae can serve as valuable indicators of water quality and general degradation of the Danube and can be reliably applied to the assessment of its ecological status. Not only the diatom indices, but also the diatom guilds proved to provide a reliable reflection of the environmental conditions and supply an additional insight to the aquatic ecosystem functioning.

6.5 Acknowledgements

We would like to thank Corina Carpentier who kindly lend us the BenthoTorch® (bbe Moldaenke) provided by Benten Water Solution (The Netherlands).

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7 Macrophytes

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7.1 Introduction

Macropyhtes are aquatic plants that live in the littoral zone of rivers and lakes (Haslam, 2006). Taxonomically, they are composed of non-vascular plants (bryophytes – mosses and liverworts), vascular plants (angiosperms) and macroalgae (charophytes, filamentous green algae, etc.). From a life-form point of view macrophytes can be divided to emergent (helophytes) as well as free floating and submerged macrophytes (hydrophytes). Macrophyte surveillance does not stop in the river, but it goes up to the river banks because of water fluctuations. There we can find amphibious plants capable of living in and out of the water (amphiphytes), secondary water plants that prefer wet habitat or water related plants, and "chance" species originating from ruderal and nitrophilic habitats.

Littoral vegetation of rivers and lakes helps to reduce shoreline erosion by absorbing part of the wave energy and serves as habitat for all kind of animals (Kalff, 2001). Macrophytes trap particles and associated nutrients forming substrate for bacteria and periphyton. They are also feeding, breeding and hiding place for benthic invertebrates and littoral fish as well as a habitat for songbirds, amphibians, reptiles and mammals.

Through unbreakable connection with the aquatic habitat macrophytes are a very important biological element for the assessment of ecological status of rivers and lakes. Therefore they are chosen as one of five biological elements for assessment of ecological status of water bodies in the Water Framework Directive (WFD 2000). Macrophytes do not only deliver information about eutrophication, but also together with bank vegetation indicate the hydromorphological conditions of rivers and lakes and the naturalness of aquatic ecosystems.

7.2 Methods

7.2.1 Sampling

Sampling of macrophytes and other bank vegetation was conducted from a small boat on six survey units of one kilometre length at each sampling site. Three survey units were sampled on the left and three on the right side of the river. For determining survey units a Garmin Montana 600 GPS device was used. Abundance of plants was estimated according to the Kohler 5-level scale (Kohler, 1978). Survey was documented with a Pentax WP-3 waterproof digital camera with GPS. Besides species list, additional parameters were recorded for each survey unit separately: presence of impoundment, incoming tributary or discharge, current velocity and diversity, estimated turbidity and Secchi depth, shading, type of bank fixation, proportion of submerged and emerged (bank) substrate as well as the slope and proportion of vegetation type on the banks. Species data and additional parameters were recorded in field protocols.

7.2.2 Determination of species

Plant species were identified in the field when possible while others were collected for later determination. Bryophytes were stored in paper bags, while vascular plants and charophytes were

stored in 50% ethanol or in herbarium. Determination was carried out under Olympus SZ10 stereo-microscope with magnification 10-63 and Olympus BX51 microscope with magnification 100-400X. Species identification was followed by adequate literature (Atherton et al., 2010; Casper, 2008a; Casper, 2008b; Frey et al., 2006; Jäger et al., 2000; Krause, 2008; Martinčič et al., 2007; Smith, 1990; Smith, 2004; Van de Weyer et al., 2011a; Van de Weyer et al., 2011b). Species names were updated according to Hill et al. (2006) for mosses and The Plant List (2013) for liverworts, ferns and angiosperms.

7.2.3 Data analysis

Bray-Curtis similarity (Bray and Curtis, 1957) was calculated for River Sections based on log transformed species data. Nonmetric multidimensional scaling ordinations (NMDS) were performed on resultant matrices with River Sections defined by Moog et al. (2006) and used as a grouping variable (Clarke 1993). Additionally NMDS was overlaid with cluster analysis for visualisation of River Section similarity. Similarity Percentage Analyses (SIMPER) (Clarke, 1993) was also conducted with Bray-Curtis similarity measures on log transformed data to determine contributions of individual taxa to overall dissimilarity among River Sections. Bray-Curtis similarity, NMDS and SIMPER analysis were performed in Primer 6.1.6. (Clarke and Gorley, 2006) and Column Charts were performed in Microsoft Office Excel 2013.

7.2.4 Assesment of ecological status

In order to assess the ecological status of the Danube according to the WFD three different macrophyte-based assessment systems were tested. These were the Austrian system "AIM for rivers" (Austrian Index Macrophytes for rivers; Pall & Mayerhofer, 2013), including the adapted version for the Bavarian part of the Danube (Pall, 2013), the Slovakian system "IBMR-SK" (Macrophyte Biological Index for Rivers; Baláži & Tóthová, 2010), which is the adapted French system "IBMR" according to Haury et al. (2006) and the Hungarian system "RI-HU" (Hungarian Reference Index; Lukács, 2009), which is the adapted German system "Reference Index" according to Schaumburg et al. (2004).

7.3 Results

7.3.1 Completeness of macrophyte survey

Most of the sampling sites were sampled according to standard procedure (354 km out of 408 km). Sampling sites JDS12, JDS23, JDS29 and JDS56 were not sampled at all because of technical difficulties (e.g. low water level). On sampling sites JDS14 and JDS28 it was possible to sample only one side (due to danger of the landmines). On sampling sites JDS1, JDS37, JDS48, JDS51, JDS54 and JDS58 only one sampling section was sampled on the left and on the right side because survey out of small boat was obstructed due to low water level and it was carried out on foot.

7.3.2 Species composition

7.3.2.1 Taxonomical and life-form approach

During the whole survey 182 taxa were identified to the species level and 16 to the genus level (198 taxa in total). Identified taxa belonged to groups of bryophytes (35 taxa), ferns (4 taxa), angiosperms (150 taxa), charophytes (1 taxa) and other macroalgae (8 taxa).

Angiosperms co-dominated with bryophytes in first four River Sections and completely dominated in the rest of the Danube (Figure 63). Pteridophytes occurred in the Middle and Lower Danube, but with discernible relative plant mass only in Sections from 5 to 7 (3,3-5,5%) where the floating species *Salvinia natans* (L.) All. was the dominant species in the group. A higher proportion of macroalgae occurred in Sections from 5 to 10 (3,3-27,7%) with *Cladophora glomerata* (L.) Kützing as the

dominant species while charophytes were identified only in River Section 8 with a single species, *Nitellopsis obtusa* (N.A.Desvaux) J.Groves.

Macroalge (except charophytes) and taxa identified to the genus level were not associated with life-forms. Therefore according to this concept 44 species belonged to hydrophytes, 28 species to helophytes, 34 species to amphiphytes, 40 species to the group of water related species and 35 species comprised the chance species.

Hydrophytes and helophytes were the dominant groups throughout the whole river course. A complete dominance of hydrophytes was recorded in River Section 7 with 82,6%. Amphiphytes and chance species were represented with smallest percentage, while water related species showed an almost constant value close to 20% through the whole Danube.

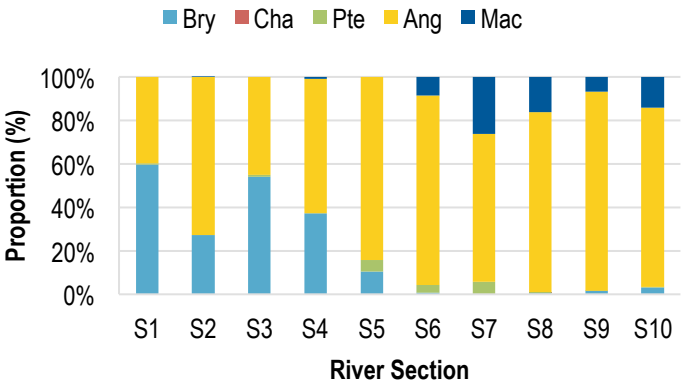

Figure 63: Proportion of plant groups in all River Sections of the Danube (Bry – bryophytes, Cha – charophytes, Pte – pteridophytes, Ang – angiosperms, Mac – macroalgae)

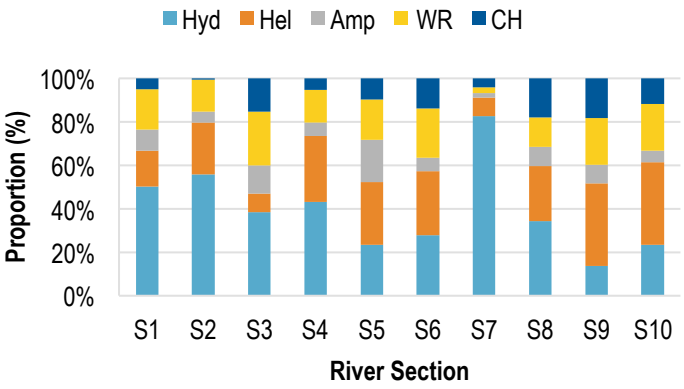

Figure 64: Proportion of life forms in all River Sections of the Danube (Hyd – hydrophytes, Hel – helophytes, Amp – amphiphytes, WR – water related plants, CH – chance species)

7.3.2.2 Characteristic species of River Sections

SIMPER analysis listed all species by their contribution to the Bray-Curtis similarity among samples within River Sections defining some species as characteristic species of each Section.

7.3.2.2.1 Aquatic vegetation

Moss species defined the aquatic plant community in River Sections from 1 to 4 where *Cinclidotus riparius* (Host ex Brid.) Arn., *Fontinalis antipyretica* Hedw., *Cratoneuron filicinum* (Hedw.) Spruce and *Amblystegium serpens* (Hedw.) Schimp. contributed the highest share to the similarity between samples in one section. River Section 5 was characterized by the aquatic moss *C. riparius* and floating

species like *Lemna minor* L. and *S. natans*, while in River Section 6 only floating species like *L. minor*, *Lemna gibba* L., *S. natans* and *Spirodela polyrhiza* (L.) Schleid. were characteristic for all samples within this section. From River Section 7 to 10 *C. glomerata* was link between samples while other species of macroalgae varied for different sections. In River Section 7 other characteristic species were *Potamogeton perfoliatus* L., *Potamogeton nodosus* Poir. and *Ceratophyllum demersum* L. while in River Section 8 other characteristic species were *Myriophyllum spicatum* L., *Butomus umbellatus* L. , *P. perfoliatus* and *Potamogeton crispus* L. *P. pectinatus* and *P. crispus* characterised River Section 9, while River section 10 was characterised by *P. pectinatus* next to the macroalga *C. glomerata*.

7.3.2.2.2 Bank vegetation

In the River Sections from 1 to 4 only *Phalaris arundinacea* L. was characteristic for all samples in these sections. Other bank species contributing to similarity between samples in each River Sections were for example *Petasites* sp. for River Section 1, *Rubus* sp. and *Lythrum salicaria* L. for River Section 2, *Eupatorium cannabinum* L. for River Section 3 as well as *Solidago canadensis* L. and *L. salicaria* for River Section 4. *Persicaria lapathifolia* (L.) Delarbre and *Persicaria hydropiper* (L.) Delarbre were two species that mostly contributed to sample similarity in River Section 5. In River Section 6 species *P. lapathifolia*, *Echinochloa crus-galli* (L.) P.Beauv. and *Bidens frondosa* L. were characteristic species as well as *P. lapathifolia* in River Section 7. *Xanthium strumarium* L. was the species with the highest contribution to similarity between samples in River Sections 8 to 10, while next to it *E. crus-galli*, *P. lapathifolia* and *Alopecurus geniculatus* L. were characteristic for River Section 8, *A. geniculatus*, *Dichostylis micheliana* (L.) Nees and *Cyperus glomeratus* L. for River Section 9 and *C. glomeratus* and *D. micheliana* for River Section 10.

7.3.2.3 Comparison with outcomes from JDS1 and JDS2

In comparison with previous Joint Danube Surveys, only the total number of identified species was compared because life-form categories were assigned differently and therefore a more detailed analysis was impossible. In comparison with JDS1 when 48 taxa were identified, 37 of them were equal taxa with JDS3 taxa list (77%). During JDS2 129 taxa were identified and 89 of them were the same species as identified in JDS3 (68%).

After accumulation of taxa in all three Joint Danube Surveys 249 taxa of macrophytes and other species related to river were identified. Final result of JDS3 was 80% identification of all three JDS taxa list.

7.3.3 Similarity of Danube River sections

NMDS analysis of River Sections based on the relative plant mass of taxa and overlaid with cluster analysis showed two main groups separating the surveyed Danube course into two parts with a border in Kliská Nemá (Figure 65). River Sections from 1 to 5 grouped with a similarity of 20%. Inside this group River Sections 4 and 5 showed a higher similarity with 40%. The second group was formed with River Sections from 6 to 10 with a group similarity of 40%. Inside this group, a subgroup was formed with River Sections 9 and 10 with a similarity of 60%.

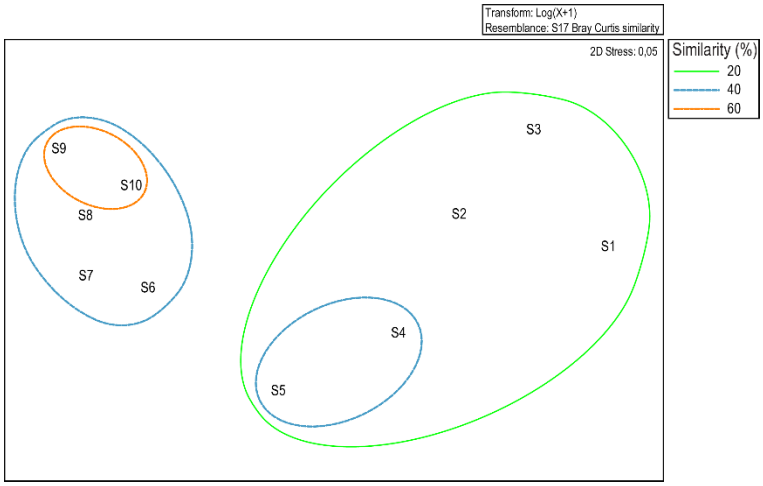

Figure 65: NMDS analysis of River sections performed after Bray-Curtis similarity on taxa relative plant mass overlaid with cluster analysis

Dissimilarity of River Sections based on SIMPER analysis performed after Bray-Curtis similarity on relative plant mass of taxa showed that dissimilarity increased with distance between River Sections (Table 9). This analysis formed two groups of River Sections. One group covers River Sections from 1 to 4 with a dissimilarity between 70,67 – 82,28% and another group with river Sections from 6 to 10 with a dissimilarity between 70,88 – 86,54%. River Section 5 had the lowest dissimilarity range in comparison with other River sections (79,62 – 93,16), but still high enough not to group with upstream or downstream River sections.

Table 9: Dissimilarity (%) of River sections based on SIMPER analysis performed after Bray-Curtis similarity on taxa relative plant mass

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	-									
S2	70,67	-								
S3	71,25	79,96	-							
S4	75,28	74,15	82,28	-						
S5	89,44	85,68	90,95	80,85	-					
S6	96,27	93,44	96,51	89,91	79,62	-				
S7	99,54	93,82	98,48	96,56	88,24	76,94	-			
S8	98,78	94,74	98,30	93,71	87,96	75,83	76,71	-		
S9	99,54	97,53	99,17	96,34	92,54	80,59	86,54	70,88	-	
S10	97,72	92,81	96,45	94,77	93,16	83,44	80,27	78,53	76,53	-

7.3.4 Indication of an ecological status

The three assessment systems are basically different. The Slovakian system "IBMR-SK", which is an adaption of the French system "IBMR", solely is based on the trophic indication of different species. The Austrian system "AIM for rivers" respects furthermore the indicative value of some species concerning hydro-morphological alterations as flow velocity or embankment. Both systems include only hydrophytes (submersed or floating-leafed species) and amphiphytes (species which can live as well submersed as occasionally in the dry at the banks). The Hungarian system "RI-HU", which is an adaption of the German "Reference Index" assigns the species to the three groups: A = reference species, B = indifferent species and C = degradation indicators, independent of a specific pressure (eutrophication or hydro-morphological alteration). In difference to the German original version the Hungarian adaption includes also helophytes (reed-vegetation) which enhances the explanatory power of the system especially with regard to hydro-morphology.

The Slovakian and the Austrian assessment systems show a decrease of ecological status from the source to the mouth of the Danube (Figure 66). This finding cannot be interpreted by the typical pressure data macrophytes are regarded to be indicative for. Neither the nutrient concentrations

(Phosphorus- and Nitrogen-compounds; see chapter 17 by Hamchevici et al. in this report) nor hydro-morphological impairments (see chapter 3 by Schwarz et al. in this report) show a significant increase along the Danube stretch. These results demonstrate clearly that the indicative value of species, especially concerning trophic conditions, changes within different regions and river-types and underline the necessity for type-specific adaptations of assessment systems.

The results of the Hungarian system generally show only small differences along the course of the Danube. Most of the sites are assessed in “good status”. “Moderate status” is assigned especially to the Gabcikovo reservoir, the sites around Budapest and the Iron Gate reservoir. However, in spite of delivering the most balanced results along the river course, even in the Hungarian system region- and river-type-specific adaptions would have to be made to achieve a sound assessment of ecological status in the sense of the WFD.

INVESTIGATED SITES			SITE ID			AIM for Rivers						IBMR-SK						Reference Index-HU						Physico-chemical Parameter								HYMO	
						Austrian assessment sysem						Slovakian assessment system						Hungarian assessment system						Nitrate		Phosphate		Diss. O2				HYMO-Assess.	
Site name	Country		left tributary	Danube	right tributary	tributary		Danube		tributary		tributary		left	right	tributary		tributary		left	right	tributary		[mg/l]		[mg/l]		[mg/l]				1 to 5	
						left	right	left	right	left	right	left	right			left	right	left	right			left	right										
Böfinger Halde	DE			JDS01				2,19	2,23					11,69	11,88					0,59	0,58			14,55		0,11							
Kelheim – gauging station	DE			JDS02				2,21	2,26					10,72	11,54					0,79	0,72			11,45		0,12		8,20					
Geisling power plant (upstream)	DE			JDS03				3,17	3,23					6,99	7,08					0,52	0,44			11,83		0,17		7,60				5	
Geisling power plant (downstream)	DE			JDS03				2,44	2,17					9,67	11,72					0,52	0,81			11,83		0,17		7,60				5	
Deggendorf	DE			JDS04				2,38	2,19					8,94	9,05					0,60	0,58			12,43		0,22		7,10				3	
Mühlau	DE			JDS05				2,35	2,93					10,86	11,07					0,67	0,70			11,53		0,23		7,35				4	
Jochenstein	DE	AT		JDS06				1,70	1,85					13,52	13,36					0,73	0,70			6,73		<0.02		8,70				4	
Upstream dam Abwinden-Asten	AT			JDS07				1,50	1,39					13,96	15,51					0,63	0,58			7,31		0,11		8,70				4	
Oberloiben	AT			JDS08				2,21	2,40					12,19	12,15					0,89	0,75			7,04		0,10		9,38				3	
Klostemeuburg	AT			JDS09				2,30	3,15					12,40	11,22					0,63	0,53			7,15		<0.02		10,54				5	
Wildungsmauer	AT			JDS10					2,35					9,00	11,73					0,11	0,51			7,28		<0.02		9,36				3	
Upstream Morava (Hainburg)	AT			JDS11				2,50	2,50					10,28	10,89					0,54	0,56			7,32		<0.02		8,85				3	
Bratislava	SK		JDS12	JDS13		n.i.	n.i.	2,50	2,10			n.i.	n.i.	11,28	13,04			n.i.	n.i.	0,61	0,59			3,89	7,32		0,79	0,12		7,83	9,33	3	4	
Gabcikovo resevoir	SK	HU		JDS14				3,06	3,27					8,78	9,02					0,37	0,35				6,94			0,16			8,89		5	
Medvedov/Medve	SK	HU		JDS15				2,50	2,50					11,04	11,42					0,55	0,56				6,61			0,10			8,40		4	
Klizska Nema	SK	HU		JDS17	JDS16			2,83	3,27	4,00	4,00			9,59	9,32	6,17	6,17			0,39	0,59	0,38	0,38		6,75	7,18		0,14	0,14		8,76	8,15	3	3
Iza/Szony	SK	HU	JDS18	JDS19			2,80	2,67	3,86				8,89	10,44	6,39			0,50	0,50	0,66	0,38			5,15	6,81		0,17	0,10		8,82	8,74	4	3	
Szob	HU			JDS20				2,78	2,50					8,95	8,22					0,48	0,42				6,56			0,13			8,62		2	
Budapest upstream - Megyeri Bridge	HU			JDS21				3,20	3,20					7,33	7,54					0,40	0,45				6,14			<0.02			9,39		4	
Budapest downstream - M0 bridge	HU			JDS22				3,29	2,50					7,74	7,92					0,45	0,44				6,47			0,17			8,54		4	
Dunafoldvar	HU		JDS23	JDS24		n.i.	n.i.		3,00			n.i.	n.i.		9,17			n.i.	n.i.	0,45	0,52			0,17	6,68		0,21	0,13		4,29	7,52	3	3	
Paks	HU			JDS25				2,50	2,50					8,43	10,31					0,44	0,49				6,68			0,17			7,89		3	
Baja	HU			JDS26				2,50						8,44	9,00					0,46	0,87				6,61			0,15			8,32		2	
Hercegszanto	HU			JDS27				2,50						10,76						0,82	0,62				6,49			0,16			8,41		3	
Upstream Drava	HR	RS		JDS28				3,50						9,22						0,63					6,51			0,15			8,47		2	
Downstream Drava (Erdut/Bogojevo)	HR	RS		JDS30	JDS29			3,50		n.i.	n.i.			9,31	10,00	n.i.	n.i.			0,61	0,55	n.i.	n.i.		5,93	3,80		<0.02	<0.02		8,95	8,63	3	2
Ilok/Backa Palanka	HR	RS		JDS31					2,63						10,15					0,78	0,58				6,17			0,14			9,29		3	
Upstream Novi-Sad	RS			JDS32				2,38	4,00					10,95	6,00					0,69	0,65				6,42			0,13			8,58		4	
Downstream Novi-Sad	RS			JDS33				3,88	2,50					8,70	10,50					0,51	0,56				6,23			0,13			7,94		3	
Upstream Tisa (Stari Slankamen)	RS			JDS34				3,44	3,43					7,47	7,70					0,63	0,60				6,43			0,13			8,22		3	
Downstream Tisa/Upstream Sava	RS		JDS35	JDS36		3,96	3,91	3,97	3,08			6,08	7,77	6,95	9,40			0,38	0,46	0,47	0,64			1,41	6,13		0,25	0,13		5,17	7,85	3	3	
Upstream Pancevo/Downstream Sava	RS			JDS38	JDS37			3,86	3,83	3,68	3,47			6,33	6,41	7,15	6,45			0,60	0,48	0,44	0,48		5,30	2,73		0,14	0,15		7,38	7,74	4	5
Downstream Pancevo	RS			JDS39				3,74	3,54					6,37	6,35					0,43	0,45				5,55			0,14			7,35		4	
Upstream Velika Morava	RS			JDS40				3,51	3,55					7,02	6,79					0,49	0,42				5,33			0,18			7,07		4	
Downstream Velika Morava	RS			JDS42	JDS41			3,54	3,49	4,00	4,00			6,72	5,96	7,81	7,98			0,44	0,38	0,46	0,38		5,46	4,88		0,14	0,20		7,30	4,26	4	4
Banatska Palanka/Bazias	RS	RO		JDS43				3,58	3,31					6,92	6,93					0,36	0,50				5,54			0,18			6,36		4	
Irongate reservoir (Golubac/Koronin)	RS	RO		JDS44				3,48	3,28					6,15	5,86					0,40	0,39				5,84			0,16			6,52		4	
Irongate reservoir (Tekija/Orsova)	RS	RO		JDS45				3,52	3,14					6,04	6,87					0,40	0,59				4,61			0,14			7,30		5	
Vrbica/Simijan	RS	RO		JDS46				2,81	3,17					7,53	7,61					0,66	0,46				4,66			0,13			5,90		4	
Upstream Timok (Rudujevac/Gruia)	RS	RO		JDS47				3,47	3,27					6,61	7,53					0,40	0,48				4,42			0,33			6,47		3	
Pristol/Novo Selo Harbour	RO	BG		JDS49	JDS48			3,01	3,14	2,90				7,22	7,45	6,50				0,58	0,58	0,40			4,55	16,02		0,18	<0.02		6,40	8,90	3	3
Downstream Kozloduy	BG	RO		JDS50				3,33	3,19					5,78	6,84					0,61	0,48				4,36			0,13			8,28		3	
Downstream Olt	RO	BG		JDS52	JDS51			3,72	3,41	2,50	3,36			7,30	6,72	8,00	6,80			0,51	0,46	0,50	0,49		4,30	4,55		0,17	0,28		8,60	8,17	2	2
Downstream Zimnicea/Svishtov	RO	BG		JDS53				3,39	3,18					6,89	6,86					0,49	0,49				4,22			0,14			9,02		3	
Downstream Jantra	RO	BG		JDS55	JDS54				3,50					6,20	7,38	7,00	5,00			0,64	0,51	0,48	0,50		3,94	4,23		<0.02	0,22		8,48	7,31	2	3
Downstream Ruse/Giurgiu	BG	RO	JDS56	JDS57		n.i.	n.i.		3,17			n.i.	n.i.	5,00	8,67			n.i.	n.i.	0,65	0,56			23,04	5,68		1,09	0,14		7,27	8,68	4	3	
Downstream Arges, Oltenita	RO	BG	JDS58	JDS59			3,50	2,50	3,30			5,00	8,50	8,00	8,89			0,50	0,60	0,59	0,48			6,11	4,49		1,54	0,14		6,66	8,53	0	3	
Chiciu/Silistra	RO	BG		JDS60					3,60					5,00	8,07					0,58	0,51				4,34			0,14			8,71		2	
Giurgeni	RO			JDS61				3,55	4,00					6,65	8,00					0,53	0,63				4,38			0,14			8,24		2	
Braila	RO		JDS63	JDS62		4,00	4,00		3,87			9,87	9,67		6,86			0,52	0,49	0,57	0,48			6,28	4,40		0,12	0,19		8,85	8,00	4	3	
Reni	RO	UA	JDS64	JDS65			4,00		3,93				8,58	9,00	3,72			0,50	0,49	0,50	0,39			4,50	4,42		0,17	0,19		8,12	8,18	3	3	
Vilkova - Chilia arm/Kilia arm	RO	UA		JDS66				3,11	2,88					7,53	8,09					0,53	0,51				4,00			0,15			8,08		2	
Sulina - Sulina arm	RO			JDS67				4,00						2,00	2,00					0,58	0,49				4,70			0,18			8,04		2	
Sf.Gheorghe - Sf.Gheorghe arm	RO			JDS68				3,85	3,88					4,97	7,20					0,51	0,58				4,77			0,19			8,21		3	

Figure 66: Ecological status of the investigated JDS-sites according to the Austrian, Slovakian and the Hungarian assessment systems.

Blue = high, green = good, yellow = moderate, orange = poor and red = bad status, grey = not assessable, n.i. = not investigated.

Physico-chemical parameter and hydromorphology results according to Hamchevici et al. and Schwarz et al. (see chapters 17 and 3 in this report), colored from green to red with increasing ecological impairment

7.4 Conclusions

The JDS3 survey of macrophytes was completed successfully because all sampling sites on the main river were sampled according to standard procedure. Sampling sites sampled with shorter survey length or sites not sampled because of technical difficulties were those located in the tributaries.

A total of 198 taxa were identified belonging to bryophytes (35 taxa), ferns (4 taxa), angiosperms (150 taxa), charophytes (1 taxon) and other macroalgae (8 taxa).

In general, angiosperms were the dominant plant group in all River Sections. Bryophytes were the subdominant group in River Sections from 1 to 4 and macroalgae were the subdominant group in River Sections from 6 to 10.

The cumulative number of identified taxa in all three Joint Danube Surveys was 249 taxa. 80% of all taxa were identified in JDS3 because of previous extended experience and because of the aim to identify bank vegetation in details with regard to the hydromorphological status.

Bryophytes were the dominant aquatic species in River Sections from 1 to 4, with *Cinclidotus riparius* as the most representative species. River Section 5 was a transitional section where both *Cinclidotus riparius* and floating species (*Lemna minor*, *Salvinia natans*) were present, while River Section 6 was mainly characterized by floating species (*Lemna minor, Lemna gibba, Salvinia natans, Spirodela polyrhiza*). In River Sections 7 to 10 characteristic species were species of the genus *Potamogeton*, as well as *Ceratophyllum demersum* and *Butomus umbellatus*.

NMDS analysis based on species composition separated two main groups of River Sections with a clear division at Kližská Nemá.

Whether macrophytes should be used for large river assessment according to the WFD or not is under discussion at present. There are controversial points of view within the European Union. Whereas e.g. the Austrian and the Slovakian systems are officially used for the Danube in the respective countries, the Hungarian system officially is regarded as not applicable for large rivers (Lukács, pers. comm.).

The results of this study on the one hand clearly demonstrate that a macrophyte-based quality assessment of large rivers is possible. On the other hand it could be shown that the systems deliver plausible results only for the river-types or regions they were developed for. For enabling an assessment on a larger scale in all systems tested river-type and region-specific adaptations would have to be performed. In this context the findings of dissimilarities and similarities between river-sections, as presented before, can support the necessary region- and river-type-specific adaptions when dealing with ecological quality assessment.

As a further outcome of this study the importance of including helophytes and selected bank-vegetation in a macrophyte-based quality assessment could be shown, especially with regard to hydro-morphology.

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8 Phytoplankton

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8.1 Introduction

An essential quality element in all lakes and larger rivers is the autotrophic phytoplankton. Photosynthetic processes by primary producers are important in the cycling of carbon and in the oxygen budget. The accumulated biomass can serve as food for other trophic levels. The composition of the phytoplankton assemblage and the biomass produced primarily indicates the trophic status of the water body. Within the framework of the EC-WFD, metrics have been developed to evaluate the trophic situation (Mischke and Oppitz, 2005). Besides species composition, biomass and chlorophyll-a, additional parameters are necessary to assess the trophic level correctly. In most cases, phosphorus (P) is limiting and therefor is the most relevant nutrient for phytoplankton growth. Among the different forms, total phosphorus (TP) is assumed to most relevant. To judge any deficiency of nitrogen (N) relative to phosphorus, total inorganic nitrogen concentration is needed. Chlorophyll-a is used as an additional measure of biomass. Development of diatoms can be estimated from concentration of dissolved silica. Availability of under-water light, important for photosynthesis, can be calculated from suspended solids.

Species composition of phytoplankton may also be used to evaluate impacts from certain chemicals or to evaluate changes in hydromorphology which affect phytoplankton assemblages. Regulated stretches decrease retention time resulting in reduced biomass development. Impounded or artificial deepened river sections are more similar to lakes indicated by an increase in species more common in standing waters and a reduction in the contribution from benthic taxa usually common in free flowing rivers.

Within the Danube River Basin phytoplankton assessment is particularly relevant because the River Danube as well as several of the larger tributaries have a great potential to produce large amounts of phytoplankton biomass. Some stretches may even carry self-sustaining plankton populations (potamoplankton). Monitoring of phytoplankton diversity will help to assess changes in nutrient input and pollution control. The development of the nutrient levels and the associated phytoplankton biomass in the Danube River Basin finally has a large impact on the Black Sea.

8.2 Methods

Samples were taken from the surface of the river on the left (L), middle (M) and right (R) side with a black bucket (8 l) and used for all further analysis. A qualitative sample was taken with a plankton net (10 µm mesh size), Secchi-depth was measured at each point. On-board analysis included the immediate measurement of 'active' chlorophyll-a by delayed fluorescence (DF, Gerhardt and Bodemer, 2000). Sub-samples were filtered onto GF/C filters for total chlorophyll-a analysis, stored at -35°C until analysis in the laboratory. Filters have been extracted and analysed in the spectrophotometer according to DIN 38412 later in the laboratory replacing 90% Methanol by 90% Acetone to allow HPLC analysis for the assessment of pigment composition. Quantitative samples (100-200 ml) for phytoplankton counting and sizing were fixed with Utermöhl's acetic acid Lugol solution, preserved with a few drops of formalin in brown screw cap glass bottles and stored in a cool dry place (Utermöhl, 1958, Hillebrand et al., 1999,) . These samples were counted in the laboratory on a NIKON Diaphot applying the sedimentation technique (Brierley et al., 2007, Benskin, 2009). Algae

were largely determined on board using the unpreserved concentrated 10 µm-net samples. Fresh-weight biomass was calculated from chlorophyll-a concentrations using three independent conversion equations (not intercalibrated):

(1.) Chl-a = 0.5% fresh-weight biomass (Reynolds 2006)

(2.) Chl-a = 0570 + 4.131*B (derived from JDS1 and 2 data, Dokulil unpubl.))

(3.) Chl-a = $4.063*B^{0.66}$ (Felip and Catalan 2000)

8.3 Results

During the observation period, samples were taken at 68 locations (53 in the Danube and 15 from tributaries) resulting in 159 river samples (L/M/R) plus 15 from the inflows. Results from the variables measured are shown in Fig. 67.

Secchi-disk (SD) readings were 1.6 to 1.8 m in the German river section and dropped to values between 0.7 and 0.9 m after the confluence with the River Inn. Visibility remained moderate in the Austrian section reaching 1.3 m in Wildungsmauer (rkm 1,895). Secchi depth varied between 0.6 and 1.3 m throughout Slovakia, Hungary, Croatia and Serbia. Higher values of 1.3 to 1.7 m were reached after the Sava (SD 1.8 m) has entered and remained high until both Iron Gate reservoirs. Maximum Sechi depth of 3.6 and 3.1 m occurred below the Iron Gate at Vrbica/Simijan, rkm 926 and upstream of the Timok (km 849). The rather turbid Timok (SD 0.9m) reduced Secchi depth which was further diminished by the inputs from the tributaries leading to readings of 0.8-0.9 m downstream of km 235.

Both chlorophyll-a and phytoplankton biomass concentration remained below 10 µg l^{-1} chl-a or 2 mg l^{-1} algal biomass in the upstream section until km 1,942 (Klosterneuburg, AT), below Budapest, HU (rkm 1,632 – 1,533) and downstream of rkm 1,151 (Pancevo) as indicated in Fig.67, panel 2 &4. Values higher than those occurred from Klosterneuburg (AT, km 1,942) till upstream Budapest (HU) and between Baja (HU, km 1,481) and downstream of the Sava (SR, km 1,159). Highest concentrations of up to 31 µg l^{-1} chl-a or 9.9 mg l^{-1} biomass were reached in the Drava/Tisa region between km 1,384 and km 1,262.

Chlorophyll-a input from tributaries to the river Danube ranged from 3 µg l^{-1} chl-a in the Jantra to the exceptional high 53 µg l^{-1} from the Morava (Fig. 67, panels 2 & 4). Similarly, biomass ranged from 0.5 to 20 mg l^{-1}.

The phytoplankton of the River Danube was dominated by diatoms (Bacillariophyceae) and green algae (Chlorophyceae) with significant contribution from Cryptophyceae (Fig. 67, 3rd panel from top). Their average contribution was 55.8, 22.3 and 16.5% respectively. Cyanobacteria were of minor importance in the river (4.6% contribution). In the region of greatest phytoplankton development, diatoms and green algae together contribute about 90% to total biomass. Centric diatoms are most abundant and quantitatively most important among the Bacillariophyeae, such as *Aulacoseira granulata, Skeletonema potamus* and *Melosira varians*. Although numerous benthic diatom species were identified their contribution to total biomass is negligible. A wide variety of green algal species from the order Chlorococcales (particularly the genera *Kirchneriella, Monoraphidium, Ankistrodesmus* and *Scenedesmus*) quantitatively contribute to phytoplankton biomass. Cyanobacteria are of greater importance in several of the tributaries such as Drava (8.5%) and Timok (7.2%). In the river Arges 41.6% of the biomass originated from the Cyanobacterial species *Microcystis aeruginosa* and *Microcystis flos-aquae*

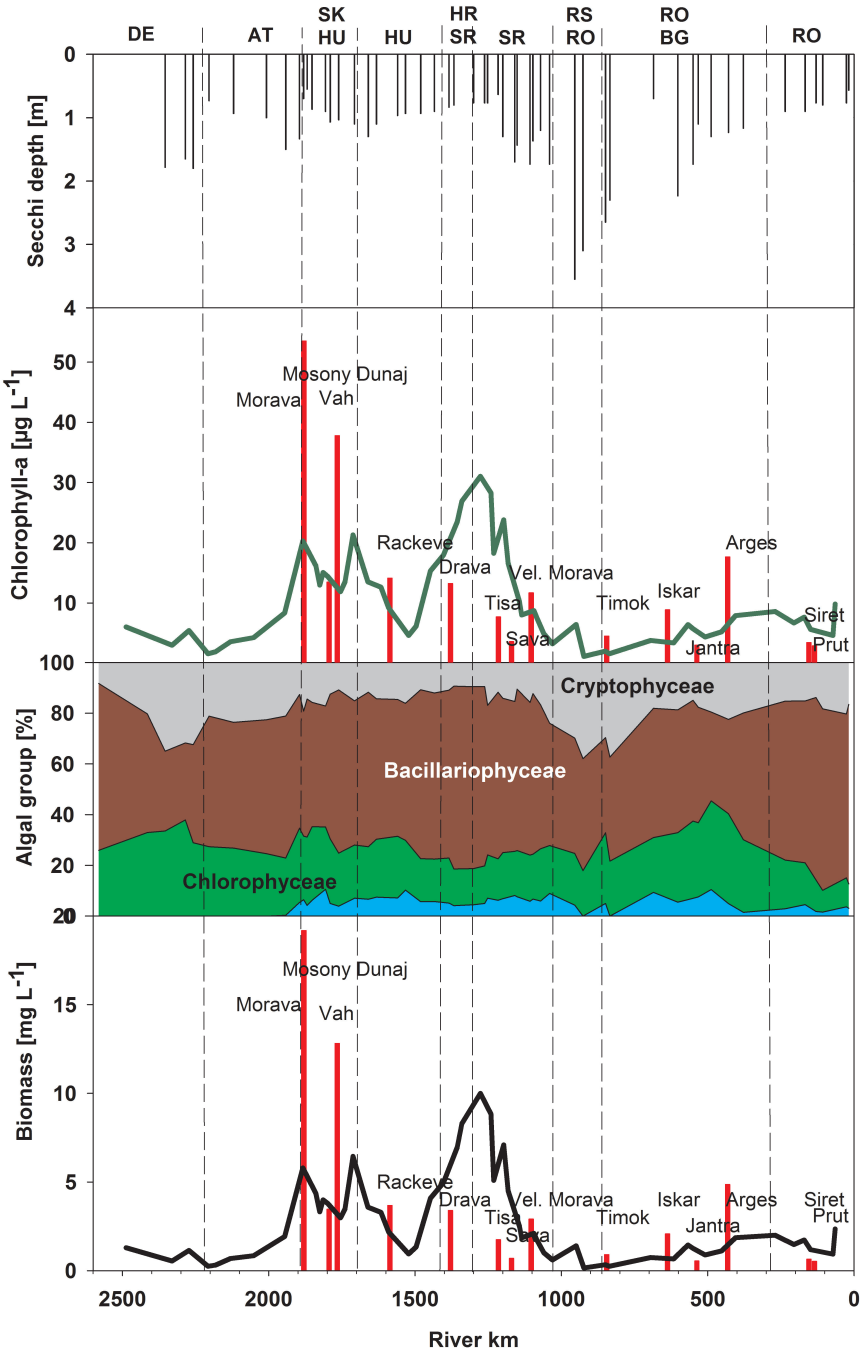

Figure 67: Longitudinal transect of the River Danube from river km 2,600 to the Black Sea obtained during JDS3, August/September 2013. Variables from top to bottom: Secchi depth (SD), Chlorophyll-a in the river (green solid line) and in the tributaries (red bars); Contribution of the main algal groups (%); phytoplankton biomass in the river (black solid line) and in the mouth of the tributaries (red bars). Units are indicated on the axes. Delineations and abbreviations of countries inserted

8.4 Conclusions

The distribution of phytoplankton chlorophyll-a and biomass along the river corridor was significantly different from previous investigations. From the findings during JDS1 and JDS2 three river sections were defined: An upstream section with low values, a middle section where values increased to a maximum and a downstream section with generally low values. During the 2013 survey, this distinct sections were somewhat replaced by alternating sections of low and high concentrations. As

previously, the highest chlorophyll and biomass concentrations occurred in the middle section of the river between km 1,481 (Baja) and 1,159 (downstream Sava). Different from earlier observations however, chlorophyll-a and biomass concentrations exceeded threshold values between Klosterneuburg (km 1,942) and upstream of Budapest (km 1,660). These high values most likely were a reflection of the heat wave preceding the investigation period and low discharge associated with.

According to the TNMN quality classification (Table 10, UNDP/GEF 2003) most chlorophyll-a concentrations in the river belonged to water quality class I (<25 µg l^{-1}). Moderate values of quality class II were observed at three sites from: km 1367, downstream Drava to km 1262, upstream of Novi Sad. Water quality class I was exceeded in two of the 15 tributaries sampled, in the Morava and Vah with 53.39 and 36.68 µg l^{-1} chlorophyll-a (Table 11). River phytoplankton was largely characterized by centric diatoms and Chlorococcales were also playing a major role in most stretches. Cyanobacteria have never shown mass occurrence at any sampling site in the River Danube while they sometimes dominated the plankton of tributaries. The increased cyanobacterial biomass made centric diatoms less important in the in-flows.

Table 10: Classification scales according to TNMN (2003) and Miscke & Oppitz (2005)

Classification	Scale	I	II	III	IV	V
TNMN (2003)	TP µg L^{-1}	<100	100-200	200-400	400-1000	>1000
	Chl-a µg L^{-1}	≤25	≤50	≤100	≤250	>250
M&O (2005)	Chl-a µg L^{-1}	<10	10-20	20-30	30-50	>50
	TP µg L^{-1}	<50	50-150	150-200	200-300	>300
	Scale	**High**	**good**	**moderate**	**poor**	**bad**

The type specific WFD criteria for large rivers established in Germany by Mischke and Oppitz (2005) using the metrics total phosphorus (TP) and chlorophyll-a (chl-a) for trophy assessment were applied additionally (Table 10). Chl-a indicated high to good status (water quality class 1-2) in most the upper and the lower reach of the Danube (Fig. 68). Moderate status was assigned to the river section from km 1,384, upstream Drava to km 1216, upstream Tisa. The 15 investigated tributaries are in high to good status except Morava in bad state and Vah in poor status. Classification based on TP was not possible because TP and chl-a were not correlated. TP concentrations were available in surplus for algal growth at all sites.

Both the concentrations of chlorophyll-a and the phytoplankton biomass are higher compared to 2007 at JDS2 particularly in the section between Vienna and Budapest. It must be emphasized however, that direct comparison of chemical and biological concentrations of the two investigation periods might be inconclusive because of different hydrological discharge situations. The smaller concentrations during JDS2 can partly be a reflection of dilution due to higher run-off.

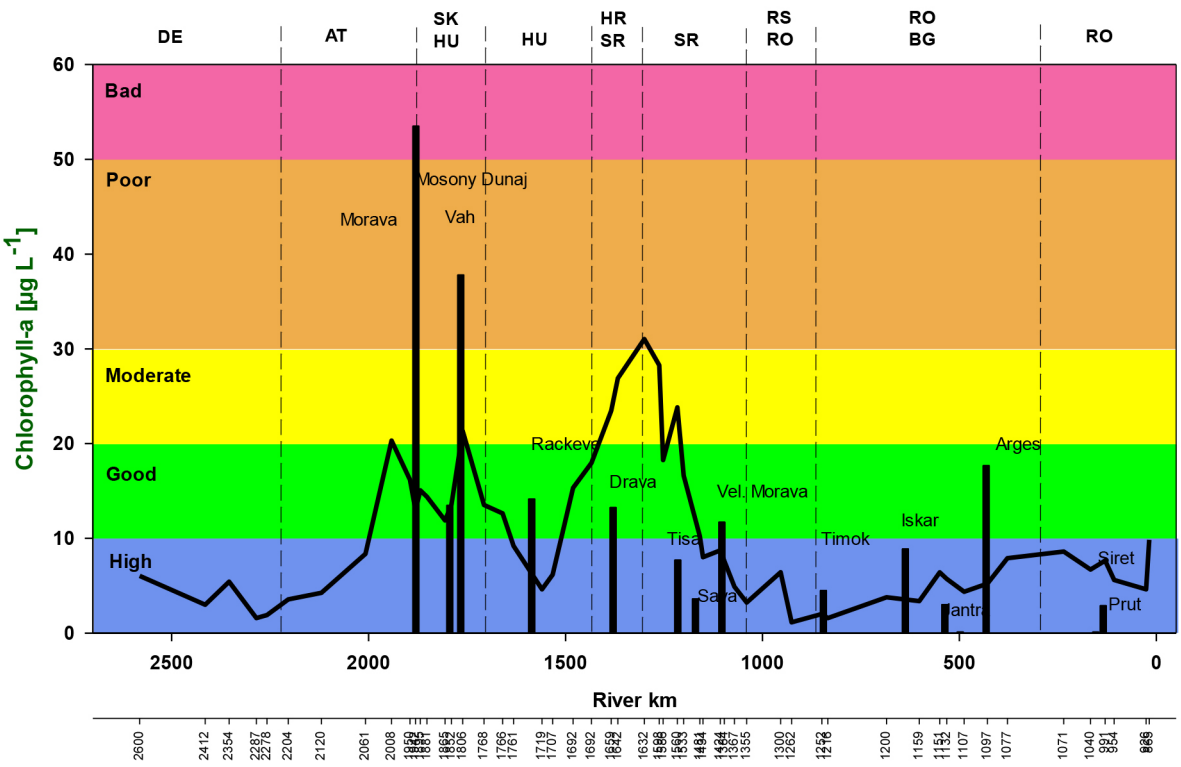

Figure 68: Chlorophyll-a concentrations in the River Danube and selected tributaries obtained during JDS3, August/September 2013 as shown in Fig. 67 but related to WFD criteria proposed by Mischke and Oppitz (2005). See also Table 10 and 11

Table 11: Water quality classification according to TNMN (2003) and Mischke & Oppitz (2005) for all river and tributary sites during JDS3 (mid-stream samples)

JDS No.	Site	Chl-a L^{-1}	TNMN 2003	M & O 2005
JDS1-M	Böfinger Halde	6.04	I	High
JDS2-M	Kelheim – gauging station	2.99	I	High
JDS3-O-M	Geisling power plant above dam	4.88	I	High
JDS3-U	Geisling power plant below dam	3.55	I	High
JDS4-M	Deggendorf	2.16	I	High
JDS5-M	Mühlau	1.88	I	High
JDS6-M	Jochenstein	3.49	I	High
JDS7-M	Upstream dam Abwinden-Asten	4.82	I	High
JDS8-M	Oberloiben	8.53	I	High
JDS9-M	Klosterneuburg	21.66	I	Moderate
JDS10-M	Wildungsmauer	17.51	I	Good
JDS11-M	Upstream Morava (Hainburg)	11.36	I	Good
JDS12	/Morava (rkm 0.08)	53.49	III	Bad
JDS13-M	Bratislava	14.63	I	Good
JDS14-M	Gabcikovo reservoir	15.73	I	Good
JDS15-M	Medvedov/Medve	10.75	I	Good
JDS16	/Moson Danube Arm – end (rkm 0.1)	13.46	I	Good
JDS17-M	Klizska Nema	13.24	I	Good
JDS18	/Vah (rkm 0.8)	37.78	II	Poor
JDS19-M	Iza/Szony	13.57	I	Good
JDS20-M	Szob	11.97	I	Good
JDS21-M	Budapest upstream – Megyeri Bridge	19.50	I	Good

JDS22-M	Budapest downstream – M0 bridge	11.25	I	Good
JDS23	/Rackeve-Soroksar Danube Arm – rkm 59	14.13	I	Good
JDS24-M	Dunafoldvar	5.60	I	High
JDS25-M	Paks	7.59	I	High
JDS26-M	Baja	14.85	I	Good
JDS27-M	Hercegszanto	17.84	I	Good
JDS28-M	Upstream Drava	24.15	I	Moderate
JDS29	/Drava (rkm 1.4)	13.24	I	Good
JDS30-M	Downstream Drava (Erdut/Bogojevo)	25.10	II	Moderate
JDS31-M	Ilok/Backa Palanka	31.63	II	Poor
JDS32-M	Upstream Novi-Sad	27.26	II	Moderate
JDS33-M	Downstream Novi-Sad	18.95	I	Good
JDS34-M	Upstream Tisa (Stari Slankamen)	24.27	I	Moderate
JDS35	/Tisa (rkm 1.0)	7.70	I	High
JDS36-M	Downstream Tisa/Upstream Sava (Belegis)	18.28	I	Moderate
JDS37	/Sava (rkm 7.0)	3.60	I	High
JDS38-M	Upstream Pancevo/Downstream Sava	11.58	I	Good
JDS39-M	Downstream Pancevo	7.42	I	High
JDS40-M	Upstream Velika Morava	9.81	I	High
JDS41	/Velika Morava	11.69	I	Good
JDS42-M	Downstream Velika Morava	10.25	I	Good
JDS43-M	Banatska Palanka/Bazias	3.93	I	High
JDS44-M	Irongate reservoir (Golubac/Koronin)	2.60	I	High
JDS45-M	Irongate reservoir (Tekija/Orsova)	7.92	I	High
JDS46-M	Vrbica/Simijan	1.16	I	High
JDS47-M	Upstream Timok (Rudujevac/Gruia)	2.27	I	High
JDS48	/Timok (rkm 0.2)	4.49	I	High
JDS49-M	Pristol/Novo Selo Harbour	1.72	I	High
JDS50-M	Downstream Kozloduy	5.43	I	High
JDS51	/Iskar (rkm 0.3)	8.86	I	High
JDS52-M	Downstream Olt	5.48	I	High
JDS53-M	Downstream Zimnicea/Svishtov	9.14	I	High
JDS54	/Jantra (rkm 1.0)	2.99	I	High
JDS55-M	Downstream Jantra	6.81	I	High
JDS57-M	Downstream Ruse/Giurgiu	4.71	I	High
JDS58	/Arges	17.65	I	Good
JDS59-M	Downstream Arges, Oltenita	4.99	I	High
JDS60-M	Chiciu/Silistra	9.58	I	High
JDS61-M	Giurgeni	8.59	I	High
JDS62-M	Braila	7.20	I	High
JDS63	/Siret (rkm 1.0)	3.43	I	High
JDS64	/Prut (rkm 1.0)	2.88	I	High
JDS65-M	Reni	7.48	I	High
JDS66-M	Vilkova – Chilia arm/Kilia arm	10.47	I	High
JDS67-M	Sulina – Sulina arm	4.65	I	High
JDS68-M	Sf.Gheorghe – Sf.Gheorghe arm	5.71	I	High

8.5 References

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9 Fish

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9.1 Introduction

In total 102 species of freshwater fish inhabit the Danube along its entire course, covering various ecological and functional guilds (Schiemer & Waidbacher, 1992, Schiemer, 2003, Eros et al. 2005). This comparatively high number is a result of its remarkable importance as an east-west migration route after the end of the last ice age (Banarescu. 1960; Balon et al., 1986), which led to the genesis of many endemic species like the three Danube percids *Zingel streber, Zingel zingel* and *Gymnocephalus schraetser*. The autecology of many species, however, is still poorly known (Bammer, 2010). A high proportion of the original fish community is still existent, although species abundances are remarkable low as shown in Joint Danube Survey 2 (Wiesner et al., 2007). Migratory sturgeon species are of main concern for conservation purposes as well as for fisheries (Reinartz, 2002, Bloesch et al., 2006, Reinartz et al., 2012, Schmall & Friedrich, 2013). The introduction of new species is an ongoing process with about ten new species that have been recorded since 1992 upstream the Iron Gates as immigrants from the Lower Danube (Schiemer, 2003). The appearance of new species is known to cause negative impacts on autochthonous species due to new parasites, diseases but also to drastic changes in fish communities and food chains as a consequence of increased predation, competition for food and ecological requirements (Brander et al 2013, Wiesner, et al. 2010, Essl & Rabitsch 2003). Danubian fish stocks are declining (Schiemer, 2003) and many species are at the edge of extinction or even beyond that point (Spindler, 1997), nevertheless, fish are still of great economic importance, as an important food source and a valuable target of recreational fishery. Beside this importance, fish populations are a good indicator for human pressures, in particular for hydromorphological alterations, which are the main cause for declining fish stocks in the Upper Danube (e.g. Spindler, 1997). Various studies (e.g. Wiesner et al., 2007) have shown, that the loss of connectivity due to the use of hydropower and the resulting deterioration of habitat quality can be seen as the main reason for ecological deficits of the fish fauna in the Upper Danube, whereas bad water quality and the exploitation of fish stocks both by legal fishery and poaching are the most considerable causes in the middle and lower course (Schmall & Friedrich, 2014).

Links to the Water Framework Directive (WFD)

As fish react very sensitive to various human impacts, they play a remarkable role as one of four biological quality elements used for the assessment of the ecological status of running waters according to the EU Water Framework Directive (WFD, 2000). The most crucial assessment parameters are composition, abundance and age structure of fish fauna, which have to be implemented in the evaluation index of each EU member state. Especially in large rivers, appropriate fish sampling methods are still a challenging task and there is no agreed standardised procedure yet. This, however, would be a prerequisite for comparing national data sets. This is also the case with assessment tools like fish indices which have partially been developed by some EU member states, whereas other countries still lack a suitable method for evaluating fish data in this context. The EU wide harmonisation and intercalibration of the assessment process for large rivers will be a challenging task for the near future. Although fish samples had been taken for the first time along the whole stretch of the Danube during the JDS2, the consistent standardised effort from the fish core team was then limited by budgetary restrictions and by a rising water level in the lower Danube. The standardised

representative data set, which has been collected by the core team during the JDS3, combining two quantitative sampling methods, provides a sound basis for the comparison of different sampling methods and different assessment approaches.

9.2 Methods

The investigation of the Danube fish fauna followed the joint approach of JDS2, combining fish sampling efforts of the core team with field investigations of the various national teams. The core team sampled the littoral area by electric fishing and the river bottom using an electrified benthic frame trawl net, the national teams mainly focused on additional electric fishing in the littoral zones and also used different sampling methods (e.g. trammel nets) at some sites.

9.2.1 Littoral electric fishing

Electric fishing is the most used non-lethal method worldwide to sample fish in smaller rivers or shallow waters with the best efficiency in water depths below 2 meters. The sampling effort in inshore areas was based on the Austrian national guideline for fish sampling in running waters (Haunschmid et al., 2010) for assessing the ecological status according to the WFD (2000) and the European Standard "Water Analysis – Fishing with Electricity" (EN 14011, CEN, 2003) for wadeable and non-wadeable rivers.

The standardised sampling procedure for each site followed the habitat specific approach (strip fishing method) published by Schmutz et al. (2001) and consisted of 10 sampling strips during daylight and 5 strips during night, as fish assemblages in large rivers show different spatial distribution in the course of day and night (e.g. Reynolds, 1993 or Potyó et Guti, 2012).The German sites at Kelheim and Niederalteich have been sampled only by the Bavarian team due to legal restrictions, the methodology, however, was similar. Unfortunately, the following sites could not be sampled with the standardised effort due to bad weather conditions: Downstream Kolzduj (JDS51), Downstream Iskar (JDS72), Downstream Ruse (JDS57) and Downstream Silistra (60).

Fishing in general was conducted using an EFKO 11.00 KW DC generator with a 1,20 m long floating copper cathode. For the sampling of structured littoral habitats i.e. rip-rap shore lines, a 3 m long handheld anode (47 cm in diameter, net mounted; approx. 280V, 12A) was used moving upstream while catching the paralysed fish both with the handheld anode and a dip net. All other areas were sampled using a boom mounted anode (2.2 m width, 11 steel cables, 1 m each; approx. 580V, 20A) going downstream. The speed of the 5.1 m long electro fishing boat, equipped with a four stroke Honda 40 hp engine, during electrofishing was slightly higher than the flow velocity in order to generate an efficient electric field that leads to electro-taxis in fish individuals. The fishing team on the sampling boat consisted of two persons in the front catching the paralysed fish and one person steering the boat. For sampling purpose the electric field was activated by pushing the switch in irregular intervals. All fish showing electro-tactic movement towards the anode or paralysis were sampled with dip nets, put in a fish tank and afterwards determined to species level, measured (+/- 0,5 cm total length TL) and released alive. The coordinates of the start and end of each fishing strip were recorded by a GARMIN etrex VIST HC GPS and the sampled stretches were categorised according to its littoral habitat type. The length of each strip depended on the littoral structure and varied from approx. 100 to 200 m when the handheld anode was used and from approx. 200 to 400 m when the boom mounted anode was applied. Thus, a total length of 3000 m shoreline was sampled at each site. Littoral fish sampling by the national teams was done quite similar to this procedure.

9.2.2 Electrified benthic frame trawl

The electrified benthic frame trawl consisted of a stainless steel frame (2 m long × 1 m high, 3,4 cm tube diameter) with a drift net attached. The drift net was 5 m long and consisted of an inner mesh bag with 5 mm mesh size and an outer mesh bag with 8 mm mesh size. Weighted metal wheels were attached to the frame to keep the device close to the bottom and to keep the frame 6 cm above the bottom thus preventing the net from filling with substrate material. The frame was electrified with a 40

m long electrode cable which was connected to a Hans-Grassl EL65 II GI electrofishing device operated by a VANGUARD HP21 14.9 kW generator. A 6 m long copper cathode cable was attached to the tow rope and was hanging freely approximately 2 m before the electrified frame (Figure 69). Fishing (hereafter called trawling) was conducted along the flow direction with a 6,3 m long boat powered by a 50 hp outboard Mercury four stroke engine. The main sampling team consisted of three people, two handled the trawl net and the electrofishing device and one operated the boat. Occasionally an additional person, usually from a national fishing team, assisted the crew during sampling. The sampled stretches were measured by a GPS right after the trawl reached the bottom and the electroshocking started. The direct current (approx. 350 V, 33 A) was given for 5-8 sec. with 3-5 sec. breaks to minimize fright bias and injury of fish. Each time the electroshocking restarted, water depth was measured by a LOWRANCE X-50DS fishfinder. The applied trawling speed was slightly higher than the current velocity of the river (approx. 60 cm sec.$^{-1}$). The length of trawling stretches was usually 500 m. Sometimes the trawl got stuck due to large rocks or logs on the bottom thus shorter trawling stretches also occurred. Trawling was carried out only during daytime.

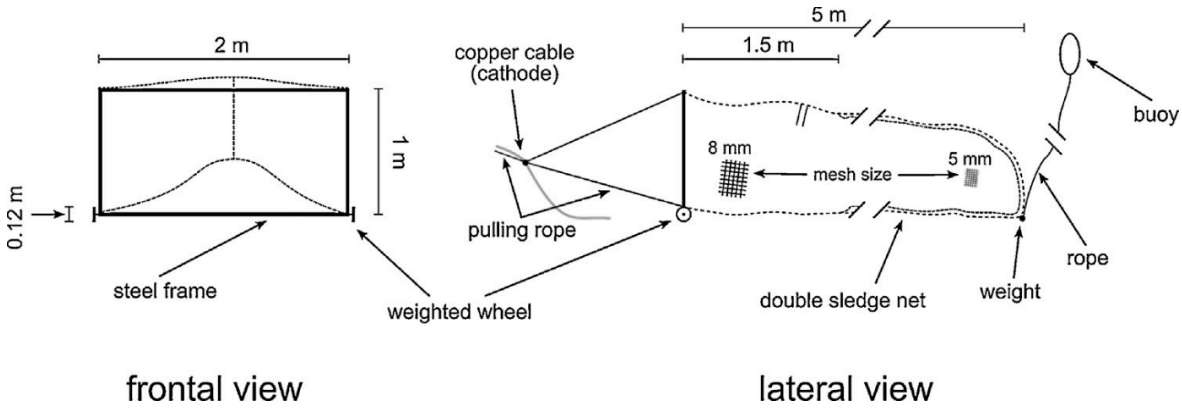

Figure 69: Schematic picture and parameters of the electrified benthic framed trawl (after Szalóky et al. 2014)

A total of 154 samples were collected at 22 sampling sites (Table 12) from the 32 previously designated river sections. Exceptions were at Kelheim (JDS2) and Niederalteich (JDS5) in Germany, where only one member of the core team joined the sampling of the national team, between Grocka (RS) and downstream Iskar (BG) (JDS52, JDS53, JDS57, JDS60, JDS62, JDS65, JDS67) bottom trawl sampling could not be done due to serious technical problems with the engine and also downstream Ruse, Giurgiu (JDS57) due to bad weather conditions. Seven side arms, however, were additionally sampled, one at Oberloiben (JDS8), two at Hainburg (JDS11) and one at Bratislava (JDS13), at Novi Sad downstream (JDS33), at Belegis (JDS36) and at Svishtov (JDS53). In each of the 22 sampling sites a minimum of two transects were selected randomly, perpendicular to the bank. Along every transect, across the width of the main channel, generally 3-5 trawl stretches were evenly distributed depending on the width of the river, excluding the littoral, less than 2 m deep shoreline zone.

Fish were identified and measured to the nearest mm standard lengths (SL) and then released back to the river. From some selected species (zingel, schraetser, streber, barbel, white-finned gudgeon), fin clip samples from 6 individuals per site if possible were collected for future genetic analysis.

9.2.3 Sampling effort

The final data set includes 32 sampling sites along the Danube. Due to legal restrictions, sampling in the German stretch (sampling sites Kelheim and Niederalteich) were conducted by the national Bavarian team, in the presence of the JDS3 Core Team leader. Furthermore, strong wind and technical problems led to a reduction of the sampling effort in the lower Danube section. As indicated in Table 12, the sampling effort of the various national teams differed significantly. On sites containing “JDS” in their code, fish was the only parameter that has been sampled during JDS3. More details about the

sampling sites are provided in the chapter 2. Most additional strips have been sampled by the Austrian national team, as part of their national monitoring program, whereas Serbia could not provide any national fishing team.

Table 12: Sampling effort (strips) of each JDS3 site

		Core team			National team							
site name	site code	electr. day	electr. night	bottom trawl	Electr. day	Electr. night	beach seine	gill net	Long-line	trammel net	bottom trawl	total effort
Kelheim	2				8	3						11
Niederalteich	JDS5				8	3						11
Jochenstein	6	10	5	3	25	10		8	10		2	73
Ybbs Persenbeug	JDS9	10	5	7	25	9		8	10		2	76
Oberloiben	8	10	5	6	34	8		8	10	2	2	85
Wildungsmauer	10	10	5	8								23
Bratislava	13	10	5	10	3							28
Cunovo	JDS17	10	5	8								23
Medvedov	15	10	5	9								24
Szob	20	10	5	5		5						25
Downstr. Budapest	22	10	5	6		5						26
Hercegszanto Mohacs	27	10	5	6		5						26
Upstream Drava	28	10	5	3	5	4						27
Ilok/Backa Palanka	31	10	5	9	6	5						35
Novi Sad downstream	33	10	5	9								24
Downstr. Tisa/Belegis	36	10	5	9								24
Upstr. Pancevo Downstr. Sava	38	10	5	6								21
Grocka	JDS54	10	5									15
Upstr. Velika Morava	40	10	5		5	3						23
Golubac/Koronin	44	6	5		3							14
Upstr. Timok (Radujevac/Gruia)	47	10	5		7	4						26
Downstr. Timok	48	10	5		5	2	2					24
Downstr.Kozloduj	51				4	3	1					8
Downstr.Iskar	JDS72		5		1							6
Downstr. Olt	52	10	5	7			1					23
Downstr. Svishtov	53	10	5	8	4	2						29
Downstr. Ruse	57		5			2	1	1				9
Downstr. Silistra	60	10		8	8	1						27
Downstr. Braila	62	10	5	6	8	3						32
Reni	65	10	5	6	1					17		39
Chilia Arm-Valcov	JDS93a	10	5	7						1		23
Sulina Arm	67	10	5	8		1						24
Total		**266**	**140**	**154**	**160**	**78**	**5**	**25**	**30**	**20**	**6**	**884**

9.2.4 Ecological Quality Assessment

According to the requirements of the Water Framework Directive, all EU-member states have to establish a monitoring network and develop assessment methods for all 4 biological quality elements in all natural water bodies. As the methodological approach for small and medium-sized rivers is well known and widely used, there are lots of reliable datasets which provide a sound basis for the development of appropriate assessment tools. There is a wide range of fish indices for such running waters from various EU member states, but until now only the German FiBs (fish based assessment approach) and the Austrian FIA (fish index Austria) constantly deliver reliable results also for large

rivers (Wiesner et al., 2007). For the evaluation of potential impacts on the fish fauna at the JDS3 sites we calculated the FIA and the EFI using the Danufishbase at the Institute for Water Ecology, Fisheries and Lake Research in Austria. The sampling for the Austrian national monitoring requires a certain minimum effort and includes additional methods (e.g. longlines, gillnets, trammel nets, etc.) which could not be applied during JDS3 due to the tight time schedule. For calculating the quantitative FIA parameters "abundance" and "biomass" all littoral strips sampled by the core team were used, all other data were included in the parameters "species and guilds" and the assessment of age structure.

Alterations of hydromorphological parameters such as impoundments are the main human impacts on the Austrian river stretches. The FIA was developed to detect the reactions of the fish fauna to these anthropogenic stressors. In accordance with Hughes and Oberdorff (1998) the basis for each WFD assessment method is the reference fish coenosis which reflects the historic species composition of a river in absence of human impacts. For that reason we used the reference coenoses from JDS2 which have been reviewed and – if necessary- adapted by the leaders of the JDS3 national teams.

For stretches in the upper and middle Danube section, additionally the FIS (Fish index of Slovakia) was calculated by Vlado Kovacs, an index, which also takes into account the presence of allochthonous species as a special metric. The FIA, as well as some EFI linked indices, detects the presence of alien species only by changes in dominance, absence of native species or low biomass values but not by a separate metric. As the FIS requires sampling of connected side arms (if existent in the sampling stretch) whereas the focus of JDS3 sampling was in the main channel, the FIS results may contain some uncertainties (Kovacs, personal communication).

9.2.5 Tissue samples

Tissue samples of the following fish species had been taken at each site for the analysis of specific biomarkers and organic substances: *Abramis brama, Alburnus Alburnus, Neogobius melanostomus, Neogobius kessleri, Aspis aspius*. Detailed procedures and results are described in the chapters 23 and 28.

9.3 Results

9.3.1 Total catch

In total more than 139.000 individuals representing 67 fish taxa and one jawless species (ammocoetes larvae) were caught during the JDS3 sampling by the core team (littoral and benthic) and national teams. In total two species, namely *Alburnus alburnus* and *Neogobius melanostomus* dominated the catches by far (see Figure 70) with a relative proportion of 46% and 26% of the total catch respectively. Most abundant (>55%) were eurytop individuals, followed by allochthonous species (>30%). Please note that for reasons of clearability and comprehensibility in Figure 70 only the 20 most abundant species are shown and the occurrence of Neogobius species is expressed separately for the Danube downstream the Iron Gate dam, where it is autochthonous (indicated by a single star) and upstream, where it is allochthonous (indicated by a double star). Until now, there is no clear classification of the katadromous *Anguilla anguilla* concerning its rheophilic preference. The same is the case for *Neogobius melanostomus*. A comparison with the total catch results of JDS2 shows, that there has been a drastic shift of the total species frequency: allochthonous species, most notably *Neogobius melanostomus* have been caught more often during JDS3 than JDS2 outside of their range of natural occurrence (31,491 vs. 3,389 specimens). This results show a dramatic, active distribution of the round goby in the Danube basin. Other smaller changes most probably derive from the different sampling effort of the two surveys.

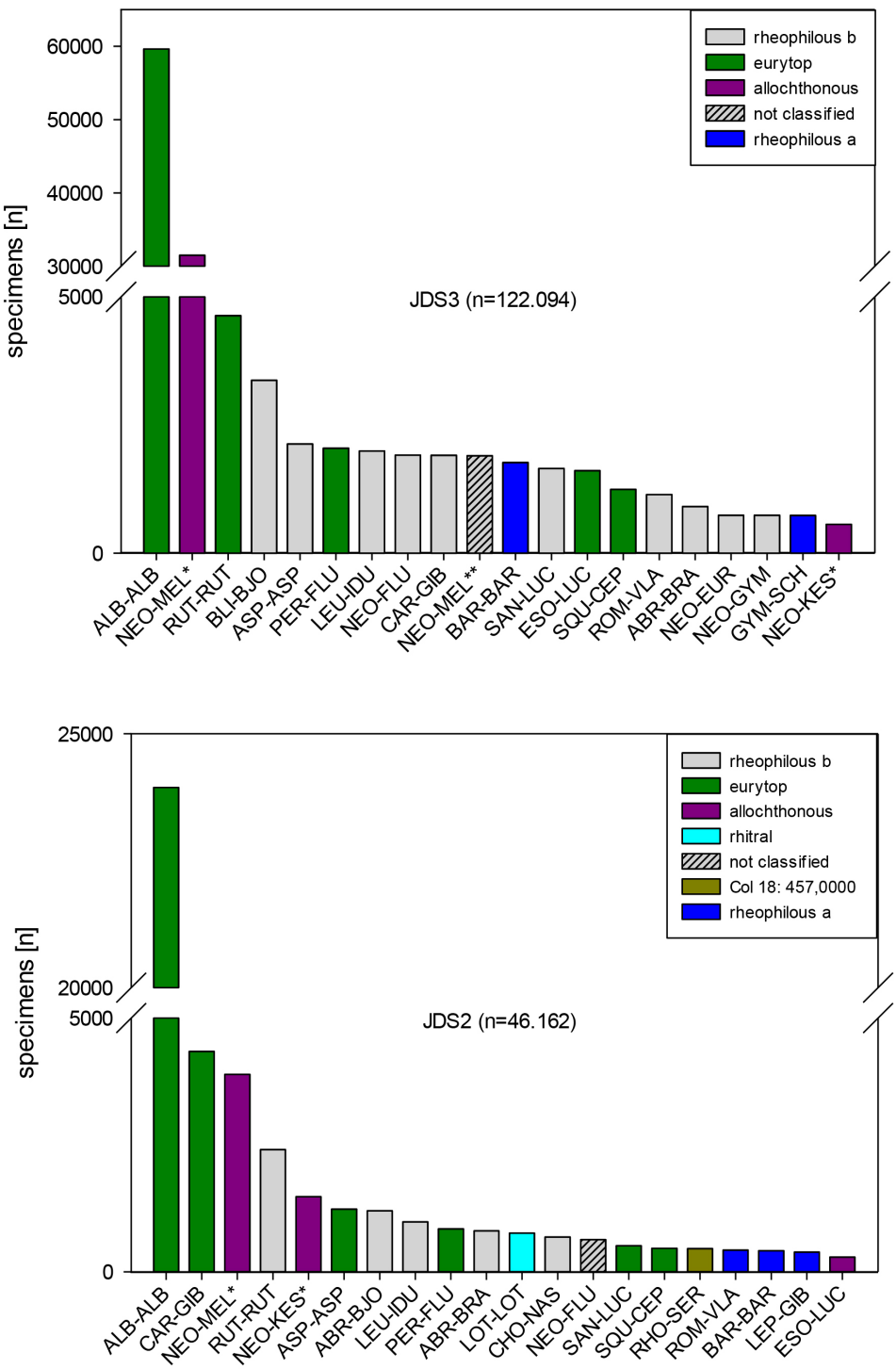

Figure 70: 20 most abundant species and their numbers caught during JDS3 (above) and JDS2 (below)

Table 13: Absolute [n] and relative [%] number of species detected by different teams and methods; ct=core team, nt=national team, day=littoral sampling at day, night=littoral sampling at night, bottom=sampling with the electrified bentic frame at day

	ct [n]				nt [n]	species overall	ct [%]				nt [%]
river km	day	night	bottom	total	total		day	night	bottom	total	total
2420					29	29					100,0
2278					28	28					100,0
2215	11	13	5	15	25	27	40,7	48,1	18,5	55,6	92,6
2072	4	9	5	10	23	27	14,8	33,3	18,5	37,0	85,2
2010	9	16	15	19	39	40	22,5	40,0	37,5	47,5	97,5
1894	16	17	15	20		25	64,0	68,0	60,0	80,0	0,0
1876	17	14	16	20	19	30	56,7	46,7	53,3	66,7	63,3
1852	20	16	6	22		24	83,3	66,7	25,0	91,7	0,0
1807	18	26	13	27		29	62,1	89,7	44,8	93,1	0,0
1705	13	25	11	28	25	29	44,8	86,2	37,9	96,6	86,2
1632	12	22	14	24	22	29	41,4	75,9	48,3	82,8	75,9
1446	16	22	11	23	25	32	50,0	68,8	34,4	71,9	78,1
1380	19	21	13	25	22	32	59,4	65,6	40,6	78,1	68,8
1303	17	18	13	22	28	33	51,5	54,5	39,4	66,7	84,8
1252	15	17	14	20		26	57,7	65,4	53,8	76,9	0,0
1202	13	14	14	20		25	52,0	56,0	56,0	80,0	0,0
1163	13	16	16	20		24	54,2	66,7	66,7	83,3	0,0
1132	11	14		17		17	64,7	82,4	0,0	100,0	0,0
1107	20	18		25	31	38	52,6	47,4	0,0	65,8	81,6
1046	16	18		22	24	27	59,3	66,7	0,0	81,5	88,9
1027	20	24		27	29	35	57,1	68,6	0,0	77,1	82,9
850	15	13		15	26	27	55,6	48,1	0,0	55,6	96,3
690					28	29	0,0	0,0	0,0	0,0	96,6
634		20		20	9	21	0,0	95,2	0,0	95,2	42,9
602	20	22	9	25	12	28	71,4	78,6	32,1	89,3	42,9
557	17	17	11	21	31	35	48,6	48,6	31,4	60,0	88,6
485		19		19	17	27	0,0	70,4	0,0	70,4	63,0
383	17		13	17	31	36	47,2	0,0	36,1	47,2	86,1
172	18	24	19	28	27	37	48,6	64,9	51,4	75,7	73,0
136	18	21	21	23	22	31	58,1	67,7	67,7	74,2	71,0
60	14	20	19	23		29	48,3	69,0	65,5	79,3	0,0
21	24	15	20	24	19	35	68,6	42,9	57,1	68,6	54,3

Table 13 shows the absolute numbers of species detected with the different methods by the core team and national teams and their relative proportion of the overall number of species found at the sampling sites during the survey. The largest number of species (n=40) has been found near Oberloiben (rkm 2010), which is due to a relatively high sampling effort by the national team. Despite the relatively low sampling effort (8 strips during day and 3 strips during night) by the national team in the 2 German sites (Kehlheim and Niederalteich) the number of species caught, especially during day, was comparably high. This might be due to the fact, that these sites were quite "natural" with few hydro-morphological deficits, and a high depth of visibility which explains the high efficiency of daytime electrofishing. Table 13 points out the importance of the additional sampling done by the national teams concerning the completion of the species inventory.

9.3.2 Electrified benthic trawl

During JDS3 a total of 4445 specimens from 38 species could be collected by electrified benthic frame trawl sampling (Figure 71). The results show, that *Neogobius melanostomus* (36.5% relative abundance) is the dominant species even in benthic habitats, followed by *Romanogobio vladykovi* (14.7%) and *Blicca bjoerkna* (10.1%). The relative abundance of the other species was below 10%. Please note that in Figure 71 occurrence of Neogobius species is shown separately for the Danube downstream the Iron Gate dam (indicated by a single star) and upstream (indicated by a double star) and only the 20 most abundant species are illustrated.

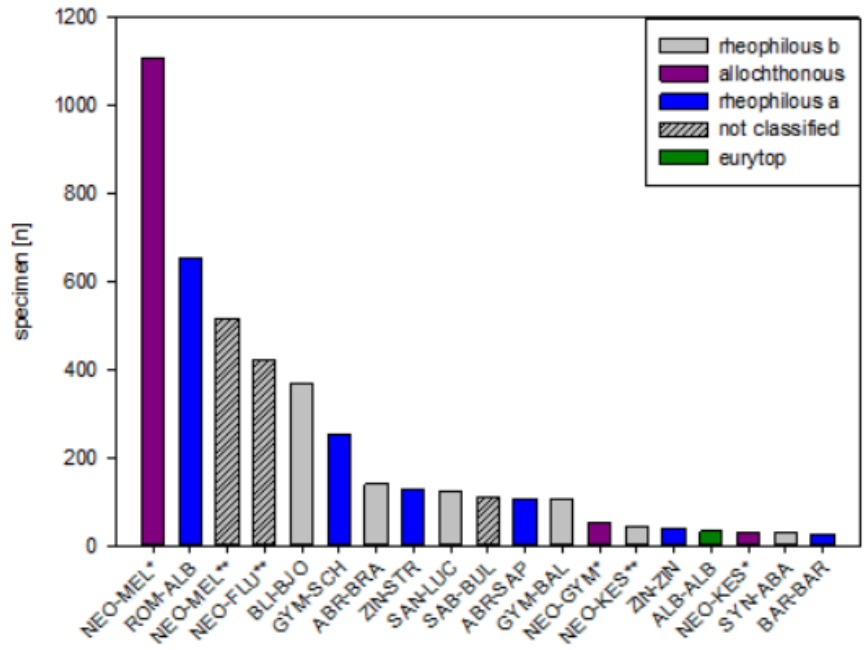

Figure 71: 20 most abundant species caught by electrified benthic frame trawl (n=4.270)

Electrified benthic frame trawls detected *Acipenser ruthenus* L. (Figure 72) at three sampling sites with three individuals at Belegis (JDS36), Reni (JDS65) and Valcov in the Chilia Arm (JDS93a). This species could not be detected by shoreline surveys, despite the high sampling effort. Similarly *Alburnus mento* (Heckel) (Figure 73) was detected only by trawling at Ilok/Backa Palanka (JDS31) close to the inflow of River Drava. Moreover, the electrified benthic frame trawling could detect the monkey goby (*Neogobius fluviatilis*) for the first time in the Austrian section of the River Danube.

Figure 72: *Acipenser ruthenus*

Figure 73: *Alburnus mento*

9.3.3 Ecological guilds

The composition of rheophilic guilds of JDS3 in comparison to JDS2 is shown in Figure 74. Eurytopic species were most dominant at both surveys, although their proportion decreased between 2007 and 2013. Neogobius species are categorised as "not classified" in Figure 74, because no common ecological guild classification was done until now.

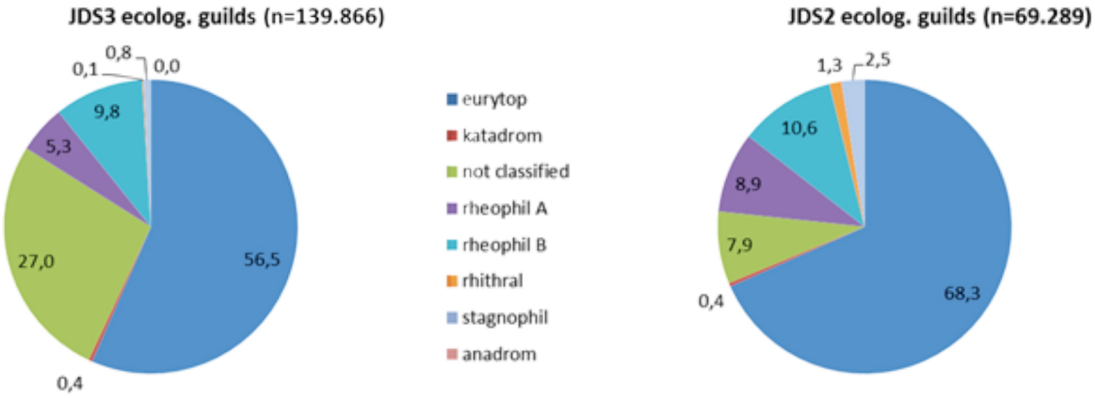

Figure 74: Ecological guilds (according to Schiemer & Waidbacher (1992) as proportion of the total catch; JDS3 left, JDS2 right

9.3.4 Allochtonous species

As shown in Figure 75, the proportion of allochthonous species to the total number of species differs significantly between sampling sites upstream and downstream of the Iron Gate Dam. Between 2007 and 2013 this value has more than doubled from 17.8 to 37 percent at sampling sites upstream the migration barrier, whereas downstream a decrease from 2.6 to 0.3% could be detected. Concerning the entire river course an increase from 19.9 to 24.95% was found.

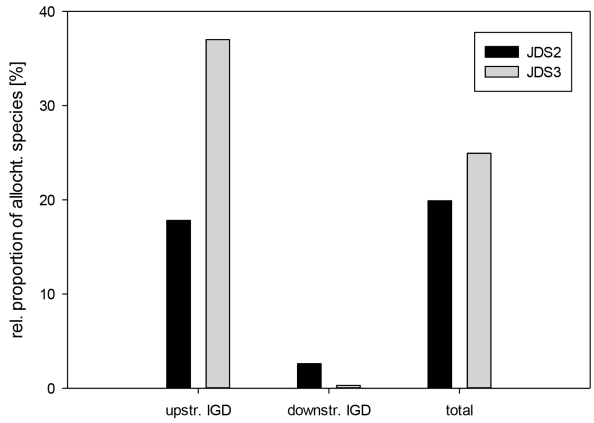

Figure 75: Proportion of alien species to the total catch for the entire Danube River and sections up-and downstream the Iron Gate Dam (IGD)

For a better understanding, Table 14 shows the species considered allochthonous in the specified Danube river sections according to Wiesner et al. (2007).

Table 14: Danube fish species considered allochthonous in specified Danube river sections

stretch	species name
entire course	*Ameiurus melas, Ameiurus nebulosus, Anguilla anguilla, Gasterosteus aculeatus, Hypophthalmichthys molitrix, Lepomis gibbosus, Oncorhynchus mykiss,*
upstream Iron Gate dam	*Neogobius spp., Syngnatus spp., Perccottus sp.*
upstream Morava river	Additionally *Protherorhinus semilunaris*

Table 15 shows the relative proportion of single alien species to all species classified as allochthonous in the entire Danube River as well as upstream and downstream of the Iron Gate Dam that have been detected during JDS2 and JDS3. During both studies, *Neogobius melanostomus* was found to be highly dominant outside its natural range of occurrence with a proportion of the total catch of 56.7% during JDS2 and even 92.8% during JDS3. The proportion of the second most abundant allochthonous species, *Neogobius kessleri*, declined from 20.9 to 1.8 percent. The abundance of other species can be seen as negligible. Note the drastic rise in the numbers of allochthonous specimens caught during JDS3.

Table 15: Proportion of single species to the group of allochthonous fish upstream and downstream the Iron Gate Damn (IGD) and in total

	upstream IGD		downstream IGD		total	
	JDS2 (n=6.868)	JDS3 (n=34.800)	JDS2 (n=268)	JDS3 (n=137)	JDS2 (n=7.136)	JDS3 (n=34.937)
Ameiurus melas	0.3				0.3	
Ameiurus nebulosus	0.0				0.0	
Anguilla anguilla	3.3	1.6			3.2	1.6
Bentophilus stellatus		0.0				0.0
Gasterosteus aculeatus	1.4	0.0			1.3	0.0
Hypophthalmichthys molitrix	0.0	0.0	0.7		0.0	0.0
Lepomis gibbosus	3.5	0.3	53.7	97.8	5.4	0.6
Neogobius eurycephalus		0.7				0.7
Neogobius fluviatilis	7.1	1.1			6.8	1.1
Neogobius gymnotrachelus	2.4	0.8			2.4	0.8
Neogobius kessleri	21.7	1.8			20.9	1.8
Neogobius melanostomus	59.0	93.2			56.7	92.8
Oncorhynchus mykiss	0.0	0.0			0.0	0.0
Perccottus glenii	0.3	0.3			0.3	0.3
Proterorhinus semilunaris	0.2	0.2			0.2	0.2
Pseudorasbora parva	0.7	0.1	45.5	2.2	2.4	0.1
Syngnathus abaster	0.0				0.0	

Similar to the situation for the entire river course, the undisputed dominance of *Neogobius melanostomus* in allochthonouis species upstream the Iron Gate Dam can be seen clearly. Also the same drastic rise in its proportion from 59 to 93.2% can be noticed between 2007 and 2013. Downstream the migration barrier only three (JDS2) and two (JDS3) species respectively could be found outside their natural range of distribution: *Lepomis gibbosus, Pseudorasbora parva* and *Hypothalmichtys molitrix*. The proportion of *L. gibbosus* has risen from 53.7 to 97.8 percent, whereas *P.parva* showed a decline from 45.5 to 2.2 percent. Only two specimens of *H. molitrix* could be caught in the lower Danube section during JDS2. Compared to the number of allochthonous specimens,

caught upstream of the Iron Gate Dam (34,800) during JDS3, the 137 specimens caught downstream is remarkably low.

9.3.5 Ecological status

In Table 16 FIA, EFI and FIS statuses, calculated for each sampling site, are shown. It is obvious, that these indices react differently to various stressors and as a consequence deliver diverting results for most of the stretches. The national assessment systems used for JDS3 are not adapted for the entire Danube course, even EFI was shown as not being feasible for the assessment in certain parts of the Danube river during the intercalibration exercise. There is need to mention, that the assessment exercise done during JDS is not adequate to the national assessment due to these or even methodological restrictions.

Table 16: FIA, EFI and FIS statuses calculated for each site and the corresponding indication of the WFD classification (* insufficient data set)

site name	rkm	JDS2		JDS3		
		Status FIA	Status EFI	Status FIA	Status EFI	Status FIS
Kelheim, DE_JDS02	2420	Good	Good	Good	Good	Poor
Niederalteich, DE_JDS05	2278	Good	Good	Good	Good	Bad
Jochenstein, AT_JDS07	2215	Poor	Good	Bad	Good	Bad
Ybbs, AT_JDS09	2072	Bad	Moderate	Bad	Good	Poor
Oberloiben, AT_JDS10	2010	Poor	Good	Bad	Good	Good
Wildungsmauer – Hainburg, AT_JDS13	1894	Good	Good	Moderate	Moderate	Moderate
Bratislava, SK_JDS16	1876	Moderate	Moderate	Good	Moderate	Moderate
Cunovo, SK_JDS17	1852	Bad	Poor	Moderate	Poor	Bad
Medvedov, HU_JDS18	1807	Bad	Good	Moderate	Moderate	Moderate
Szob, HU_JDS26	1705	Moderate	Good	Good	Moderate	Moderate
Budapest downstream, HU_JDS32	1632	Good	Good	Good	Moderate	Poor
Mohacs Hercegszanto, HU_JDS39a	1446	Good	Good	Good	Moderate	Moderate
Upstream Drava, Aljmas, HR_JDS41	1380	Moderate	Moderate	Good	Moderate	Moderate
Ilok, Backa Palanka, HR_JDS45	1303	Moderate	Moderate	Moderate	Moderate	Bad
Novi Sad downstream, RS_JDS47	1252	Moderate	Moderate	Moderate	Moderate	Poor
Belegish, RS_JDS50	1202	Moderate	Moderate	Poor	Moderate	Moderate
Downstream Sava, RS_JDS52	1163	Moderate	Moderate	Moderate	Bad	Poor
Grocka, RS_JDS54	1132	Moderate	Moderate	Moderate	Poor	Bad
Velika Morava downstream, RS_JDS57	1107	Good	Moderate	Good	Moderate	Bad
Golubak Koronin, RO JDS 60	1046	Moderate	Bad	Good	Poor	
Vrbica, Simijan, RO_JDS63	1027			Good	Moderate	
Near Timok, RO JDS 65	850		Moderate	Moderate	Poor	
Downstream Kozloduy, BG_JDS69	690		Poor	*	*	
Downstream Iskar, BG_JDS72	634		Poor	*	*	
Downstream Olt, RO JDS 75	602		Moderate	Moderate	Poor	
Downstream Ruse – Giurgiu, RO JDS 82	485		Moderate	*	*	
Chiciu, Silistra, BG_JDS86	383	Bad	Poor	Poor	Moderate	
Downstream Braila, RO JDS 89	172		Moderate	Good	Moderate	
Reni, RO JDS 91a	136		Moderate	Good	Moderate	
Chilia Arm-Valcov, RO JDS 93a	60		Moderate	Good	Moderate	
Sulina – Sulina Arm, RO JDS 95	21		Moderate	Good	Moderate	

As mentioned above, the FIA strongly reacts to hydromorphological alterations which represent the main threat for the fish fauna especially in the Austrian stretch. The heavily modified sections in a chain of impounded areas in Austria (Jochenstein, Ybbs, Oberloiben) show a bad ecological status according to the FIA mainly due to the low fish biomass, whereas EFI indicates a good status as a consequence of high numbers of species and ecological guilds. The FIA values for most stretches differ only slightly between JDS2 and JDS3, which shows it is a consistent assessment of the ecological status even of large rivers. The FIS, in contrast, detects a strong negative reaction of the fish fauna at the sites "Jochenstein" and "Ybbs" especially due to a lack of benthic and piscivorous species, which could also indicate a methodological inaccuracy of the reference coenosis (Kovacs pers. comm.). For stretches in the lower Danube the FIA mostly indicates a good or moderate status, which coincides with the more or less natural habitat conditions in the lower course. In this area EFI detects an impact on the fish communities which leads to mainly moderate results. This could be explained by the deterioration of the water quality, especially downstream of larger cities or by the influence of fishery. The results clearly show deficits in the Danubian fish fauna and indicate the demand for action and the urgency for a harmonisation of the sampling methods as well as of the WFD assessment process for the biological quality element fish in large running waters.

Table 17: Percentage share of the 5 ecological status classes of the calculated WFD indices

Ecol. status	JDS2		JDS3		
	Status FIA	Status EFI	Status FIA	Status EFI	Status FIS
High	-	-	-	-	-
Good	28.6	30.0	50.0	17.9	5.3
Moderate	42.9	53.3	32.1	60.7	36.8
Poor	9.5	13.3	7.1	17.9	26.3
Bad	19.0	3.3	10.7	3.6	31.6

Table 17 shows the percentage share of the five ecological status classes according to the WFD separately for FIA, FIS and EFI based on results of JDS2 and JDS3. Not one sampling site showed a high ecological status during both surveys. Interestingly, the percentage of sites with a good ecological status according to the FIA increased from 28.6% during JDS2 to 50% during JDS3, whereas the "good" sites according to EFI decreased from 28.6% to 17.9%. In the case of FIA, this phenomenon might reflect the higher biomass recorded in 2013 which acts a K.O. criterion and also explains the higher percentage of poor and bad sites during JDS2. According to the FIS, only 5.3% of the validated sites showed a good status. In total, all assessment methods of JDS3 indicate a call for action as 50% of the sites according to FIA, 72.1% (EFI) and 94.7% (FIS) respectively show a value worse than "good" and do not meet the goal of the WFD.

9.4 Conclusions

The planned sampling effort appeared to be feasible for a fish ecological investigation of a large river, despite a tight time schedule, some technical problems and unsuitable weather conditions. Due to the high sampling effort (day & night sampling), the fish core team had to be independent from the lab ships. While the core team generated consistent data providing a solid basis for assessing the ecological status of the river Danube in compliance with the Water Framework Directive, the additional sampling effort conducted by the national teams was essential for a concise description of the Danubian fish fauna. The high species numbers detected by the national Bavarian team could be explained by methodological variations: most strips in Kelheim and Niederalteich were samples by using handheld anode, whereas at other sampling sites the boom anode was used more often.

The electrified benthic frame trawl indicated the commonness of specific benthic species along the Danube and added valuable information which would have remained hidden using only shoreline surveys. It revealed the common occurrence and relatively high abundance of Zingel species, especially of *Zingel streber* which occurred at 16 sampling sites with 127 individuals (cf. with all the other methods only 84 individuals were caught at 8 sites). To emphasize the importance of the application of the electrified benthic frame trawl, note that the JDS2 survey, without this method, could not prove the occurrence of *Zingel streber* in the Hungarian river section of the Danube (Wiesner *et al*. 2007). This large scale spatial survey revealed that benthic offshore areas are intensively used by a variety of species which are distributed relatively homogenously along the entire river course. Their abundance and species composition, however, can vary largely within the standardised sample stretches.

Both the abundance and quantitative proportion of invasive and non-native species change along the Danube depending on the habitat types and shoreline structure (e.g. rip rap). Thus, allochthonous Neogobius species were found in high or even dominating abundance along the rip-rap protected banks in the upper and middle course of the Danube, while downstream the Iron Gate, where this habitat is not frequent, their abundance was much lower.

The three applied national WFD assessment indices of JDS3 indicate a call for action as 50% of the sites according to FIA, 72.1% (EFI) and 94.7% (FIS) respectively show a value worse than "good" and do not meet the requirements of the WFD.

9.5 Summary

- In total 139,866 individuals representing 67 fish taxa could be caught, by the core team and the national teams which underlines the importance of the Danube river as ecosystem for a wide range of fish species;
- The electrified benthic frame trawl proved to be a great additional sampling method, detecting species not caught by littoral sampling;
- The Danubian fish fauna is heavily influenced by non-native species which can be found in all habitats, even close to the river bottom and partly in remarkable densities. It appears that the dominance of Neogobius species in the Upper Danube has dramatically increased since JDS2, especially in altered littoral structures as rip rap;
- In the upper course of the Danube the fish fauna mainly reflects hydromorphological alterations and damming as most important human impacts, but also the lack of connectivity along the whole river stretch;
- The use of waterpower in the upper Danube, which consequently leads to an impoverishment of aquatic habitats can be detected easily by the absence of sensitive species and certain age classes and is clearly indicated by the applied national WFD assessment indices FIA and FIS;
- The lower course of the Danube seems to be influenced by professional & recreational fishery and poaching, which was obvious during JDS3 sampling;
- The applied national fish indices (FIA, FIS, EFI) deliver inconsistent results for the whole river course and indicate, that they react on different stressors (hydromorphology vs. water quality) and are hence applicable for restricted river stretches only;
- The assessment exercise done during JDS is not adequate to the national assessment due to certain restrictions;
- Especially in the Lower Danube additional sampling methods (e.g. trammel nets) are required to complete the data set;
- A methodological harmonisation for large European rivers is an important task to meet the goals of the WFD.

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10 Invasive species

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10.1 Introduction

Aquatic ecosystems are exposed to the influence of non-indigenous (non-native, alien or exotic) species. The Danube River is not an exception. Non-indigenous species were recorded among algae, aquatic macrophytes, macroinvertebrates and fish. Also, introduction of allochthonous fish species caused introduction of new fish parasites (Djikanovic et al. 2012).

The pressure caused by biological invasions has already been documented for the Danube River and its main tributaries (Literáthy et al. 2002, Csányi 2002, Csányi & Paunović 2006, Liška et al. 2008). The Danube River is a part of the Southern Invasive Corridor (Panov et al. 2009). The Southern Corridor links the Black Sea with the North Sea basin *via* the Danube-Main-Rhine waterway including the Main-Danube Canal. Thus, the Danube River is a part of one of the main routes for the migration of aquatic organisms in Europe, including the non-native species and consequently the river is exposed to high potential pressure from biological invasions.

The aim of this chapter is to present the state of the art in respect to presence of non-native aquatic species (aquatic macrophytes, aquatic macroinvertebrates and fish) in the Danube River based on Joint Danube Survey 3 (JDS3) results. Also, the present situation is compared with prior, based on previous Danube Surveys.

10.2 Methods

The study on presence and abundance of non-indigenous taxa was done based on additional analysis of datasets obtained for each biological quality element surveyed during Joint Danube Survey 3 – for details, please see Chapters 5, 7 and 9 of this report.

In addition, free diving collection was done to collect information on relative abundance of non-indigenous freshwater mussel species.

For supplementary collection of crayfish species, LiNi crayfish traps with appropriate bite (small fish, wet cat food or fresh liver, etc.) were used. At selected sites, 5-15 traps were positioned for more than 5 hours during the night at different depths and bottom types, thus covering majority of possible finding places (activity areas).

The review of neophytes along the Danube River comprised, beside non-native aquatic macrophytes, the vegetation of bank habitats.

The evaluation method presented below is not accomplished to estimate the level of biological invasion in the Danube countries and is therefore not comparable to national data on the invasion of alien species. The evaluation of invasive species is not included in the ecological assessment of surface waters according to Water Frame Directive.

In order to estimate the level of biological invasions we used the Site-specific Biological Contamination (SBC) Index (Arbačiauskas et al. 2008). SBC index estimates biological contamination (means the presence of alien species regardless of their abilities to cause negative ecological and/or socio-economic impacts) of the specific sites. It is used for comparison of biological contamination of

different locations and for estimation. Site-specific Biological Contamination (SBC) involves both the specific value of number of alien species and the specific value of an abundance of alien species in the total community by using the formula:

$$SBC = (n_a / n_{sum} + \log N_a / \log N_{sum}) / 2,$$

where n_a is a number of alien species, n_{sum} a number of all species in the sample, N_a abundance of alien species and N_{sum} total abundance of fish in the sample. For the calculation of SBC, the results of macroinvertebrate and fish JDS3 surveys were used. JDS2 datasets on macroinvertebrates (Liška et al. 2008) were additionally used to calculate SBC and compare the level of biological contamination over the time.

SBC index was calculated using ranking presented in Table 18, based on samples from the shore region only, in order to make the data comparable with JDS2 results. The index ranges from 0 to 4 and the following classification scale was used (modified original scale proposed by Arbačiauskas et al. 2008):

0 (no biocontamination, no pressures caused by biological invasions)

1 (low biocontamination, minor pressures caused by biological invasions)

2 (moderate biocontamination, moderate pressures caused by biological invasions)

3 (high biocontamination, high pressures caused by biological invasions)

4 (severe biocontamination, high pressures caused by biological invasions).

Table 18: Scoring scheme for SBC

Taxa Richness Contamination%	Abundance Contamination%				
	0	>0 – <10	>10-20	21-50	>50
0	0				
>0 – <10		1	2	3	4
>10-20		2	2	3	4
21-50		3	3	3	4
>50		4	4	4	4

The results were discussed in relation to basic sectioning of the Danube River to Upper, Middle and Lower Danube, as defined in Literáthy et al. (2002) and Liška et al. (2008).

10.3 Results

During the JDS3 a considerable number of non-native aquatic species was recorded. Non-indigenous taxa were identified within aquatic macrophytes, macroinvertebrates and fish.

10.3.1 Neophyta

Neophytes are plant species which are non-native to a geographical region but they were introduced in recent history. They are present all around the world and the Danube is certainly not a river that neophytes would avoid. On the contrary, historical investigation shows presence of neophytes throughout the whole stretch of the Danube. They invade main channel, side channels and standing water next to the main river (Janauer and Steták 2003, Sipos et al. 2003, Valchev et al. 2006).

Besides of many individual researches of the Danube vegetation where neophytes were recorded, they were identified in both Joint Danube Survey 1 and 2 expeditions as well (Literáthy et al. 2002, Liška et al. 2008).

During Joint Danube Survey 3, out of 25 recorded neophytes, four of them belonged to aquatic macrophytes: *Azolla filiculoides* Lam., *Elodea nuttallii* (Planch.) H.St.John, *Lemna turionifera* Landolt and *Vallisneria spiralis* L. (Table 19). Other species belonged to the bank vegetation as species of open and ruderal habitats like *Xanthium strumarium* L. that was dominating on Romanian and Bulgarian banks of the Danube and sometimes pushed out other helophytes, or *Amaranthus blitum* L. dominating on the banks downstream Belgrade.

Most of the listed species are aggressive and fast spreading. Two species of neophyte trees recorded during JDS3 share the same characteristics, *Robinia pseudoacacia* L. and *Ailanthus altissima* (Mill.) Swingle. Hanover and Mebrahtu (1991) reported that *R. pseudoacacia* is adaptable to environmental extremes such as drought, air pollutants and high light intensities and that it easily propagates by seed, coppice and root suckers. It is an aggressive, thorny pioneer species and as such presents a threat to native riparian vegetation.

Among macrophytes, *V. spiralis* was the most abundant neophyte during this survey, while other species were found only occasionally. It was first recorded in Hungary upstream Budapest in Szob. Later on it was sporadically present in Danube around the tributary Velika Morava, but also very abundant downstream the Iron Gate (Đerdap) Reservoir.

Table 19: List of neophyte species recorded during JDS3

Species	Origin
Abutilon theophrasti Medik.	Southern Asia
Ailanthus altissima (Mill.) Swingle	East Asia
Amaranthus blitum L.	Mediterranean region
Ambrosia artemisiifolia L.	North America
Amorpha fruticosa L.	North America
Asclepias syriaca L.	North America
Azolla filiculoides Lam.	Subtropical America
Bidens frondosa L.	North America
Buddleja davidii Franch.	East Asia
Datura stramonium L.	North and South America
Echinocystis lobata (Michx.) Torr. & A. Gray	North America
Eclipta prostrata (L.) L.	Tropics, Subtropics
Elodea nuttallii (Planch.) H.St.John	North America
Fallopia japonica (Houtt.) Ronse Decr.	East Asia
Helianthus annuus L.	North America
Impatiens glandulifera Royle	Asia
Impatiens parviflora DC.	Eurasia
Lemna turionifera Landolt	North America, East Asia
Robinia pseudoacacia L.	North America
Rudbeckia hirta L.	North America
Solidago canadensis L.	North America
Symphyotrichum lanceolatum (Willd.) G.L.Nesom	North America
Vallisneria spiralis L.	Tropics, Subtropics
Xanthium spinosum L.	South America
Xanthium strumarium L.	North America

10.3.2 Non-native aquatic macroinvertebrates

The list of non-native macroinvertebrate species recorded during the JDS3 is presented in Table 20.

Out of 34 non-native taxa recorded during the JDS3, crustaceans are the most numerous, with 19 species – Amphipoda 13, Mysida 3, Isopoda 1 and Decapoda 2. Eight alien molluscs (Bivalvia 5 and Gastropoda 3) and four annelids (Oligochaeta 2 and Polychaeta 2) were recorded.

A considerable number of alien species were recorded in the Upper and Middle section of the Danube – 24 and 27 species, respectively. Having in mind that the majority of the species identified as non-native for the Upper and Middle section of the Danube are of Ponto-Caspian origin, those species are considered as native for the Lower stretch of the Danube and thus, only seven non-native taxa were found for the Lower Danube section (marked with * – Table 20).

Table 20: Non-native macroinvertebrates recorded during the JDS3

Species	Origin
Bryozoa	
Pectinatella magnifica (Leidy 1851)*	North America
Turbellaria	
Dendrocoelum romanodanubiale (Codreanu 1949)	Ponto-Caspian
Polychaeta	
Hypania invalida (Grube, 1860)	Ponto-Caspian
*Manayunkia caspica (*Annenkova, 1928)	Ponto-Caspian
Oligochaeta	
Branchiura sowerbyi (Beddard, 1892)*	Indo-Pacific
Potamothrix moldaviensis (Vejdovsky and Mrazek, 1902)	Ponto-Caspian
Bivalvia	
Corbicula fluminea (O. F. Müller, 1774)*	East Asia
Corbicula fluminalis (O. F. Müller, 1774)*	East Asia
Sinanodonta woodiana (Lea, 1834)*	East Asia
Dreissena polymorpha (Pallas, 1771)	Ponto-Caspian
Dreissena bugensis (Andrusov, 1897)*	Pontic
Gastropoda	
Physella acuta (Draparnaud, 1805)*	North America
Potamopyrgus antipodarum (J. E. Gray, 1853)*	New Zealand
Potamopyrgus antipodarum f. carinata (J. T. Marshall, 1889)	New Zealand
Decapoda	
Orconectes limosus (Rafinesque, 1817)	North America
Pacifastacus leniusculus (Dana, 1852)	North America
Amphipoda	
Chelicorophium robustum (G. O. Sars, 1895)	Ponto-Caspian
Chelicorophium curvispinum (G. O. Sars, 1895)	Ponto-Caspian
Chelicorophium sowinskyi (Martynov, 1924)	Ponto-Caspian
Echinogammarus ischnus (Stebbing, 1899	Ponto-Caspian
Echinogammarus trichiatus (Martynov, 1932)	Ponto-Caspian
Gammarus roeseli (Gervais, 1835)	
Obesogammarus obesus (G. O. Sars, 1894)	Ponto-Caspian
Obesogammarus crassus (G. O. Sars, 1894)	Ponto-Caspian
Pontogammarus sarsi (G. O. Sars, 1894)	Ponto-Caspian
Dikerogammarus villosus (Sowinsky, 1894)	Ponto-Caspian
Dikerogammarus haemobaphes (Eichwald, 1841)	Ponto-Caspian
Dikerogammarus bispinosus (Martynov, 1925)	Ponto-Caspian
Mysida	
Limnomysis benedenii (Czerniavsky, 1882)	Ponto-Caspian
Katamysis warpachowsky (G. O. Sars, 1877)	Ponto-Caspian
Paramysis lacustris (Czerniavsky, 1882)	Ponto-Caspian
Niphargus hrabei (S. Karaman, 1932)	Ponto-Caspian
Isopoda	
Jaera istri (Vieuille, 1979)	Ponto-Caspian
Trichoptera	
Cladotanytarsus conversus (Johannsen, 1932)	Southeast Asia

Among non-native macroinvertebrates, taxa of North American (4), Asian (4) of New Zealand (2) and Indo-Pacific (1) origin were identified, but spreading of Ponto-Caspian species from the Lower to the Middle and Upper Danube was found to be the most frequent case – 22 taxa of Ponto-Caspian original distribution were identified during the JDS3.

During the JDS3, the North American freshwater bryozoans species *Pectinatella magnifica* (Leidy 1851) (Bryozoa: Phylactolaemata: Plumatellida; common names: magnificent bryozoan or moss animal) was recorded for the first time in the main course of the Danube (Zorić et al. 2014). After the initial detection of the magnificent bryozoan in the Rackeve-Soroksar Danube side Arm in 2011 (Szekeres et al. 2013), the species rapidly colonized a 900 km long stretch of the Danube, between river kilometres 1586 (Hungary, downstream Budapest) and 685 (Romanian-Bulgarian stretch of the Danube). Beside, spreading of *Manayunkia caspica* Annenkova, 1929, a Ponto-Caspian species, was recorded in the Middle Danube – sites 16-21.

Crustaceans of Ponto-Caspian origin *C. curvispinum, D. villosus* (Amphipoda) and *J. istri* (Isopoda), as well as molluscs species of Asian origin *C. fluminea* (Bivalvia) were found to be the most abundant and frequent non-native macroinvertebrate taxa along the entire Danube. Thus, mean abundance of *D. villosus* was 529 ind./m^2 for the Upper Danube and 431 ind./m^2 for the Middle Danube, while the abundance of *C. curvispinum* was 247 ind./m^2 for the Upper Danube and 310 ind./m^2 for the Middle Danube (both species native for the Lower Danube).

The significant influence of non-native taxa to the Danube ecosystems is well illustrated by mean percentage participation of alien macroinvertebrates within the three main Danube sections (Figure 76).

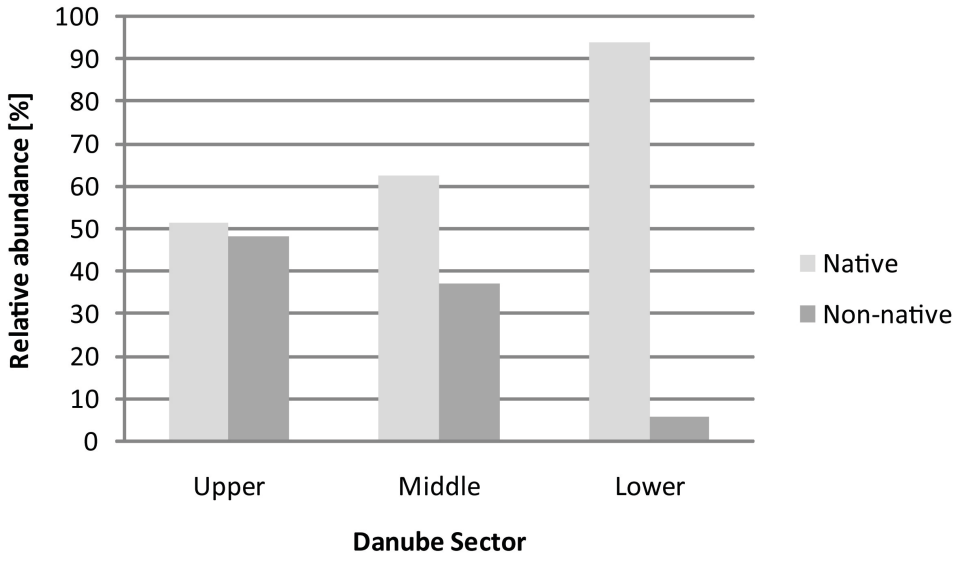

Figure 76: Mean percentage participation of native and non-native aquatic macroinvertebrate species within the three main Danube sections

10.3.3 Non-native fish species

During the JDS3, a total of 12 non-native fish species were recorded (Table 21).

Eight alien taxa were recorded for the Upper Danube, 9 for the Middle, while only 4 species that are non-native were identified in the Lower section of the Danube (marked with * – Table 21).

As in the case of aquatic macroinvertebrates, fish species that are non-native for the Middle and Upper Danube of the Ponto-Caspian origin were the most numerous – 5 species. Beside, species of Asian (4 taxa) and North American origin (3 taxa) were recorded.

Based on the share of non-native species in total fish community abundance (Figure 77), the Upper stretch of the Danube is exposed to higher pressure of biological invasions.

Table 21: Non-native fish species recorded during the JDS3

Species	Origin
Carassius gibelio (Bloch, 1783)*	Asia
Gasterosteus aculeatus (Linnaeus, 1758)	North America, Europe
Hypophthalmichthys molitrix (Valenciennes, 1844)	Asia
Lepomis gibbosus(Linnaeus, 1758)*	North America
Neogobius fluviatilis (Pallas, 1814)	Ponto-Caspian
Babka gymnotrachelus (Kessler, 1857)	Ponto-Caspian
Neogobius kessleri (Günther, 1861)	Ponto-Caspian
Neogobius melanostomus (Pallas, 1814)	Ponto-Caspian
Oncorhynchus mykiss (Walbaum, 1792)	North America
Perccottus glenii (Dybowski, 1877)*	Asia
Proterorhinus semilunaris (Heckel, 1837)	Ponto-Caspian
Pseudorasbora parva (Temminck et Schlaegel, 1842)*	Asia

10.3.4 Assessment of the level of biocontamination

Data on aquatic macroinvertebrates and fish from the JDS3 were used to evaluate the present situation, while results of macroinvertebrate survey from the JDS2 were also employed in order to compare the situation over the time – six year period, 2007-2013.

The results of calculation of SBC were presented in Table 22.

Table 22: SBC Index results

	SBC MZB 2013 (JDS3)	SBC Index fish 2013 (JDS3)	SBC MZB 2007 (JDS2)
Overall Range	0-4	1-2	0-4
Overall mean	3	2	4
Upper Danube Range	2-4	2-4	0-4
Upper Danube Mean	3	4	4
Middle Danube Range	1-4	1-3	3-4
Middle Danube Mean	3	2	4
Lower Danube Range	0-4	1-4	2-4
Lower Danube Mean	1	1	3

Based on the SBC calculation for macroinvertebrate dataset, the index ranged from 0 (no biocontamination) to 4 (severe biocontamination).

Based on calculated SBC Index for 2013 (JDS3) for macroinvertebrates and fish, the level of biocontamination of entire section of the Danube River covered by the investigation could be assessed as moderate to high. Both communities that were used for calculation (macroinvertebrates and fish) showed higher level of biocontamination for the Upper (high to severe biocontamination) and Middle Danube (moderate to high biocontamination), in compare to the Lower Danube (low biocontamination).

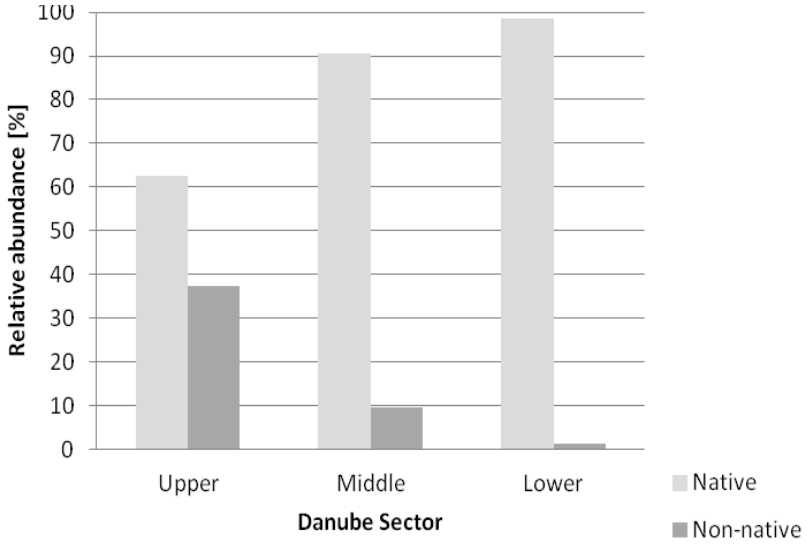

Figure 77: Mean percentage participation of native and non-native fish species within the three main Danube sections

10.3.5 Comparison with previous results

The number of non-native species recorded during the previous Danube Surveys is presented in Table 23. As it could be seen from Table 23, considerable number of non-indigenous taxa is recorded at each sampling occasion. The rise of number of identified alien macroinvertebrate taxa over the period 2001-2013 is evident. Having in mind that Danube Surveys have been planned with aim to provide comparable datasets, the recorded pattern concerning the number of non-indigenous macroinvertebrate taxa over the time realistically illustrates the situation.

The number of non-native taxa within other biological quality elements is relatively constant over the period covered.

Although the number of recorded alien MZB taxa is higher in 2013 (JDS3) when compared to those recorded in 2007 (JDS2), the comparison of the level of biocontamination based on the results of JDS3 (2013) and JDS2 (2007) surveys (Table 22) indicated lower level of biocontamination in 2013, mostly due to reduced participation of non-native taxa in total abundance of macroinvertebrate community.

Table 23: Review of number of non-native taxa recorded during the previous Danube Surveys: JDS1 (Literáthy et al. 2002), Aquaterra Danube Survey (ADS – Csányi & Paunović 2006) and JDS2 (Liška et al., 2008) and JDS3. Number in brackets denote additional criptogenic species

Quality element	JDS1 (2001)	ADS (2004)	JDS2 (2007)	JDS3 (2013)
Aquatic macrophytes	3	-	6	4
Macroinvertebrates	12	13	20	34
Fish	-	-	14 (1)	12 (1)

The results of comparison of the results of the Danube expeditions in period 2001-2013 showed that there is a continuous influence caused by biological invasions. New species of alien aquatic macroinvertebrates have been recorded over the time. Incomers sometime occupy considerable area over a short time period – e.g. in the case of *P. magnifica*. Bioinvasion process is complex and presented results pointed to variation of relative participation of non-indigenous taxa in total community, which is illustrated by the values of SBC index.

10.4 Conclusions

Based on the results of JDS3, the Danube River is significantly exposed to influence of non-native species.

Twenty five neophytes (4 aquatic), 34 non-native aquatic macroinvertebrates and 12 non-native fish species were recorded during the JDS3 survey.

The level of biocontamination of the section of the Danube River covered by the investigation was estimated as moderate to high, with higher level of biocontamination for the Upper (high to severe biocontamination) and Middle Danube (moderate to high biocontamination), in comparison to the Lower Danube (low biocontamination).

The overview of the situation of bioinvasions over the period 2001-2013, based on the results of four Danube Surveys (JDS1 – 2001, ADS – 2004, JDS2 – 2007 and JDS3 – 2013), clearly showed constant influence of alien species to native biota and considerable rise of number of non-native aquatic macroinvertebrate species. Thus, during the JDS3 (2013), 22 more alien macroinvertebrate species were recorded when compared with JDS1 (2001). Although the number of recorded alien species raised over the time, non-native species are found to be less dominant in 2013 (JDS3) when compared to 2007 (JDS2), which resulted in lower level of biocontamination in 2013.

The results of JDS3 show that biological invasions within the Danube River Basin should be properly managed. This implies that further work has to be done in the fields of collecting of basic information on the distribution of alien species and their influence on native biota, developing effective tools for the assessment of the level of pressures caused by the bioinvasions, as well as designing the appropriate mitigation measures.

It is important to evaluate accurately and rationally the real pressure of each invader to native ecosystems, because its influence on the native biota should not be considered *a priori* as negative.

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11 Zooplankton

Katalin Zsuga

11.1 Introduction

Zooplankton is a fundamental component of the pelagic food web. It is the main link between small phytoplankton and larger carnivores, primarily young fish.

Several studies have been organized in River Danube so far, which investigated shorter or longer sections of the river: Rotifera dominance and the similarly high proportion of nauplius and copepodit larvae among Crustacea were proved by Bothár (1974), Naidenow and Schewzowa (1990), Naidenow et al. (1991) and Gulyás (1994, 1995). The most frequent occurrence has been observed by species typical of still or slow-flowing eutrophic waters. According to earlier results, the dominant species of the river are: *Brachionus calyciflorus, Keratella spp., Synchaeta spp*., *Bosmina longirostris, Thermocyclops crassus, Acanthocyclops robustus* (Reckendorfer et al. 1999, Zsuga 2008). Bothár (1974) pointed out that the joining of Drava and Tisza did not have an effect on Crustacea plankton.

Naidenow (1998) laid the qualitative and quantitative proportions of the Danube zooplankton in a comprehensive work, on the grounds of the results of 164 studies. In the aspect abundance the Upper Section until Slovakia proved to be the poorest, the amount increased in Hungary, but then dropped in Croatia, Serbia and Bulgaria.

Gulyás (2002) reported the zooplankton survey made on the section between Neu-Ulm and Tulcea in summer 2001 (JDS1). According to the results the individual numbers were the lowest in the German, Austrian, Romanian and Bulgarian sections of the river, the highest on the section below Budapest and in Croatia and Serbia. Low rates of individual numbers were also observed at Neu-Ulm-Tass, as well as on the section between Iron Gate Reservoir and Danube Delta. The primary reasons for these results are the higher water flow velocity in the Upper Section, and the high turbidity in the Lower Section.

According to the surveys of Sandu and Kutzenberger (2008) in consequence of the global climate change the water level decreases and the temperature increases in Danube Delta lakes. The rise in temperature would be the cause of the increase of abundance and would grow the rate of presence of the warm stenotherm organisms.

Vadadi-Fülöp (2012) demonstrated that the flow regime has an important role for the development and abundance of zooplankton of the Danube.

11.2 Methods

During JDS3 53 sampling sites were investigated in the Danube River – from each profile from left, middle and right side zooplankton samples (sum total 159) were collected. Among of the tributaries 11 were examined in the middle profile. Out of zooplankton the three main characteristic groups, Rotifera, Cladocera and Copepoda community were investigated in details. For the analysis of zooplankton 50 litres water were filtered through plankton net with 50 μm mesh size at all three (left, middle, right) profiles. The samples were preserved in the field in formaldehyde 4-5% concentration. The quantity and qualitative composition of zooplankton was determined with light- and stereomicroscope. For the purpose of exact identification of some Rotifera species their trophi was prepared with sodium hypochlorite solution. The abundance was given in ind/100 litres unit. We

investigated the ratio of Rotifera, Cladocera, Copepoda, and the characteristic, dominant species or taxa in the different reaches of the Danube.

11.3 Results

In the Danube River and its tributaries 149 zooplankton taxa have been identified: 107 Rotifera, 33 Cladocera and 9 Copepoda. These values are a little higher than JDS1 and JDS2 values (Zsuga 2008; Gulyás 2002). The majority of the species maintain planktonic living, however, in some sampling areas tychoplanktonic elements, which penetrate the plankton from aquatic plant environment or from the surface of the sediment through mud-mixing, have been found.

The distribution of zooplankton in the whole longitudinal profile of the Danube presents the following features: Rotifera and Copepoda have the most numerous populations; Cladocera populations are less abundant (Fig.78-80). Earlier Gomoiu et al. (1997) demonstrated similar abundance relations in the lower section and Iron Gate of Danube.

11.3.1 Rotifera plankton of the Danube River

In the Upper Danube to the rkm 1800 (German, Austrian, Slovak section) the density of zooplankton is very low. From the Rotifera *Brachionus angularis, Br. calyciflorus, Keratella tecta* and *Synchaeta* spp. are dominant, their ratio is changing.

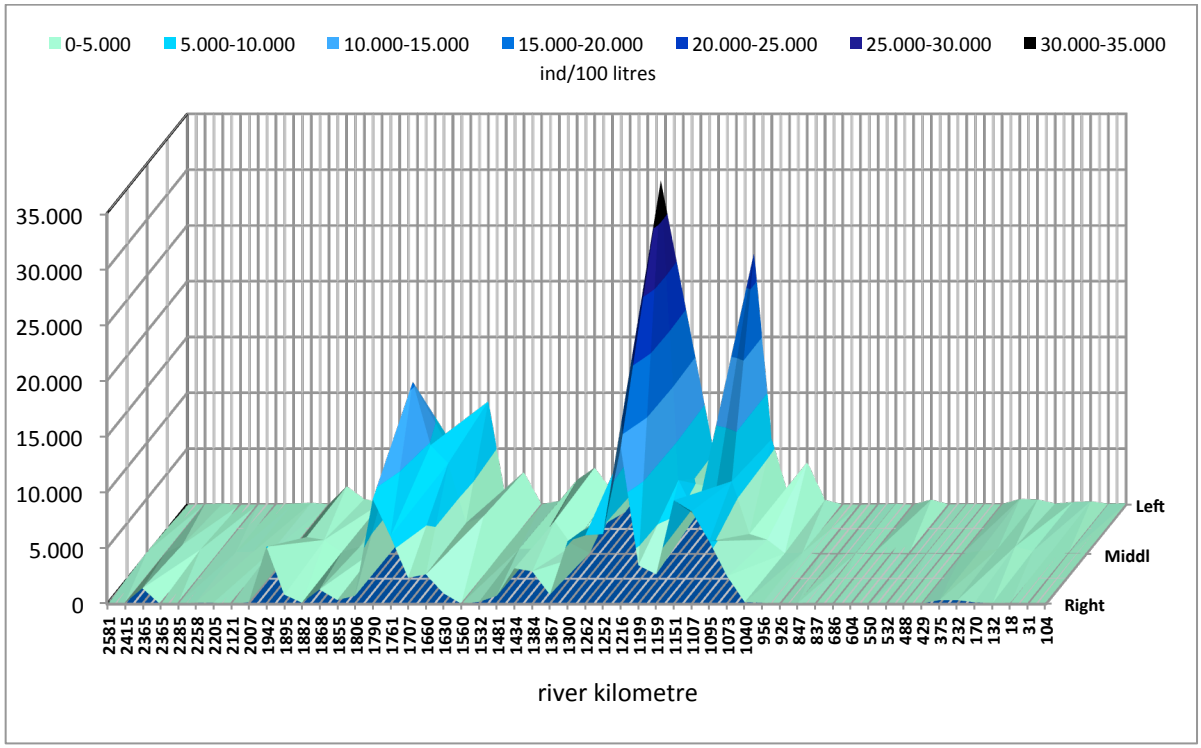

Figure 78: Rotifera abundance in the longitudinal profile of the Danube River

Between Klizska Nema (rkm 1790) and Szob (rkm 1707) zooplankton has a medium abundance, further downstream the Hungarian Danube section the abundance decreases. In the Serbian reach between rkm 1300 and 1216 the increase of zooplankton abundance is observed, which is the most intensive in the area of Stari Slankamen (Fig.78). This situation corresponds to JDS2 results (Zsuga 2008). Along the whole river the highest individual numbers of Rotifera plankton density can be found in the rkm 1216 (Stari Slankamen) and rkm 1151 (Downstream Pancevo). The value of abundance measured here is about four times higher than the JDS2 values. This result calls attention to an increased nutrient load and eutrophication of this section. In the rotifers community the proportion of *Synchaeta oblonga* and *S. kitina* being 80-95% indicates the eutrophic state of the Danube River. The

main food is phytoplankton for the most rotifer species, therefore the density of rotifers is closely connected with the phytoplankton and chlorophyll-a value (compare with chapter 8 on Phytoplankton). In the Iron Gate Reservoir and the Lower section of the Danube River the abundance of Rotifera plankton is low, but the diversity is higher than in the previous section. In the lateral profile of the river the middle segment has the highest abundance at most of the sites, and the Rotifera plankton of the left and right side does not differ significantly from each other (Fig. 78).

11.3.2 Cladocera plankton of the Danube River

The density of cladocerans in the upper stream is very low. In the rapid streaming circumstances the conditions are not prosperous to the development of the Cladocera community. In the middle Danube section the abundance is elevated, the highest values were observed at Serbian section (rkm 1330-1073), the maximum was in the left profile at area of Pancevo (rkm 1151) (Fig. 79). This value is about 1,5-2 times higher than that observed during JDS2 (Zsuga 2008). In this Danube reach the most diversified composition (9 species) can be seen. In the area of the Iron Gate Reservoir and the Lower Danube the abundance decreased, and the change of species composition can be registered. The characteristic *Disparalona rostrata* of the previous sections becomes rarer, but the high proportion of *Bosmina longirostris* is continued. At the same time the ratio of euplanktonic *Daphnia cucullata* and *Diaphanosoma brachyurum* increases in the river. In the lateral profile of the Danube the left side had the higher cladocerans density, the middle and the right side did not differ significantly from each other, and no difference was observed in the composition as well (Fig. 79).

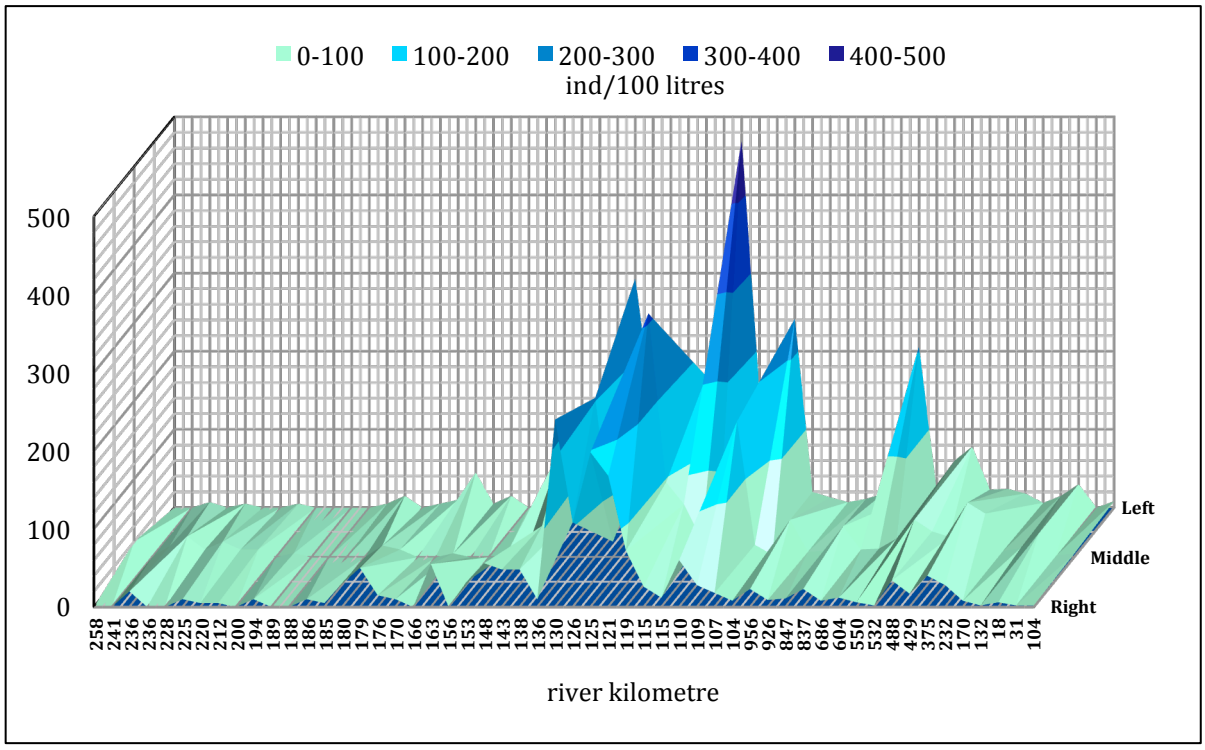

Figure 79: Cladocera abundance in the longitudinal profile of the Danube River

11.3.3 Copepoda plankton of the Danube River

In the Copepoda community the nauplii forms are dominant in the whole longitudinal section. The tendency of copepods abundance is similar to cladocerans. In the rapid streaming of the upper Danube the tychopanktonic copepodits of Harpacticoida group were also found. The highest abundance is characteristic in the middle region of the river downstream Pancevo (Fig.80) and it is higher than the value observed during JDS2. In the Lower Danube reach down to the Delta there was a significant change observed, the quantity of copepods becomes the highest among the three zooplankton groups. The adult copepods *Thermocyclops crassus* and *T. oithonoides* are characteristic species, and the

Eurytemora velox can be found in some sections as well. In the lateral river profile the higher density in the left side can be observed, while the density of middle and right segment copepods community is similar. The reason for this would be the different hydromorphological conditions in the lateral profile of the river (different water movement, different turbulence, etc).

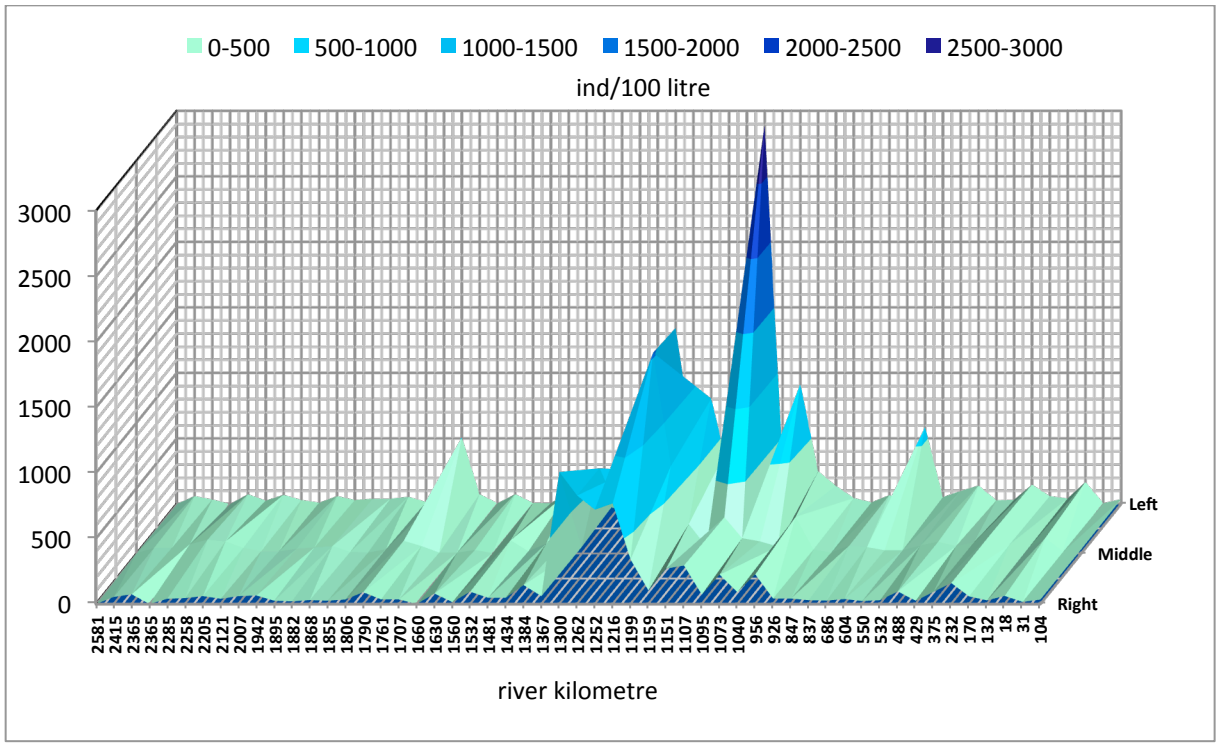

Figure 80: Copepoda abundance in the longitudinal profile of the Danube River

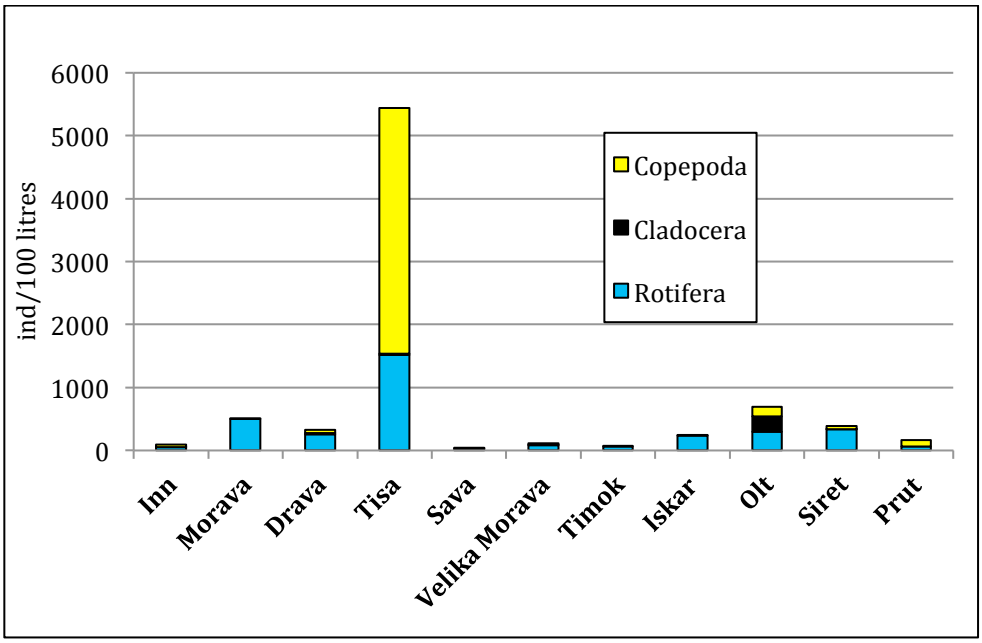

Figure 81: Abundance of the zooplankton groups in the tributaries of the Danube River

11.3.4 Zooplankton of the tributaries

In the tributaries the greatest diversity and highest abundance are recorded also for the group Rotifera. These results are similar to the data of Ostojic et al (2004). Only in the River Tisa the Copepoda abundance was higher than that of Rotifera, this was caused by an increased number of nauplii (Fig. 81). In the most cases the tributaries did not influence significantly neither the quantity nor the composition of the Danube zooplankton, the only exception being the Danube downstream Drava showing an increase of zooplankton diversity.

11.3.5 Other organisms in the plankton of the Danube River

The characteristic *veligera larvae,* similarly to JDS2, were found in high proportion in several sections of the Danube, but their species identification was not done. We suggest investigating the *veligera larvae* in zooplankton of the Danube River, because of the importance of spreading and invasive bivalve species (i.e. Corbicula) along the Danubian water way.

11.4 Conclusion

In the Danube River the density of zooplankton varied substantially. Water velocity and the amount of turbidity both had significant effects on the zooplankton density. The high numbers have evolved in the slow flowing Middle Danube reach. A number of sections can be identified along the river with zooplankton abundances considerably differing from each other.

In the Danube River and its tributaries 149 zooplankton taxa have been discovered, out of which 107 Rotifera, 33 Cladocera and 9 Copepoda have been registered. There are tychoplanktonic elements among planktonic community, coming from aquatic plant stocks or from the sediment. The zooplankton composition of the main branch shows that in some cases the dead arms and side arms have an effect to the Danube plankton as well (tychoplanctonic, metaphytic elements, which live mainly in the shallow waters *Macrothrix, Leydigia* sp.).

The proportion of the dominant species was the same as in former researches (Gulyás 1992, Zsuga 1998). The density of zooplankton was in general higher than in 2007 (JDS2), the reason would be for this the rise of temperature and the increase of nutrient load. The maximum individual number was registered in the Serbian reach, where the most eutrophic-polytrophic environment was found (*Brachionus calyciflorus, Keratella tecta, Synchaeta oblonga, S. tremula, Bosmina longirostris, Daphnia cucullata, Diaphanosoma brachyurum, Thermocyclops crassus, T. oithonoides)*. There was no increased abundance or species number observed in reservoir sections and in the Danube Delta.

During the previous surveys (Zsuga 2008) a Rotifera species, *Brachionus forficula* was found only in some sections of the Danube, but during JDS3 this species was found almost in the whole longitudinal profile of the Danube. This is a warm stenotherm organism (Koste 1978, Kutikova 1970); its stabile presence may refer to the rise temperature.

In the lateral profile of the Danube the density of the three main zooplankton groups is different. In the middle segment the Rotifera plankton species had the major abundance while the Crustacea plankton density was elevated in the left side. The reason would be for this the different hydromorphological conditions in the lateral profile of the river (different water movement, different turbulence, etc).

The tributaries did not have a significant effect neither on the quantity nor on the composition of Danube zooplankton.

The further investigation of veligera larvae in zooplankton is suggested along the River Danube, due to the importance of invasive alien Bivalvia species.

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12 Bacterial Faecal Indicators

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12.1 Introduction

12.1.1 Background

Escherichia coli and intestinal enterococci are used worldwide as sensitive indicators for the assessment of faecal pollution in the aquatic environment. Faecal indicators are excreted by humans and warm blooded animals in high concentrations and survive for a certain time in aquatic systems. Faecal pollution can be caused by point sources like discharges of sewage from human sources or livestock enterprises and by non-point sources like pasture, urban and agricultural run-off or water fowl. Faeces frequently contain pathogenic microorganisms like bacteria, viruses and parasites. Therefore intestinal indicator bacteria like *E. coli* and enterococci indicate the potential presence of pathogens and are especially well appropriate to indicate faecal pollution in surface waters.

Because of the hazard to humans caused by aquatic faecal pollution quality regulations for water intended for irrigation, water for recreation (e.g. bathing), aquaculture and water for human consumption have been established. According to the Water Framework Directive (WFD, 2000) protected bodies of water – like recreational waters, including areas nominated as bathing waters – can be designated. Surface water for bathing has to fulfil the requirements of the EU Bathing Water Quality Directive (2006). Information on the bathing water quality of official EU Bathing sites along the Danube can be found at the website http://ec.europa.eu/environment/water/water-bathing/.

Faecal pollution and microbiological contamination from anthropogenic sources have been shown to be a crucial problem throughout the Danube River Basin (Kavka & Poetsch 2002, Kirschner et al 2008, Kirschner et al. 2009). The river and its tributaries receive incompletely treated waste water e.g. from urban areas, animal farms and pasture leading to serious debasement of water quality. Thus detailed knowledge on the extent (see this chapter) and the origin of microbiological faecal pollution (see chapter 13 on "Microbial Faecal Source Tracking") is crucial for watershed management activities in order to maintain safe waters according to their quality targets.

12.1.2 Aims of the study

Data of microbial faecal pollution were collected during the Joint Danube Survey 3 (2013) along the longitudinal stretch of the River Danube from the upper section (rkm 2581) to the Delta (rkm 18) for the following aims:

- analysis of the extent and variation of faecal pollution on the basis of standard bacterial faecal indicators along the longitudinal stretch of the River Danube, in branches and main tributaries
- classification of faecal pollution according to a classification scheme developed in Kavka et al. (2006) and Kirschner et al. (2009)
- identifying hot spots of faecal pollution of the Danube River basin
- comparison of the data collected during JDS2 (2007)

12.2 Methods

12.2.1 Sampling and storage

Water samples were collected by hand from small boats at a water depth of approx. 20 to 30 cm in two sterile 1 l glass flasks from all JDS3 sampling stations and two additional stations at the Inn (upstream confluence with Danube) and downstream Vienna (after inflow of wastewater treatment plant effluent). At all Danube stations (except station JDS1) and the tributaries Inn, Drava, Tisza, Sava, and Siret samples were taken from left, middle and right of the river. The other tributaries and branches were sampled only at the middle of the river. All samples were immediately processed in the on-board laboratory.

12.2.2 Escherichia coli

E. coli concentrations were determined with Colilert 18 (IDEXX, Ludwigsburg, Germany), a most probable number technique, using two volumes (100 ml, 1 ml). Samples were incubated at 36 ± 2°C for 18 – 22 hours and analysed in a UV-cabinet. Quantitative values were obtained by comparison with the MPN table provided by the manufacturer.

12.2.3 Intestinal enterococci

Enterococci concentrations were determined by the standard method according to ISO 7899-1:1998 (ISO, 1998) using commercially available MUD/SF microtiter plates (BIORAD, Vienna, Austria). The method represents a most probable number technique. Two dilutions were applied (1:2 and 1:20). The microtiter plates were incubated at 43 ± 2°C for 32 – 40 hours and analysed in a UV-cabinet. Quantitative values were obtained by means of the MPN table given in ISO 7899-1:1998.

12.2.4 Classification system

To enable the assessment of faecal pollution levels, faecal indicators were classified by a system of 5 microbiological water quality categories after Kavka et al. (2006) and Kirschner et al (2009) (Table 24). For setting up this scheme, two concentrations derived from the EU Bathing Water Quality Directive 2006 (European Parliament and the Council of the European Union, 2006) were used as anchor points (1000 CFU/MPN for *E. coli* and 400 CFU/MPN for enterococci). Faecal pollution levels of quality class I and II are below, quality classes III, IV, and V exceed these values. The EU Bathing Water Directive and the assessment of bathing water quality could not be applied for the JDS data set since the data of bacterial indicators of faecal pollution generated during the Joint Danube Survey are single measurements. It can thus be considered only as a snapshot analysis of faecal pollution. According to the EU Bathing Water Directive the assessment of bathing water quality shall always comprise at least 16 samples compiled in relation to that bathing season and the three preceding bathing seasons, based upon a 95-percentile and 90-percentile evaluation, respectively.

Table 24: Microbiologically based classification system of water quality according to faecal pollution (Kavka et al 2006; Kirschner et al 2009). Faecal indicator concentrations are given in colony forming units (CFU) or most probable numbers (MPN) per 100ml

Classification of faecal pollution		Class				
		I	II	III	IV	V
Parameter	Faecal pollution	little	moderate	critical	strong	excessive
Escherichia coli	in 100ml water	≤ 100	> 100 - 1 000	> 1 000 - 10 000	> 10 000 - 100 000	> 100 000
Intestinal Enterococci	in 100ml water	≤ 40	> 40 - 400	> 400 - 4 000	> 4 000 - 40 000	> 40 000

12.3 Results

12.3.1 Variation in E.coli concentrations

In contrast to JDS1 and JDS2 microbiological sampling was performed not only in the middle of the Danube but also at the left and right river side. It had been observed that the water in the middle of the river was often unaffected by high concentrations of microbial faecal indicator bacteria, entering the Danube from untreated wastewater, wastewater treatment plants, or polluted tributaries. Thus, for JDS3, at some sampling sites, significantly higher concentrations at the river sides were expected.

E.coli concentrations are shown in Figure 82 and expressed in most probable numbers (MPN) per 100 ml. In the upper part of the Danube most *E.coli* concentrations corresponded, with only a few exceptions, to class I and II (little to moderate pollution). Downstream Vienna (right river side) the limit value of moderate pollution was exceeded due to the influence of the wastewater treatment plant of the city of Vienna (2.400 MPN per 100 ml). After a few kilometres (JDS10), right side values were again already below 1.000 MPN per 100 ml.

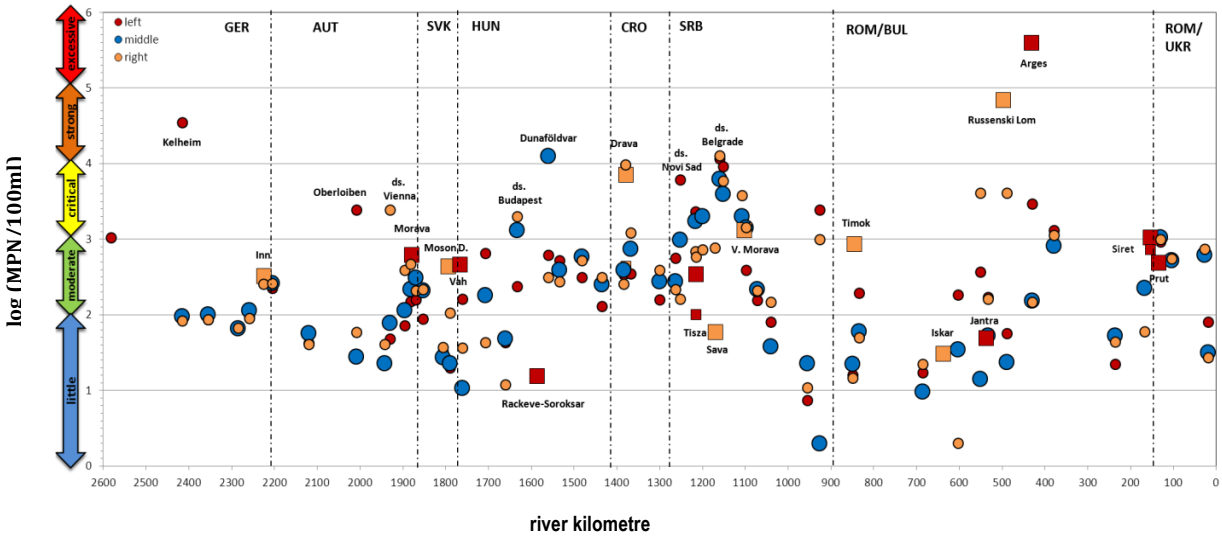

Figure 82: *E. coli* concentrations along the Danube (circles) and in selected tributaries (squares). Data were log – transformed: 1 = 10 MPN per 100 ml, 2 = 100 MPN per 100 ml, 3 = 1.000 MPN per 100 ml, 4 = 10.000 MPN per 100 ml, 5 = 100.000 MPN per 100 ml, 6 = 1.000.000 MPN per 100 ml. Samples were taken left (red), middle (blue, large symbols) and right (orange) at all Danube stations (except station 1) and at the tributaries Inn, Drava, Tisza, Sava and Siret. Left side tributaries are marked with red, right side tributaries are marked with orange. Coloured arrows along the y-axis indicate the pollution status according to Table 24, from little (blue) to strong (excessive) pollution

Surprisingly, at Kelheim (strong pollution) and Oberloiben (critical pollution) elevated values were observed at the left river side. The high *E.coli* concentrations at Kelheim even were the highest measured during the whole survey in the Danube samples. Measurements from additional samples taken concomitantly with the JDS samples performed by the German national team (Dr. Margit Schade) corroborated this finding. No clear source for these high values could so far be identified. At Oberloiben, the presence of several touristic ships in the shipping pier near to the sampling site may have had an influence on the microbiological water quality. During the passage through Slovakia, *E. coli* concentrations in the middle of the Danube dropped significantly by one order of magnitude, despite the merging of the tributaries Morava and Moson Danube, both of which displayed much higher concentrations than the respective stretch of the Danube. Elevated levels of pollution, however, were displayed after the merging with these two tributaries at the respective river sides. *E.coli* concentrations remained at little to moderate levels in Hungary until Budapest. At sampling site "Downstream Budapest", critical pollution levels were observed in the middle and at the right river side. Yet, *E.coli* concentrations were only slightly higher than the limit value for moderate pollution, despite the fact that the effluent of the new wastewater treatment plant is situated in the middle of the

Danube. Surprisingly, 72 km downstream (Dunaföldvar), a massive increase in *E.coli* concentrations (12500 MPN per 100 ml) was observed only in the midstream sample, the only JDS site where the midstream showed a more than 0.5 log higher value than both river sides and the midstream site with the highest concentrations of the whole Danube. No explanation could be found for this phenomenon. After Dunaföldvar, moderate pollution levels could be observed until the mixing of the Drava tributary in Croatia, which lead to an increase of the *E.coli* concentrations at the right river side of the Danube to critical pollution levels. After Novi Sad (RS) pollution levels of the Danube were generally critical, and even reached the level of strong pollution after Belgrade. Both, Tisza and Sava tributaries, showed much lower *E.coli* concentrations than the Danube and the increase of microbial-faecal pollution in the Danube can be traced back to influence of the large cities in this stretch. After Pancevo, pollution levels started to decrease significantly to values below 10 MPN per 100 ml in the Iron Gate reservoirs (Orsova, RO), caused by sedimentation processes in these lake-like habitats. Already at the first station after the Iron gates (Vrbica/Simijan) critical pollution levels were observed at both river sides, while in the midstream the values were still below 10 MPN per 100 ml. Until rkm 600 *E.coli* concentrations showed little to moderate pollution, but downstream Svishtov (BG) and especially after Ruse (BG) and the merging of the tributary Arges, receiving the untreated wastewater from Bucharest (RO), critical pollution levels were obtained in the Danube at the respective river sides. Both, Russenski Lom and Arges exhibited the highest *E.coli* concentrations of all samples investigated during JDS3. Towards the Delta, faecal pollution was little to moderate without a significant influence of the tributaries Siret and Prut.

12.3.2 Variation in enterococci concentrations

Enterococci concentrations are shown in Figure 82 and expressed in most probable numbers (MPN) per 100ml. In general, the concentrations of enterococci followed the patterns observed for the *E.coli* concentrations and the same hotspots of faecal pollution were identified. A highly significant correlation between these two parameters was observed (rho = 0.672, $P < 0.001$). Therefore, those results are reported where marked differences to the *E.coli* results were observed. It has to be mentioned here, that with the method used for determination of enterococci, several values below the detection limit of 15 MPN per 100 ml were observed. Those are indicated as “0-values” in Figure 83. For 5 out of 186 samples a marked difference between the *E.coli* and enterococci results was obtained that had a significant influence on the classification of the sample. At sites 40 (left and middle), 41 (tributary) and 42 (middle) as well as at site 60 (left), enterococci results indicated little pollution, while *E.coli* concentrations indicated critical levels of pollution (analysis was done from the same sample!). Apparently, samples from sites 40 to 42 were analysed and incubated on the same day, suggesting that the low enterococci results in a stretch with basically high levels of faecal pollution (after Belgrade), may be severe underestimations most probably due to processing errors on board and could be excluded from the data set. In the Arges enterococci results lead to a classification of critical pollution, while *E.coli* showed excessive pollution. Vice versa, only one *E.coli* result led to a markedly better classification than the enterococci data. At station 53 the *E.coli* based classification was moderate, while enterococci led to strong (nearly excessive) levels of pollution. No explanation for these discrepancies was found.

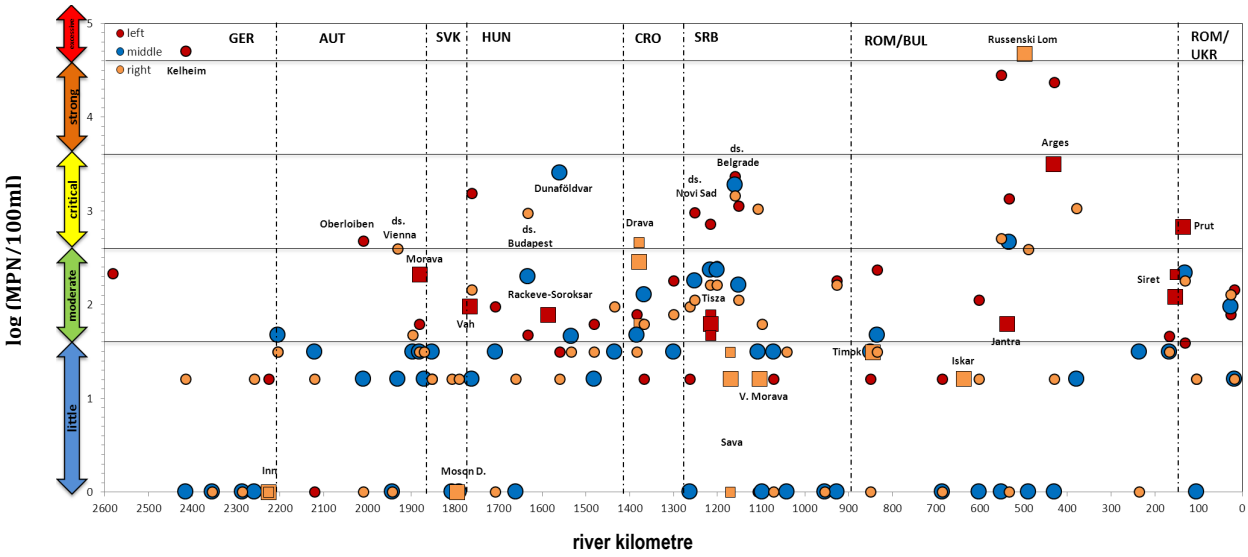

Figure 83: Enterococci concentrations along the Danube (circles) and in selected tributaries (squares). Data were log – transformed: 1 = 10 enterococci per 100 ml, 2 = 100 enterococci per 100 ml, 3 = 1.000 enterococci per 100 ml, 4 = 10.000 enterococci per 100 ml, 5 = 100.000 enterococci per 100 ml. "0"-values are values below the detection limit of the method (15 MPN/100 ml). Samples were taken left (red), middle (blue, large symbols) and right (orange) at all Danube stations (except station 1) and at the tributaries Inn, Drava, Tisza, Sava and Siret. Left side tributaries are marked with red, right side tributaries are marked with orange. Coloured arrows along the y-axis indicate the pollution status according to Table 24, from little (blue) to strong (excessive) pollution

12.3.3 Comparison to JDS2

For a comparison with the previous JDS only data from 2007 (JDS2) were included, because the same methods were applied during the two surveys. However, only midstream samples could be compared, as in 2007 no samples were taken from the left and the right river side. Table 25 provides an overview of *E.coli* and enterococci data from both surveys for the entire data set, for the Danube and for the tributary samples.

Table 25: Median and range of log transformed faecal indicator concentrations in the Danube and the tributaries obtained during JDS3 (2013) and JDS2 (2007)

Year	Faecal indicator	all data	Danube	Tributaries
2013	*E.coli*	2.35 (0.30 – 5.59)	2.29 (0.30 – 4.10)	2.68 (1.19 – 5.59)
	Enterococci	1.49 (0 – 4.67)	1.49 (0 – 3.40)	1.84 (0 – 4.67)
2007	*E.coli*	2.57 (0.9 – 6.18)	2.57 (0.3 – 4.21)	2.49 (1.28 – 6.18)
	Enterococci	1.59 (0.0 – 5.55)	1.59 (0 – 3.38)	2.08 (0 – 5.55)

In general, very similar results for *E.coli* and enterococci concentrations were obtained in both years. There was a slight tendency towards lower median values in the Danube in JDS3 compared to JDS2 for both *E.coli* and enterococci and also for the total data set. For tributaries, *E.coli* median concentrations were slightly higher, enterococci concentrations slightly lower than in 2007. However, the comparison between the two years is based only on two snap-shot microbiological analyses and is biased by the fact that not the same sites were sampled. Thus, an improvement of the microbiological water quality in the Danube and the tributaries cannot be deduced from this data.

12.4 Conclusions

- The longitudinal study of the entire course of the Danube River and its tributaries by applying uniform methods in the on-board laboratory allowed for a reliable quantitative estimation of the presence of faecal indicators and thus faecal pollution levels.
- Both, the Colilert system for *E.coli* detection and the ISO microtiter plate technique with 2 dilutions for the enumeration of enterococci were appropriate and robust microbiological methods for the enumeration of faecal indicator bacteria.
- Through the application of a "5-level" classification system, the assessment of the microbiological water quality regarding to faecal pollution based on a single event sampling was possible. However, a classification according to the EU Bathing Water Directive is not directly possible since the bathing water quality assessments comprise at least 16 samples and a percentile evaluation.
- Fourty-two JDS sampling points (35 Danube samples and 7 tributaries/branches) out of 186 were classified as critically (34), strongly (5) or excessively (3) polluted. As hot spots of excessive polution the tributary Arges and the branch Russenski Lom were identified. Surprisingly, the highest contamination in the Danube with excessive pollution levels was measured in Kelheim (DE), in the uppermost stretch, with otherwise little to moderate faecal polution levels. Other hot-spots of faecal pollution in the Danube (strong pollution or high critical pollution levels) were the stretch between Novi Sad and downstream Belgrade (RS), downstream Budapest (HU, right side) and Dunaföldvar (HU, midstream!), downstream Zimnicea (RO, left side) and downstream Arges (RO, left side).
- Sampling at the left, middle and right river sides enabled a much deeper view into the microbial faecal pollution patterns of the Danube. At many JDS sampling sites the influence of a wastewater input (from a point source or a tributary) could only be detected at one of the two river sides, most prominently at Kelheim (DE), downstream Russenski Lom (BG) and downstream Arges (RO), but also at Oberloiben and Vienna (AT), downstream Vah (HU) or after the Iron gates at Vrbica/Simijan (RS/RO). Thus sampling at both river sides in addition to the midstream is a prerequisite for assessing the microbiological-faecal status of the river.
- A comparison with data from 2007 revealed very similar median values for both faecal indicators *E.coli* and enterococci. Although a slight tendency towards lower values was observed in the Danube, an improvement of the microbiological water quality cannot be deduced from the data, because of the selection of different sampling sites in the two surveys and the fact that the microbiological analysis is based on two snap-shots.

12.5 Acknowledgements

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13 Microbial Faecal Source Tracking

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13.1 Introduction

Faecal pollution in rivers can originate from point sources such as discharges of treated or untreated sewage containing human or livestock excreta and from non-point sources like urban and agricultural run-off or wildlife. Microbial faecal pollution of water and water resources is a significant health hazard as it can contain bacterial, viral and protozoan pathogens from human or animal intestinal origin. Standard faecal indicator bacteria like *Escherichia coli* and intestinal enterococci are routinely used for faecal pollution monitoring since they sensitively detect and quantify the presence of faecal contamination. Unfortunately, these standard indicators cannot provide information about the origin of faecal contamination (e.g. human vs. animal) as they occur in both animal and human pollution sources (i.e. used as indicators for total or general faecal pollution). Information on the origin of faecal contamination is needed for determining the responsible sources, designing effective and target-oriented management strategies, evaluating the effectiveness of the management, and, finally supporting further health risk assessment especially in large and complex river catchments such as the River Danube.

Microbial faecal source tracking (MST) methods were developed to provide this critical information for water resource management. During the last years methods for the molecular detection of source-associated bacterial and viral indicators of faecal pollution have been established as the methods of choice to identify the responsible sources of environmental contamination (Hagedorn et al., 2011). Most prominent and widely used among these approaches is the detection and quantification of genetic faecal markers targeting source-associated bacterial faecal populations from the phylum *Bacteroidetes* (Wuertz et al., 2011, Farnleitner et al., 2011). Usually these markers are detected by applying quantitative polymerase chain reaction (qPCR) on DNA extracted from water samples. Extracted DNA can be stored at -80°C before further molecular analysis is performed, supporting the collection of large DNA sample libraries.

MST investigations have been conducted on the Danube in the past. The human-associated marker BacH (Reischer et al., 2007) which was developed in an Austrian alpine study area was evaluated on samples from JDS2 (Reischer et al., 2008). It was shown that the marker was detectable in Danube and tributary samples throughout the catchment. Investigations on samples from JDS2 showed that faecal pollution in the tributaries was dominated by human sources as demonstrated by a clear relationship between the standard faecal indicator *E. coli* and the BacH parameter (Kirschner et al., 2014; Kirschner et al., 2008).

13.1.1 Aims and goals

The aims of the MST investigations in the course of the JDS3 were:

- Employ human- and animal-associated genetic faecal marker diagnostics including state-of-the – art qPCR quality assurance on all investigated Danube tributary samples and samples from corresponding upstream and downstream tributary locations of the River Danube. Analyse samples from midstream, left and right side river locations (n=69).

- For the human-associated genetic faecal markers apply two state-of-the-art Bacteroidetes qPCR assays. For the animal associated genetic faecal markers apply the currently recommended ruminant and porcine Bacteroidetes based qPCR.
- Investigate the occurrence and abundance of the selected genetic faecal markers at the samples from the respective river locations and determine its relationship to the bacterial standard indicators (see chapter 12 on "Bacterial faecal indicators") as well as to the selected chemical tracers (see chapter 26 "Chemical and immunochemical analysis of anthropogenic markers and organic contaminants").
- Determine the major faecal pollution sources of the River Danube and its tributaries as reflected by the selected samples and compare with the investigation of the JDS2 in 2007.

13.2 Methods

13.2.1 Sampling, sample storage and filtration, DNA extraction

This MST investigation was focused on the Danube tributaries and the Danube sampling sites directly upstream and downstream of the confluence with the respective tributary. In addition to the official JDS tributary sites, samples from the Inn River were also included in this investigation. In total this investigation includes 26 samples from tributaries and 43 samples from the Danube.

Water samples were collected from a small boat in sterile 1L glass bottles in the middle and on the left and right side of the river (approx. 15 m from the bank) from a depth of 20-30 cm (see also chapter 12 on "Bacterial faecal indicators"). Samples were stored in cooling boxes until filtration.

Triplicate subsamples were filtered through 0.2µm polycarbonate filters. Filtration volume was between 100 and 300 ml. Filters were immediately frozen at -20°C and after no more than 3 weeks all filters were transferred to an -80°C freezer. Clean filters were frozen and stored alongside the sample filters as filtration controls.

DNA was extracted by a phenol-chloroform extraction combined with bead-beating (Reischer et al., 2008). DNA was solved in 100µl of Tris buffer. Extraction controls were routinely run alongside each extraction batch.

13.2.2 Microbial faecal source tracking

13.2.2.1 qPCR quality assurance and inhibition control

The sample DNA was diluted 1:4 and 1:16 and the AllBac assay (Layton et al., 2006) was applied to ensure the presence of amplifiable bacterial DNA and the absence of inhibition.

13.2.2.2 Microbial faecal source tracking assays

The human-associated faecal marker BacHum (Kildare et al., 2007) and a recently modified version of the HF183II (Green et al., 2014) were enumerated by quantitative PCR (qPCR) indicating human-associated faecal pollution. The ruminant-associated BacR qPCR assay (Reischer et al., 2007) and the pig-associated Pig2Bac qPCR assay (Mieszkin et al., 2009) were included as methods for detecting animal faecal pollution sources. All these qPCR assays were adapted to run on the Rotor-Gene Q thermocycler with the Rotor-Gene Multiplex PCR mastermix (Qiagen Inc.). Quantification was achieved by running plasmid standard dilution series of known concentration. No template controls were applied at all instrument runs.

13.2.2.3 Data analysis

The recovered qPCR data were log10 +1 transformed. Graphs were produced using Microsoft Excel and SigmaPlot. Statistical analysis was performed using SPSS for Windows. Standard faecal indicator data (*E.coli*, intestinal enterococci) and selected micropollutants (carbamazepin, coffein) for statistical correlation analysis with genetic faecal marker originate from the JDS3 and were provided by authors of the chapters 12 and 26.

13.3 Results

13.3.1 Sample selection and DNA quality controls

The AllBac marker was used to check the quality of DNA extracted from the water samples. The DNA extract was diluted 1:4 and 1:16, the AllBac concentration was determined in both dilutions, and, finally results expressed to the undiluted DNA extract level.

Figure 84 shows that the AllBac marker concentrations in the two dilutions were highly correlated indicating the absence of PCR inhibition on the investigated samples.

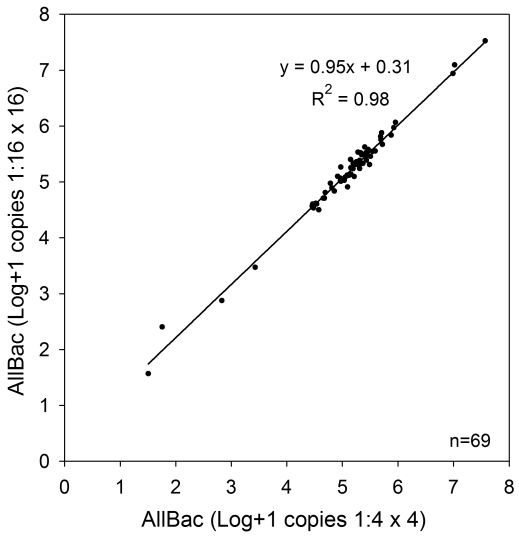

Figure 84: High correspondence of AllBac marker concentrations in 1:4 and 1:16 dilutions of DNA extracts indicated the absence of inhibition (results are expressed to the undiluted extract volume; a linear regression model with the coefficient of determination is given, $p < 0.01$).

13.3.2 Occurrence of source-associated genetic faecal markers during JDS3

The concentrations of the human-associated genetic faecal markers BacHum and HF183II were determined in the samples using quantitative PCR detection. Those markers were designed to be specific indicators of human faecal influence originating from untreated and treated sewage discharges into the environment. The genetic markers could be found in more than 90% of the investigated samples. The concentrations of the BacHum marker were higher by one order of magnitude than the closely related HF183 marker (Figure 85). Both of these markers target host-associated *Bacteroidetes* populations of faecal origin and have very similar target populations as the BacH marker, used in previous MST investigations during JDS1 and JDS2. Marker levels in the tributaries were slightly lower than in the selected Danube samples (see also Chapter 12 on "Bacterial faecal indicators").

In order to detect the possible presence of animal faecal pollution two additional MST markers were included. The BacR marker targets *Bacteroidetes* populations associated with ruminant animal faeces, while the Pig2Bac marker is targeting pig-associated *Bacteroidetes* populations. In significant contrast to the human-associated markers, the animal-associated markers were rarely detected in the investigated JDS3 samples. The BacR marker was detected in only 20% of the samples, the Pig2Bac assay only in 13% of the samples. In both cases the detected concentrations were very low and close to the limit of detection (results not shown).

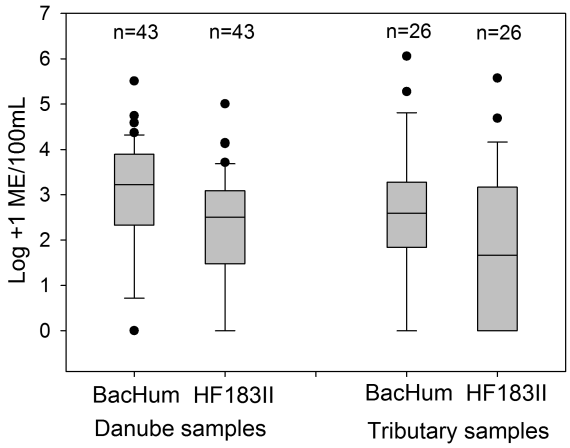

Figure 85: Distribution of BacHum and HF183II marker concentrations in tributary and selected Danube samples (ME, marker equivalents; Boxes, 25th and 75th percentile; lines within the boxes, median; whiskers, 10th and 90th percentile, respectively; n, number of samples.)

13.3.3 Comparison of genetic faecal marker levels at midstream versus river side locations

In the course of JDS3, samples for microbiological analysis were taken not only in the midstream section but also on the left and right side of the river. Figure 86 shows the levels of the BacHum and the HF183II makers in the middle and on the sides of the Danube and the tributaries. Median genetic faecal marker concentrations were slightly higher in samples from the sides of the rivers than in midstream samples, although not statistically significantly different ($p > 0.05$).

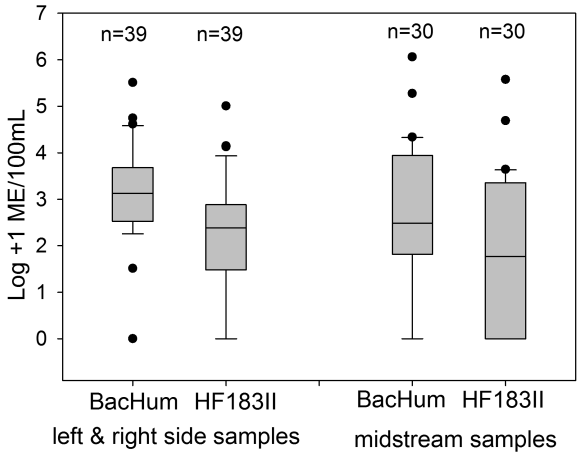

Figure 86: Distribution of BacHum and HF183II marker concentrations in tributary and selected Danube samples as determined at the left/right side versus the midstream section (ME, marker equivalents; Boxes, 25th and 75th percentile; lines within the boxes, median; whiskers, 10th and 90th percentile, respectively; n, number of samples)

13.3.4 Correlation analysis of genetic markes with faecal indicators and chemical tracers

In order to investigate for relationships between the levels of source-associated genetic faecal markers, bacterial standard indicators of faecal pollution (*E.coli*, intestinal enterococci, *Cl. perfringens*) and chemical indicators/tracers (carbamazepine, caffeine) non-parametric Spearman rank correlations were calculated. Both human-associated MST markers were highly correlated with each other ($r=0.84$, $p<0.01$, $n=69$) strongly supporting the reliability of the performed lab procedures and molecular biological analysis. Most remarkably, there were also moderate to high correlations of the human-associated genetic faecal markers with the bacterial standard faecal indicators detectable. For example, HF183II and BacHum revealed a general relationship with *E. coli* ($r=0.74$ and $r=0.66$, respectively, $p<0.01$, $n=69$). In sharp contrast, there were no correlations between bacterial standard faecal indicators and the ruminant- and pig-associated genetic faecal markers BacR and Pig2Bac detectable

(r= 0.20 and r = 0.06, p>0.10). Similar relationships were derived when comparing the MST markers with intestinal enterococci. A low but still statistically significant relationship was detectable between the levels of human-associated genetic faecal marker HF183II and the carbamazepine concentrations (r= 0.28, p<0.05, n=69). The chemical tracer caffeine did not reveal any detectable Spearman relationships with the genetic faecal markers and the bacterial standard indicators as analysed for the selected range of samples (n=69).

13.3.5 Quantifiying the contribution of human faecal pollution input

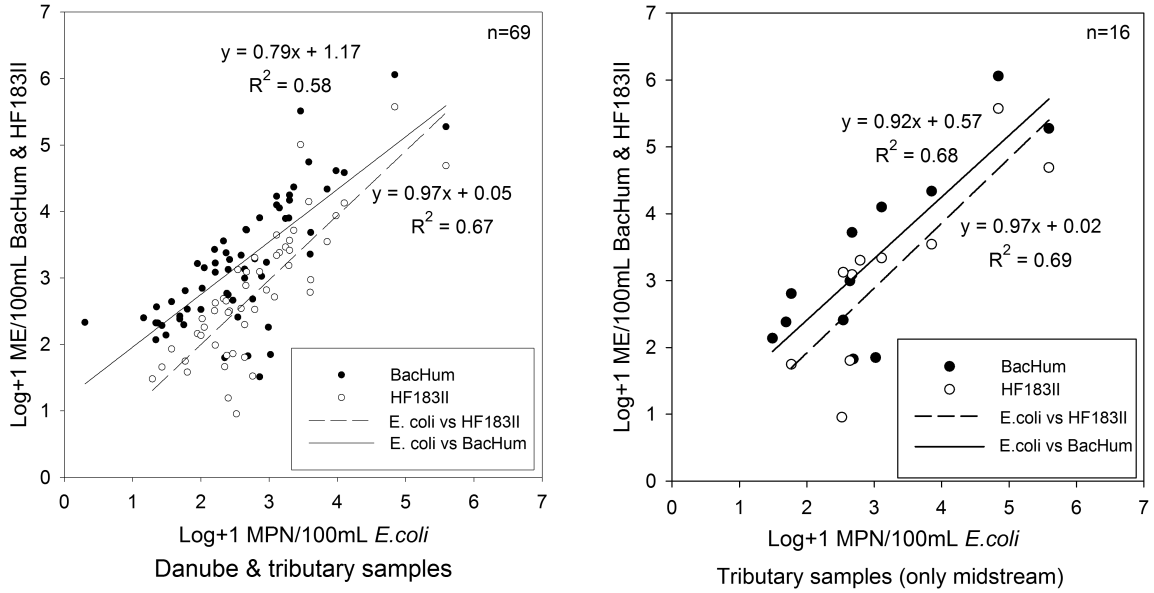

Figure 87: Regression analysis of human-associated *Bacteroidetes* genetic faecal marker versus *E. coli* levels (as indicator for total faecal pollution) in all investigated samples (left) and in midstream tributary samples (right) based on linear regression models

Linear regression analysis showed that for the whole range of selected samples from River Danube and its tributaries 67% of the variation in *E. coli* concentrations could be explained by the respective levels in the human-associated genetic faecal marker HF183II. The corresponding level for the BacHum marker was 58% (Figure 87). When making this comparison for midstream tributary samples alone, the coefficients of determination were 69% and 68% for HF183II and BacHum, respectively. Interestingly the coherence of this analysis in the Danube and the tributaries was very high, higher than it was observed in the results from JDS1 and JDS2 (Kirschner et al., 2008).

13.4 Conclusions

- The results of this microbial source tracking investigation of selected samples from JDS3 (n=69) demonstrate quite clearly that human faecal impact is the main driver for faecal pollution levels in the Danube and its major tributaries. Human-associated genetic faecal marker levels could be predicted by the bacterial standard indicator variations, such as *E.coli,* to a high extent. For the first time human-associated faecal pollution detection was complemented with animal-associated MST markers, making it possible to contrast the potentially most relevant pollution sources against each other.
- In contrast to human genetic faecal markers, ruminant and pig faecal markers could very infrequently be detected and showing very low levels (close to the detection limit of the method). This indicates that faecal pollution from ruminant and pig contamination source did not play a

significant role for faecal pollution as compared to the contamination load from human sources during the JDS3 investigation.

- One valuable addition in the future would be the application of genetic faecal markers for bird faecal pollution, but unfortunately up to date there are no such methods available that have been tested in the Central European region.
- The MST results of JDS3 are in good accordance with the results from JDS1 and JDS2. Although different markers for human-associated pollution have been used (HF183II and BacHum in contrast to BacH) the dominance of human faecal impact stayed evident.

13.5 Acknowledgements

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14 Spread of non-wild type antibiotic resistant phenotypes in the river Danube

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14.1 Introduction

Antibiotic resistant bacteria are known almost since the use of antibiotics has started. But in recent years the spread of multi-resistance, outside the hospital environment, enhanced this problem. One possible transmission route is via waste water and the water environment (Suzuki et al. 2013, Zarfel et al. 2013, Zurfluh et al. 2013, Kittinger et al. 2013).

The aim of this study was a detailed investigation of the presence of non-wild type antibiotic resistance in specific bacterial groups. The microbiological definition of wild type (or naturally susceptible) bacteria includes those that belong to the most susceptible subpopulations and lack acquired or mutational mechanisms of resistance. For this purpose one species and one genus were chosen:

- Escherichia coli as important faecal indicator bacterium with high impact in medicine. It has also a wild-type resistance pattern susceptible to a broad spectrum of antibiotics and in addition a very good ability to acquire new resistance genes.
- Pseudomonas spp with a focus on Pseudomonas aeruginosa; these bacteria have a more advanced wild-type resistance pattern. Pseudomonas spp are also in clinical settings one of the most frequent bacteria with no treatment option left and the origin for different resistance genes.

14.2 Methods

Of the samples taken for the microbiological investigations (see also chapter 12 on "Bacterial Faecal Indicators") two subsamples of 45 ml were filled into sterile non-toxic 50-ml plastic vials containing 5 ml glycerine (final conc. 10% v/v) and immediately stored at -20°C until analysis in the home laboratory.

Sampling points of the Joint Danube Survey used for this study were JDS02(left), JDS03(right), JDS10(right), JDS22(right), JDS36(left), JDS68(left).

Five ml of the thawed samples were plated in 0.5 ml portions on Pseudomonas selective agar and on Chromocult Coliform agar (CCA). Growth conditions for Pseudomonas spp were 37 ± 1 °C for 18-24 h and 41 ± 1 °C for 18-24 h for Escherichia coli. The conspicuous isolates were tested for species identification with mass spectrometry MALDI-TOF MS Axima™ Assurance (Shimadzu, Japan).

For all identified Escherichia coli and Pseudomonas spp, resistance testing was performed as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org/). The inhibition zone diameters were interpreted according to EUCAST guidelines, in addition Escherichia coli was tested for tetracycline, chloramphenicol and nalidixic acid, which were evaluated in conformity with Clinical Laboratory Standards Institute (CLSI, http://www.clsi.org) guidelines and Pseudomonas spp. for Trimethoprim/Sulphamethoxazole, which were evaluated with breakpoint of the inhibition zone diameters values for Stenotrophomonas maltophilia, since these antibiotics are considered as inappropriate drugs by EUCAST.

For Escherichia coli the following antibiotics were tested:

Ampicillin, Amoxicillin/Clavulanate, Piperacilin/Tazobactam, Cefuroxime, Cefoxitine, Cefotaxime, Ceftazidime, Cefepime, Meropenem, Imipenem, Ciprofloxacin, Moxifloxacin, Amikacin, Gentamicin, Chloramphenicol, Trimethoprim/Sulphamethoxazole, Tigecyclin, Nalidixic acid and Tetracycline.

For Pseudomonas spp. the following antibiotics were tested:

Piperacilin/Tazobactam, Cefepime, Ceftazidime, Meropenem, Imipenem, Doripenem, Amikacin, Gentamicin, Netilmicin, Ciprofloxacin, Levofloxacin, and Trimethoprim/Sulphamethoxazole.

PhenePlate™ system (Alere, Austria), which allows phenotypic differentiation on the basis of conversion of different substrates was used to avoid use of identical clones for the resistance test.

14.3 Results

A total of 123 independent Escherichia coli clones were isolated, five to 31 per sampling point. 128 Pseudomonas spp. were obtained (nine up to 30 per sampling point) belonging to the following species: Pseudomonas putida (75), Pseudomonas florescens (38), Pseudomonas oleovorans (8), Pseudomonas stutzeri (7). No Pseudomonas aeruginosa could be isolated.

14.3.1 E. coli

70 (56.91%) of the E. coli isolates showed at least one resistance against one tested antibiotic: 35 specimens against one antibiotic, 12 against two, 15 against three, and 3 isolated specimens against four and five antibiotics. One isolated specimen showed resistance against six or seven tested antibiotics, respectively. Isolates from JDS36 showed the highest multi- resistance with one strain resistant against Ampicillin, Cefuroxime, Ciprofloxacin, Trimethoprim/Sulphamethoxazole, Cefepime and Nalidixic acid; and one with Ampicillin, Amoxicillin/Clavulanate, Cefuroxime, Cefoxitine, Trimethoprim/Sulphamethoxazole and Ceftazidime. Highest resistance rates were determined for Ampicillin 41 isolates (33.34%), Tetracycline 18 isolates (14.6%), Trimethoprim/Sulphamethoxazole 15 isolates (12.1%) and Moxifloxacin 14 isolates (11.4%). All isolates were susceptible against the tested carbapenems, Amikacin and Tigecyclin (Table 26).

Table 26: Proportion of resistance against antibiotics from isolated Escherichia coli. Multiple resistance against different classes of antibiotics rises downstream

	JDS02	JDS03	JDS10	JDS22	JDS36	JDS68	Total
Ampicillin	56,7%	20,0%	10,0%	47,1%	26,7%	16,1%	33,33%
Amoxicillin/Clavulanic acid	0,0%	0,0%	0,0%	17,6%	3,3%	3,2%	4,06%
Piperacilin/Tazobactam	0,0%	0,0%	0,0%	0,0%	0,0%	3,2%	0,81%
Cefuroxim	0,0%	0,0%	0,0%	0,0%	10,0%	0,0%	2,43%
Cefoxitin	0,0%	0,0%	0,0%	17,6%	3,3%	3,2%	4,06%
Cefotaxim	0,0%	0,0%	0,0%	0,0%	3,3%	0,0%	0,81%
Cefepim	0,0%	0,0%	0,0%	0,0%	3,3%	3,2%	1,62%
Ceftazidim	0,0%	0,0%	0,0%	0,0%	3,3%	3,2%	1,62%
Imipenem	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0%
Meropenem	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0%
Nalidixic acid	3,30%	0,0%	10%	5,90%	6,60%	3,20%	4,88%
Moxifloxacin	0,0%	40,0%	0,0%	23,5%	13,3%	16,1%	11,38%
Ciprofloxacin	0,0%	20,0%	0,0%	11,8%	13,3%	6,5%	7,31%
Gentamicin	3,3%	0,0%	10,0%	11,8%	13,3%	6,5%	8,13%
Amikacin	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0%
Tigecyclin	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0%
Tetracycline	10%	0,0%	30%	17,6%	20%	9,70%	14,63%
Trimethoprim/Sulphamethoxazole	3,3%	0,0%	0,0%	11,8%	30,0%	9,7%	12,19%
Chloramphenicol	6,60%	0,0%	20%	17,6%	10%	6,40%	9,75%

14.3.2 Pseudomonas

70 Pseudomonas isolates (54.7%) showed no resistance against any tested antibiotic (exception Trimethoprim/Sulphamethoxazole, resistance against these combined antibiotics corresponds to the wild type). Only three isolates could be identified as multi-resistant (resistance in three antibiotic classes or more). The only antibiotic with no detected resistance was the aminoglycoside Netilmicin. Also the other two tested aminoglycosides (Amikacin and Gentamicin) showed very few resistances in the samples with only one isolated strain each.

Highest resistances were recorded for Meropenem (47 isolates; 36.7%), Doripenem (20 isolates; 15.6%) and Ciprofloxacin (13 isolates; 10.2%).

Trimethoprim/Sulphamethoxazole is recorded by EUCAST as natural resistance for clinical/treatment reasons, because most species do have this resistance wild-type. But nevertheless, sixteen isolates (12.5%) had prominent inhibition zones that were scored as susceptible. Resistances were high at station JDS2 and in the middle section of the Danube (as well the percentage and the occurrence of different types), and were lower at JDS3 and especially at the last two sample points (Figure 88).

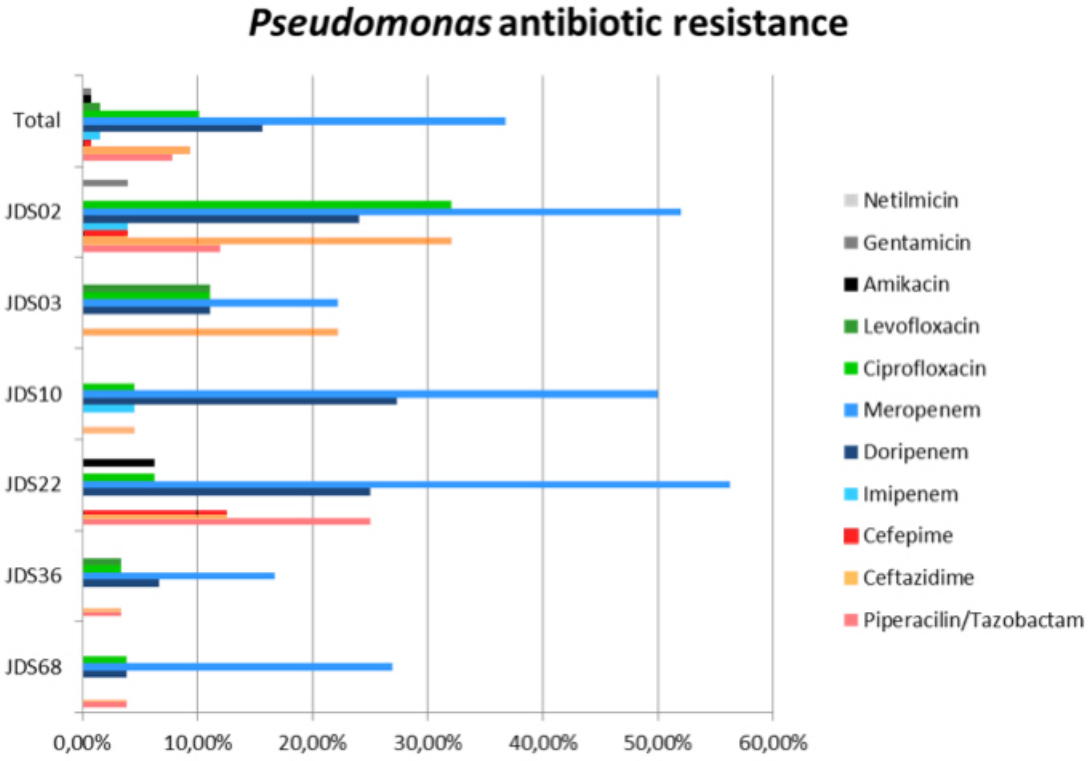

Figure 88: Proportion of antibiotic resistance of Pseudomonas in total and at the different sampling points; the same colour basis represents an antibiotic class relationship (beta-lactam-antibiotics are split in carbapenems and non- carbapenems)

14.4 Conclusions

– More than 50% of the isolated Escherichia coli showed a modified resistance pattern, but most of them (47 isolates) were only resistant against one or two tested antibiotics. Hence, multi-resistant isolates (with resistance in three or more antibiotic classes) were rare. The frequency of multi-resistance was elevated at the downstream sampling points, (including isolates with resistance against up to seven tested antibiotics). This may reflect the more problematic resistance situation in clinical settings in the downstream countries. It is known that in these countries a high percentage (50% or even more) of clinical isolates of different pathogenic bacteria show resistance

to important antibiotics. Additionally, it could also refer to a cumulative effect (http://www.ecdc.europa.eu/en/Pages/home.aspx).

- All Escherichia coli isolates were susceptible to last-line antibiotics (e.g. Amikacin, Tigecyclin, Table 26).
- Resistance findings for Pseudomonas revealed that the aminoglycoside Netilmicin was the only antibiotic to that all isolates were susceptible. Although the isolated Pseudomonas species are clinically unimportant and the overall resistance patterns of the isolates were less critical (and better than for E coli isolates), they are a possible reservoir for resistance acquisition for other species (especially Pseudomonas aeruginosa).
- Comparison of resistance data of E. coli and Pseudomonas showed no concordance in the occurrence and frequency of resistances, this includes also the frequency of multi-resistant isolates. The low number of isolates, the difference in origin and the natural antibiotic resistance may be responsible for these findings.
- The present data show that the water of the Danube represents a reservoir for antibiotic-resistant bacteria. However, this study is only a first step to assess the importance of this potential transmission route for human (and animal) health. Future studies will be necessary that include the whole data set from all JDS3 samples, genetic analyses to identify resistance mechanisms and dominant strains and studies to compare these data to data from different sources (e.g. wastewater, hospitals).

14.5 Acknowledgments

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15 Microbial Ecology

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15.1 Introduction

15.1.1 Background

River networks fundamentally differ from most other ecosystems because they are open systems with tight functional linkages to their adjacent ecosystems, and they are nested systems with their physical and ecological structure and function changing over several spatial and temporal scales (Velimirov et al. 2011). Their hierarchical organization and their tight link to adjacent terrestrial and subterranean ecosystems have stimulated the development of concepts such as the River Continuum Concept (Vannote et al. 1980) and more recent concepts on river floodplain functioning (Thorp et al. 2002, Tockner et al. 2000). Traditional perceptions have focussed on the question whether the longitudinal continuum or the lateral connectivity driven by flood pulses (Junk & Wantzen 2004) controls organic matter supply. More recent concepts discuss the importance of physical discontinuities (Battin et al. 2008) and local processes in rivers (Thorp et al. 2006) as substantial contribution to understand habitat structure, carbon fluxes and nutrient cycling in lotic ecosystems (Velimirov et al 2011).

Since the integration of the microbial loop concept for aquatic ecosystems, it has been recognized that a major part of the organic carbon from primary production is channeled through the bacterial compartment. However, this concept was developed for lentic ecosystems, and there is only limited information whether it is also applicable for lotic systems, especially for large rivers. Rivers differ from lakes in the way that allochthonous inputs of organic matter are of increased significance in comparison to primary production to fuel the bacterial compartment with carbon and energy (Battin et al. 2008). From this, it can be deduced that the microbial food web has an even higher importance in rivers and that bacterial metabolism is a key component of carbon processing (Bergfeld et al. 2008). Thus, analysis of bacterial population dynamics is critical to understanding patterns and mechanisms of material cycling and energy fluxes in large rivers (Velimirov et al. 2011). Despite its primordial importance for river system functioning, the bacterial compartment has not been considered in international regulations like the European Water Framework Directive, where biological quality elements were defined for assessing the ecological status of aquatic ecosystems (EU-WFD, 2000).

During JDS2, surprising continuous patterns of changes of the bacterial community along the Danube River were observed (Velimirov et al 2008, Velimirov et al 2011). Despite the presence of impoundments or hydropower plants, large municipalities and the discharge of large tributaries, several bacterial parameters, including bacterial numbers, morphotype succession and attached bacterial production, developed gradually, indicating that primarily broad-scale drivers and not local conditions shape and control the bacterial community in the midstream of this large river. In contrast, total bacterial activity did not follow a continuous trend but was mainly controlled by the phytoplankton bloom in the river as triggered by the impact of the large cities in the middle section (Budapest, Belgrade). These findings were also in remarkable accordance with molecular biological observations on the bacterial community dynamics and development in the Danube based on 16S rRNA gene analysis by Denaturing Gradient Gel Electrophoresis (DGGE; Winter et al 2007) and, most recently, by Next Generation Sequencing (NGS; Savio et al 2014). This accordance is all the more striking because the same patterns arose from two “snapshots” of bacterial population dynamics

along the Danube despite different methods used and a period of 6 years between the investigations. From these observations we concluded that the midstream of large rivers like the Danube exhibits a continuum of living conditions for bacterial communities, and influences of tributaries/wastewater may be visible mainly in the boundary water masses of the river (Velimirov et al. 2011).

15.1.2 Aims of the study

Data of microbial ecological parameters were collected during the Joint Danube Survey 3 (2013) along the longitudinal stretch of the River Danube from the upper section (rkm 2581) to the Delta (rkm 18) at the left, middle and right river side for the following aims:

- To monitor total bacterial numbers, the numbers of large and small bacterial cells and heterotrophic bacterial production rates to obtain an overview of the microbial-ecological status of the Danube.
- To compare the microbial ecological data from the midstream to the data from the left and right river side to detect potential influences of tributaries and/or wastewater on the bacterial community in the Danube.
- To investigate whether the patterns of the longitudinal development of the bacterial compartment in the Danube detected during JDS2 in 2007 are also observed during JDS3
- To quantitatively compare the JDS3 data set with the data obtained during JDS2.

15.2 Methods

15.2.1 Sampling and storage

Water samples were collected by hand from small boats at a water depth of approx. 20 to 30 cm in two sterile 1 l Schott-flasks from all JDS3 sampling stations and two additional stations at the Inn (upstream confluence with Danube) and downstream Vienna (after inflow of wastewater treatment plant effluent). At all Danube stations (except station 1) and the tributaries Inn, Drava, Tisza, Sava, and Siret samples were taken from left, middle and right of the river. The rest of the tributaries and branches were sampled only from the middle. All samples were immediately processed in the on-board laboratory.

15.2.2 Bacterial Numbers

Bacterial numbers were estimated by epifluorescence microscopy according to a modified protocol applied in Riepl et al (2011). Subsamples of 1.5 ml were fixed with sterile filtered formaldehyde (final concentration 1.8%) for 1 to 2 h at room temperature. A volume of 0.2 to 0.5 ml of the fixed subsamples was filtered through a 0.2-µm membrane (Anodisc 25; Whatman, Germany). The filter was mounted on a drop of SYBR-Gold (Invitrogen, Austria), freshly diluted to a final concentration of 1:400 of the stock solution. The filter membrane was incubated at room temperature in the dark for 15±3 min, rinsed three times with 1 mL sterile filtered, distilled, autoclaved water to remove excess dye and dried in the dark. A drop of anti-fading solution (Citifluor; Groepl, Tulln, Austria) was put on a microscope slide; the dry filter membrane was mounted and covered by another drop of anti-fading solution and a cover slip. The slides were stored at -20°C until analysis in the home laboratory. At least 200 cells were counted in 20 microscopic fields at a 1250 × magnification. Cells were differentiated into large cells, including rod shaped cells, curved rods, filaments and large cocci (with a cell diameter of > 1.0 µm) and into small cells (cocci with a cell diameter < 1.0 µm).

15.2.3 ^{3}H-Leucine incorporation – Heterotrophic Bacterial Production

For the determination of bacterial production rates the ^{3}H-leucine incorporation method after Kirschner & Velimirov (1999) was followed with modifications as already applied during the last JDS2 (Velimirov et al. 2011). ^{3}H-Leucine was used as a tracer for substrate uptake activity and incorporation into the bacterial protein pool. Of each sample, four 1-ml subsamples and two blanks were amended with 100 nM (final concentration) of ^{3}H-leucine. Blanks were stopped immediately

with 60-µl trichloroacetic acid (TCA, final concentration 5%). After 30-min incubation at in situ temperature in the dark, samples were stopped with TCA, and proteins were precipitated with 100 µl of 35% NaCl and purified in several extraction/centrifugation steps. All vials with the purified proteins were stored at −20°C on board until transfer to the home laboratory. There, the vials were thawed, scintillation cocktail was added, radioactivity in the proteins was measured in a scintillation counter (Perkin Elmer, TriCarb 2300 TR) and converted to units of carbon using the conversion factor of Simon and Azam (1989).

15.3 Results

15.3.1 Bacterial Numbers

Large cells can be assumed to represent the active part of a bacterial population, while small coccal cells can be assumed to be dormant or starving, when nutrient supply is reduced or inappropriate (Novitsky and Morita 1976). Alternatively, it was recently shown that typical freshwater clades also mainly consist of small cells with an oligotrophic life-style (Salcher et al 2011, Garcia et al 2013). The longitudinal development of the large cells (Figure 89, upper panel) showed a steady decline of cell concentrations in the upper stretch until rkm 2200, where the lowest number of large cells of all investigated samples was observed (2.2×10^6 cells/ml, midstream). The Inn merging at rkm 2225 showed similar concentrations as the Danube. From rkm 2000 concentrations of large cells steadily increased until upstream Budapest (rkm 1660) with the maximum value of 13.2×10^6 cells/ml (midstream) in the Danube. All tributaries in this stretch had significantly higher concentrations of large bacterial cells than the Danube but their inflow did not show visible influence on the concentrations at the respective river sides. At JDS20 (rkm 1707) a much lower value was observed at the right river side in comparison to the midstream and left river side sample; no explanation was found for this finding. After Budapest, until the merging of the river Drava, numbers of large cells decreased again down to values around 6×10^6 cells/ml. A very high value of 24.2×10^6 cells/ml was observed in this stretch in the Rackeve-Soroksar branch of the Danube. In contrast to the high concentrations of large bacterial cells in the tributaries/branches in this stretch, bacterial faecal indicators were only slightly higher or even lower than in the Danube (see chapter 12 on "Bacterial Faecal Indicators"). This discrepancy is due to the fact that the bacterial faecal component accounts only to a small extent to the bacterial populations in the Danube and its tributaries and the bacterial populations are often controlled by other factors than faecal pollution.

After the confluence with the Drava, numbers slightly increased to values around 7.5×10^6 cells/ml and remained at this level until the inflow of Velika Morava. The Drava and Sava tributaries showed similar values than the Danube in this stretch, Tisza and Velika Morava showed significantly higher concentration of large cells. After V. Morava, numbers of large cells decreased steadily towards the Iron Gates. At JDS46, the site after the Iron Gate reservoirs (rkm 928) the lowest concentration of 3.3×10^6 cells/ml (midstream) was observed in this stretch. From this site downstream, concentrations of large bacterial cells steadily increased until the inflow of tributary Arges (rkm 432). Maximum concentrations in this stretch were registered after the inflow of Arges with 11.8×10^6 cells/ml at the right (not left!) river side. The maximum concentration of all JDS sites was observed in the Arges tributary itself with a value of 30.2×10^6 cells/ml, reflecting the high levels of faecal pollution in this river (see chapter 12 on "Bacterial Faecal Indicators"). The other tributaries in this stretch had slightly higher (Iskar), similar (Russenski Lom) or lower (Jantra) concentrations of large bacterial cells. In the final stretch towards the Delta, cell numbers decreased again, with the tributaries Siret and Prut showing similar concentrations as the Danube.

Large bacterial cells were significantly correlated to bacterial production values and showed a similar longitudinal development (see next subchapter). In contrast, small bacterial cells in the Danube (Figure 89, middle panel) followed a completely other trend with steadily increasing concentrations from the uppermost stretch towards the Delta. A highly significant correlation between JDS station number and the concentrations of small cells was obtained ($r = 0.624$, $rho = 0.677$; $p < 0.001$), very similar to the trends observed during JDS2 (Velimirov et al 2011). Most tributaries showed similar or lower concentrations of small coccal cells as observed in the Danube at the respective merging site;

only two tributaries (Rackeve-Soroksar and Velika Morava) showed higher numbers of small cells. In total, this interesting and surprising observation directs towards an increasing amount of starving and dormant bacteria with increasing river size. Despite the merging of large rivers carrying new organic material, despite the input of increasing amounts of faecal pollution into the Danube and despite the development of "lake-like conditions" with increasing algal production in the middle and lower stretches of the Danube (Dokulil & Keiblinger 2008) a large proportion of the bacterial community starts to starve and to become dormant. Alternatively, the accumulation of small cells could also indicate the increase in typical freshwater clades that represent small cells adapted to oligotrophic environments, as most recently shown for the Danube during JDS2 (Savio et al 2014).

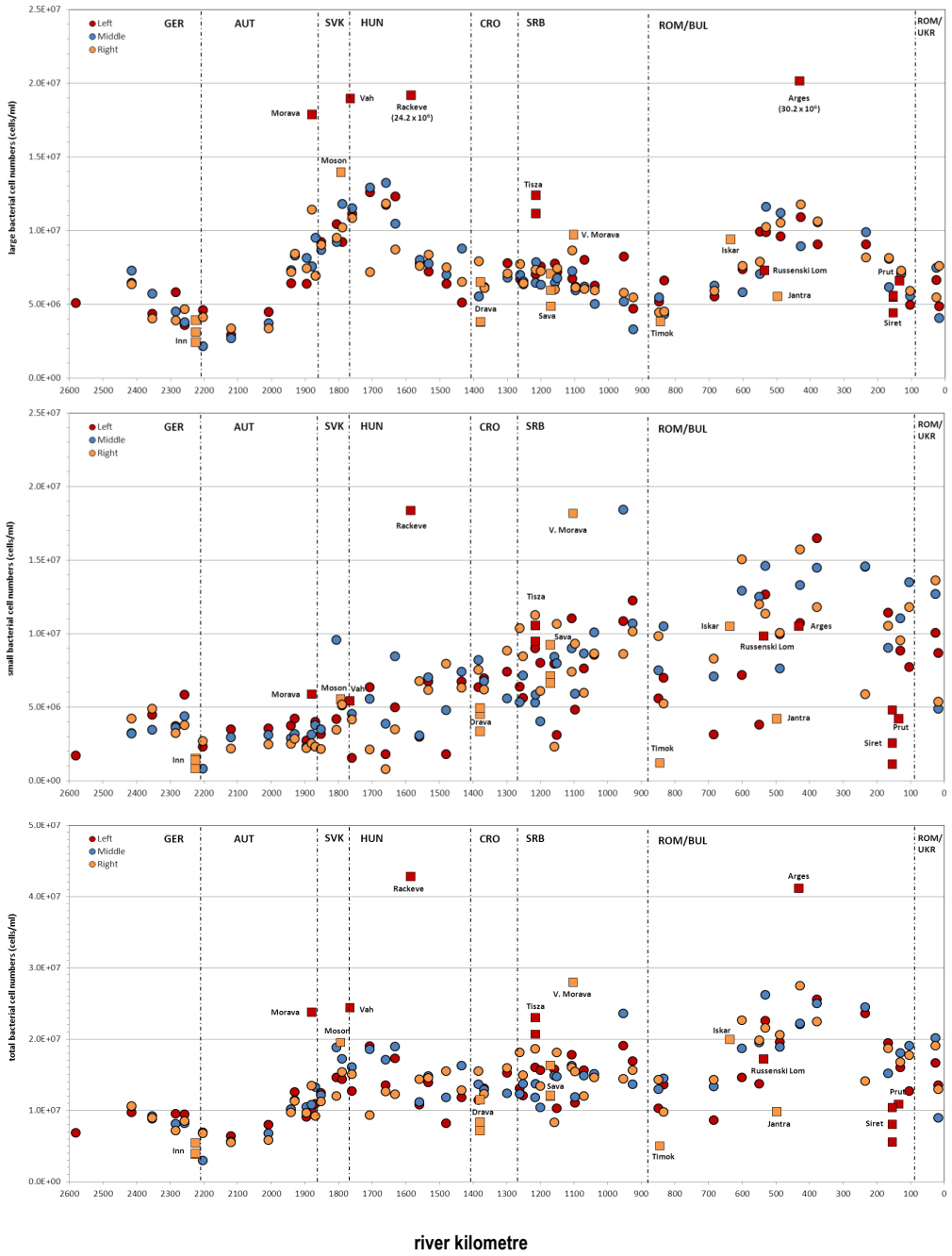

Figure 89: Total bacterial cell numbers (lower panel) and numbers of large (upper panel) and small bacterial cells (middle panel) along the Danube (circles) and in selected tributaries (squares). Samples were taken left (red), middle (blue) and right (orange) at all Danube stations (except station 1) and at the tributaries Inn, Drava, Tisza, Sava and Siret. Left side tributaries are marked with red, right side tributaries are marked with orange

The development of the total bacterial cell numbers is obviously an overlay of the trends observed for the large and the small cells and thus not of the same high meaningfulness (Figure 89, lower panel). The highest numbers observed during the JDS3 were observed in the Rackeve-Soroksar branch (42.8×10^6) and the tributary Arges (41.2×10^6). Also other tributaries (Morava, Moson Danube, Vah, Tisza, Velika Morava and Iskar) showed higher bacterial numbers than observed in the Danube at the respective merging site. The large tributaries Inn, Drava and Sava had similar concentrations than the Danube. Besides the Inn (2.4×10^6) and the JDS station downstream of the confluence site of the Inn with the Danube (2.2×10^6, midstream), the lowest concentration of all JDS sites was determined for the tributary Timok (3.8×10^6), a river that is highly contaminated with heavy metals, an observation that is in agreement with the bacterial production data (more details see below).

15.3.2 Heterotrophic Bacterial Production

Heterotrophic bacterial production (BP) rates are shown in Figure 90. Due the large range of the data (> 300 fold) a logarithmic scale is used for presentation. In the upper stretch BP rates declined steadily from ~ 2 µgC/L/h to ~ 0.5 µgC/L/h (site JDS7). No significant difference was observed between the left, middle and right side samples, except for JDS2 left (Kelheim), a site where also significantly elevated faecal pollution levels were observed (see chapter 12). Thereafter, BP values increased again to values around 2 µgC/L/h shortly upstream and downstream of Vienna. Also at JDS8 left (Oberloiben) the observed elevated faecal pollution status – most probably caused by an unknown local input of wastewater – coincided with elevated BP rates. Between Vienna and Budapest mean BP rates slightly decreased to values between 1 and 1.5 µgC/L/h. The three tributaries entering the Danube in this stretch showed significantly higher rates than observed in the Danube and led to elevated levels of BP at the respective downstream river sides. After Budapest (rkm 1632) BP rates began to increase and reached a maximum of 3.5 µgC/L/h at Dunaföldvar (rkm 1560), at the midstream and right river side. Such an elevated level in the midstream was also observed for the faecal indicator bacteria (see chapter 12). Also at the following sampling site (JDS25, Paks) a significantly higher value was observed in the middle of the Danube. Downstream Paks, until rkm 1434, BP rates decreased.

After the confluence of the river Drava BP rates started to increase again and reached a second maximum of values around 3.5 µgC/L/h downstream Belgrade. In this stretch at nearly all stations BP rates measured at the left and right river sides were markedly higher than in the midstream, indicating significant influence of untreated urban wastewaters. In contrast, the three large tributaries Drava, Tisza and Sava showed similar BP values as found in the Danube. After Belgrade a marked decrease to values below 0.5 µgC/L/h in the Iron Gates was observed that coincided with the development of faecal pollution indicators. The extremely low BP rates observed in this stretch at JDS40 are open for discussion. There are some indications that the measured low rates may be caused by inhibition through heavy metals. First, the river Timok also showed extremely low BP rates, a river which is known to be heavily contaminated with heavy metals potentially inhibiting bacterial growth (see below) and at this site (Smederevo) and the further upstream site 39 (Pancevo) there is abundant metal industry polluting the river Danube. Second, at this site, also drastically reduced Enterococci concentrations were observed (see chapter 12), a parameter that was analysed from the same sampling bottle but independently from the subsamples for bacterial production.

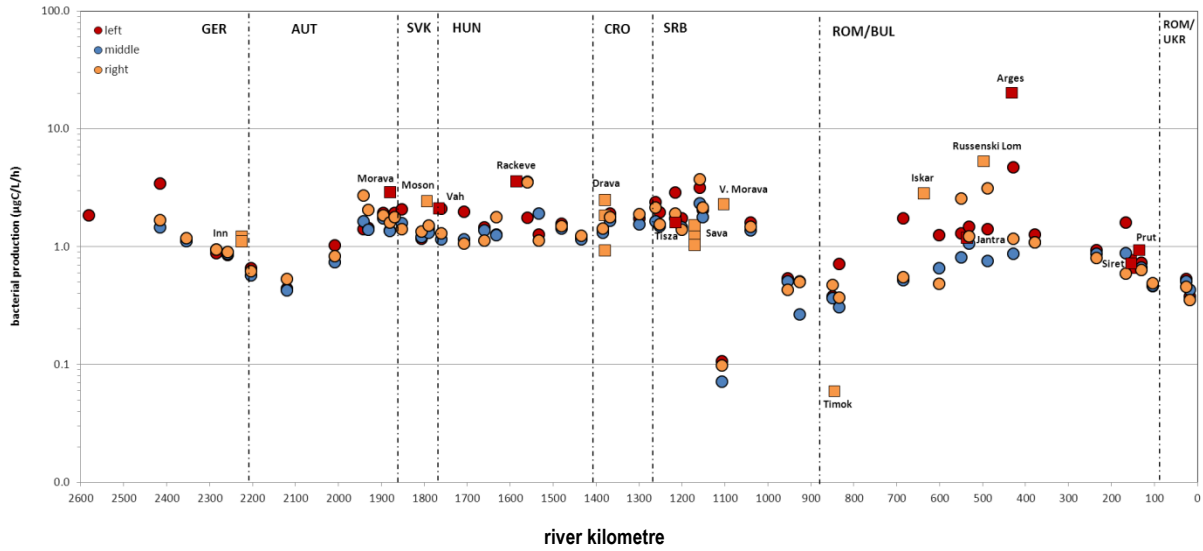

Figure 90: Total heterotrophic bacterial production along the Danube (circles) and in selected tributaries (squares). Samples were taken left (red), middle (blue) and right (orange) at all Danube stations (except station 1) and at the tributaries Inn, Drava, Tisza, Sava and Siret. Left side tributaries are marked with red, right side tributaries are marked with orange

After the Iron gates, BP rates started to increase again up to the maximum value measured in the Danube of 4.7 µgC/L/h at JDS59 (left river side) after the inflow of the river Arges. At nearly all stations in this stretch, BP values of the left and right river sides were markedly higher than the values measured in the midstream, indicating clearly the allochthonous input of organic matter/ wastewater to the lateral river zones. With the exception of the tributary Jantra all other tributaries exhibited significantly different production rates than the Danube. Bacterial production in the Timok was obviously extremely inhibited by the heavy metals (primarily copper) present in the river and lowest rates of 0.06 µgC/L/h were observed in this tributary. In contrast, BP rates in Iskar, Russenski Lom and Arges, which had the highest BP rates of all measured samples (20.2 µgC/L/h) were significantly higher than in the Danube. In case of Russenski Lom and Arges, these higher values were clearly reflected at the respective downstream JDS stations at the respective river sides. After the Arges, BP rates gradually decreased towards the Delta to values below 0.5 µgC/L/h. Values determined for the samples from the midstream and the left and right river side were not significantly different from each other with the exception of station JDS62 (Braila), where a higher value was observed at the left river side. Both tributaries (Siret and Prut) also showed similar production rates as observed in the Danube.

Taking all data together, BP rates were highly significantly correlated to the abundance of large bacterial cells ($rho = 0.415$, $p < 0.001$), but not to total bacterial cell numbers ($rho = 0.078$; $p > 0.1$) or small cocci ($rho=0.001$; $p > 0.5$). This nicely corroborates the assumption that the large bacterial cells are the active component of the bacterial compartment. BP rates were also significantly correlated to both *E.coli* ($rho = 0.374$, $p < 0.001$) and Enterococci concentrations ($rho = 0.347$, $p < 0.001$). In detail, BP rates from each river side (left, middle, right) were always and solely correlated to the concentrations of both faecal indicators measured at the respective river side (i.e. BP rates from the left side were only correlated to *E.coli* concentrations from the left side, and so on). This indicates an effect of wastewater input on the activity of the bacterial community in the Danube.

15.3.3 Comparison to JDS2

A comparison of the data obtained in 2013 to the data obtained in 2007 could only be made for the bacterial production values (Figure 91), where exactly the same protocol of analysis was followed during both surveys. For the determination of bacterial numbers a different (and more reliable) protocol was used in 2013, leading on average to significantly higher total bacterial numbers than with the protocol used in 2007. Moreover, sub-classification of bacterial numbers into small and large bacterial cells was done differently than in 2007.

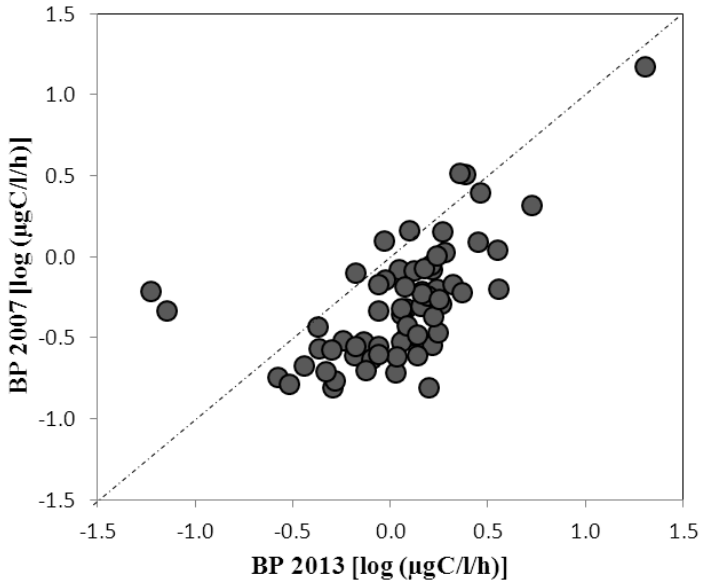

Figure 91: Scatter plot of bacterial production (BP) values determined in 2013 and 2007. To obtain normal distribution, the data were log transformed. The dotted line represents the 1:1-line. Values below that line indicate higher values in 2013, values above that line indicate lower values in 2013 compared to 2007.

Bacterial production rates in 2007 were, with a few exceptions, lower than in 2013. As can be seen from Figure 91, nearly all values were below the 1:1-line. From the seven exceptions, five were very close to the 1:1-line indicating that the values were very similar in both years. The two other exceptions were the river Timok and the station "upstream Velika Morava", where a strong inhibition of bacterial production rates due to heavy metals was hypothesized, which was obviously not observed in 2007. However, the rates observed in 2007 and 2013 were highly significantly correlated showing that the bacterial production along the Danube river and its tributaries followed the same trend in both years. Considering the whole data set, Pearson correlation coefficient r was 0.618 ($p < 0.001$), and Spearman correlation coefficient rho was 0.655 ($p < 0.001$). If the two exceptions with the extremely low values measured in 2013 were excluded from the data set, both correlation coefficients were even higher ($r = 0.768$; $rho = 0.696$; $p < 0.001$).

15.4 Conclusions

- The analysis of the development of the bacterial community via the estimation of bacterial numbers and heterotrophic bacterial production rates revealed highly interesting patterns along the Danube and its tributaries (see below).
- Heterotrophic bacterial production rates and the concentration of large bacterial cells, representing the active part of the bacterial community were significantly inter-correlated and followed a similar trend. In the Danube, lowest values were observed in the Austrian stretch, after the Iron Gates and in the Delta. The highest values were observed between rkm 1895 and 1632 and between rkm 550 and 378. The inflow of polluted tributaries and wastewater from point sources was partly reflected in the Danube at the respective river sides and partly contributed to the observed trends.
- As correlation analysis revealed, the patterns of bacterial production observed in 2013 were similar to the ones observed for JDS2. However, with the exception of only a few samples, heterotrophic production rates in 2007 were lower than in 2013.
- In many smaller tributaries and branches (Morava, Moson Danube, Vah, Rackeve-Soroksar, Velika Morava, Iskar and Arges) both the concentration of large bacterial cells and bacterial production rates were markedly higher than in the Danube at the respective merging site. Such an observation was already made during JDS2 in 2007. The highest cell concentration and production

rate were observed in the tributary Arges, most probably due to the enormous wastewater input from Bucharest in this river. The large tributaries Drava and Sava showed for both parameters similar values like the Danube, in case of the Inn (BP rates) and the Tisza (large cell numbers) only one of the two parameters was elevated in comparison to the Danube. From all sites, the tributary Timok exhibited the lowest heterotrophic production rates, most probably cause by the high contamination with heavy metals, which was also reflected in very low numbers of large, small and total bacterial cells.

– In contrast to the indicators of bacterial activity, small coccoid cells gradually and significantly increased along the Danube with an approximate 4 fold higher concentration in the Eastern Romanian lowlands and the Delta than in the upper stretches. This observation directs towards an increasing amount of starving and dormant bacteria with increasing river size. Despite the merging of large rivers carrying allochthonous organic material, despite the input of increasing amounts of faecal pollution and despite the development of "lake-like conditions" with increasing algal production in the middle and lower stretches of the Danube, a large proportion of the bacterial community starts to starve and to become dormant or/and develops into a community of typical small-sized freshwater bacteria adapted to oligotrophic conditions. This has significant consequences for models on organic matter degradation including self-purification processes, for carbon fluxes including CO_2 production and for ecosystem nutrient cycling. If the dominant proportion of the bacterial community in the large river Danube is rather inactive, only a small part of organic pollution entering the river would be effectively degraded, while the bulk of organic pollution would flow un-degraded along the river banks to the Black Sea.

15.5 Acknowledgements

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16 Microbial Metagenomics

Teresa Lettieri, Valentina Ferrero, Lourdes Duque, Armin Lahm, Alexander Kirschner, Andreas Farnleitner and Raquel N. Carvalho

16.1 Introduction

Natural microbial diversity encompasses a broad spectrum of microorganisms (bacteria, fungi, viruses) that exert a strong influence on global processes such as the carbon, nitrogen and sulphur biogeochemical cycles. Quick responsiveness to environmental changes and the rapid reproductive capacity of microorganisms allow for changes in both the qualitative and quantitative composition of a particular habitat and indices of microbial diversity are considered a sensitive measure for the ecological state and health of a habitat or ecosystem. Assessment of biodiversity therefore represents a keystone in i) understanding complex processes within ecosystems; ii) characterising the microbial communities and their relation to anthropogenic pressures like chemical pollutants; iii) identifying the microbial indicators and functional pathways for water quality management (Kisand *et al*., 2012). The possibility to investigate the microbial communities present in a water sample and without any cultivation appeared in a publication ten years ago with the name "metagenomics (shotgun sequencing)" (Venter *et al*., 2004). Metagenomics utilizes high-throughput automation of sequencing platforms to obtain (random) sequence fragments of the genetic material from the sample. The sequence information is then compared with genomic databases from known organisms in order estimate the diversity and abundance of microorganisms within the community. Importantly, metagenomics overcomes the difficulty to characterize uncultivable microbes because only genomic DNA directly obtainable from the sample is required. To date, metagenomics has been applied to many environmental samples from marine to freshwaters (Williamson & Yooseph 2012) to link the microbial communities "profile" to environmental pressures.

For the first time, a metagenomics approach has been proposed to the Joint Danube Survey as a pilot study focused on four selected sites.

16.2 Methods

16.2.1 Water Sampling

Water was collected using 10 l polyethylene containers acid washed (0.1% HCL) before sampling. Prior to submersing the containers by approx. 20 – 30 cm below the river water surface containers were pre-washed with water from the sampling site. Collected water was filtered on 0.22μm filters (1l/filter, filter diameter 9 cm). The filters were kept at -20°C during the campaign and subsequently shipped to JRC in dry ice.

16.2.2 DNA extraction from filter

In the laboratory, the next steps were performed to extract and clean the genetic material, the genomic DNA to be sequenced.

Briefly, the thawed filters were incubated and shaken (160 rpm) in 50 mM KH_2PO_4 buffer pH 7.5 overnight at 4 °C in Petri dishes to remove the microorganisms from the filters.

After washing 3 times, the buffer was transferred to new tubes while the filters were transferred to different tubes containing 50 mM KH_2PO_4 of fresh buffer at pH 7.5 and sonicated 3 times for 5 minutes at 60 °C (vortex each time) to remove additional bacteria. Extractions were always kept separated.

After sonication, the buffer was again transferred to new tubes which were treated wet with 5 U/µl of lyticase (Sigma Aldrich L2524, USA. 3770 units/mg), 4470 U/µl of lysozyme (Sigma Aldrich L6876. ≥40.000 units/mg) and finally 14 mM β-mercaptoethanol (B-ME, Sigma Aldrich). The incubation with the enzymes is required to destroy the cell wall and cellular membrane thus liberating the genetic material.

The samples containing enzymes and B-ME (either sonicated or not) were then incubated at 30 °C for 3 hours in agitation at 180 rpm.

All samples were then centrifuged at 10.000 rpm for 20 min at 5 °C to recover the pellet containing nucleic acids (genomic DNA, RNA) and proteins. The pellets were resuspended in 180µl of ATL buffer (Qiagen Kit), transferred to 1.5 ml tubes and 20µl of proteinase K was added (removal of proteins), mixed and incubated at 56 °C overnight.

Genomic DNA was extracted and cleaned using the DNeasy Blood and Tissue kit (Qiagen, UK) according to the manufacturer's instructions. Only the elution step was slightly modified: DNA was first eluted with 100 µl of AE buffer, 10 µl fresh AE buffer was added and the column was loaded again with the eluted sample.

16.2.3 DNA extraction quality control

The DNA concentration was determined with the Nanodrop and quality ratios (260/280, 260/230) were determined. DNA samples were then run on 1% agarose electrophoresis gel using a MassRulerTM High Range DNA Ladder (Fermentas, Canada).

For pyrosequencing analysis, the DNA was concentrated by precipitation with 5 M NaCl and 99.9% Ethanol and re-suspended in 100 µl of 10 mM TRIS buffer, pH 8.0

16.2.4 Direct pyrosequencing of the total community DNA

Direct sequencing of total community DNA was carried out using the equipment and tools available at LGC Genomics Centre according to the manufacturer's instructions. Library generation for the 454 FLX sequencing was carried out according to the manufacturer's standard protocols (Roche/454 life sciences, Branford, CT, USA). In short, the DNA fragments were end polished and the 454 A and B adaptors required for the emulsion PCR and sequencing were added to the ends of the fragments by ligation. The resulting DNA fragment library was then sequenced on a picotiterplate on the GS FLX using the Roche/454 FLX+ chemistry obtaining around 5×10^5 sequence reads per sample.

16.2.5 Data Analysis

The MG-RAST metagenomic analysis server (Wilke et al., 2013) was used to analyze the pyrosequencing data. In a first step all sequencing reads obtained were subjected to quality control (QC) which removed low quality and duplicate reads. In a subsequent step reads matching ribosomal RNA sequences were removed. For all remaining read sequences confrontation against both protein and DNA databases were then used to classify the metagenomic data at various levels (kingdom, domains, classes, etc) and annotate the data at the functional (protein) level. Statistical analysis of the metagenomic profiles obtained was conducted with STAMP (Parks et al., 2014) applying Fisher's Exact test and the Benjamini-Hochberg correction to adjust p-values for the false discovery rate (FDR).

Using the same reads that passed the MG-RAST QC step and had been subjected to the MG-RAST analysis, a second alternative classification method, MYTAXA was also performed (Luo *et al*., 2014).

16.3 Results

16.3.1 Water sampling and sequencing

Metagenomics analysis was performed only on four Danube water samples which had been selected based on various anthropogenic pressures. The sites were classified as JDS27M (low polluted site – Hercegszanto middle, 27), JDS33L (urban pollution – downstream Novi Sad left, 33), JDS36L agricultural pollution – downstream Tisza left, 36) and JDS39L (industrial pollution – downstream Pancevo left, 39). As can be seen from Table 27 only small differences were observed among the sites regarding the measured physicochemical sample parameters and the bacterial numbers (large cells). For what concerns the detection of main anthropogenic activities, the relatively small distance covered by the four sampling sites (about 100 km) does not allow, by comparing sites, to detect a mixture of or individual pollutants generally present, because of the absence of a "clean" reference sample. On the contrary, if a new pollutant is introduced somewhere in between the sites the resulting anthropogenic pressure should induce changes that might be detected. However, the magnitude of the expected change (global difference in microbial community composition) is presently unknown and still needs to be explored empirically by studies like the one described here. Furthermore, due to the relatively low distance (roughly 100 km between first and last sampling site) between the sampling sites chemical contaminants were expected to represent a mixture of the individual pollutants rather than being dominated by class of pollutants representing the main anthropogenic activities.

Table 27: Environmental data linked to the four selected sites. Large bacterial cell numbers were derived from the data set presented in chapter 15 on Microbial Ecology

	Sample 1	Sample 2	Sample 3	Sample 4
	Hercegszanto	downstream Novi Sad	downstream Tisza	downstream Pancevo
Sample type	Natural park	Urban	Agricultural	Industrial
Longitude (North)	45.91407	45.2605	45.0391	44.81913
Lattitude (East)	18.80612	19.8855	20.35963	20.64592
Collected (date)	Aug-30-2013	Sep-2-2013	Sep-4-2013	Sep-6-2013
pH	8.12	8.13	7.99	7.99
Conductivity [µS/cm]	397	381	406	383
Oxygen [mg/l]	8.41	7.92	7.03	7.33
Temperature	21.5	21.25	21.45	21.59
Large bacterial numbers [10^6/l]	8.8	6.4	7.6	7.2
Sample ID	JDS_C_27M	JDS_U_33L	JDS_A_36L	JDS_I_39L

16.3.2 Data analysis

16.3.2.1 MG-RAST

After the quality control and the exclusion of reads representing ribosomal RNA sequences, all remaining read sequences were matched c against both protein and DNA databases to classify the metagenomic data at various levels (kindom, domains, classes, etc) and annotate the data at the functional (protein) level.

An example of the MG-RAST results is shown in Figure 92 comparing the community composition of the four sites at the domain and class level. No major differences were observed between the samples at the domain level with bacteria, as expected, being most abundant. At the class level, Actinobacteria and Betaproteobacteria were observed most frequently followed by Alphaproteobacteria and Gammaproteobacteira and a relatively large number of unassigned bacteria.

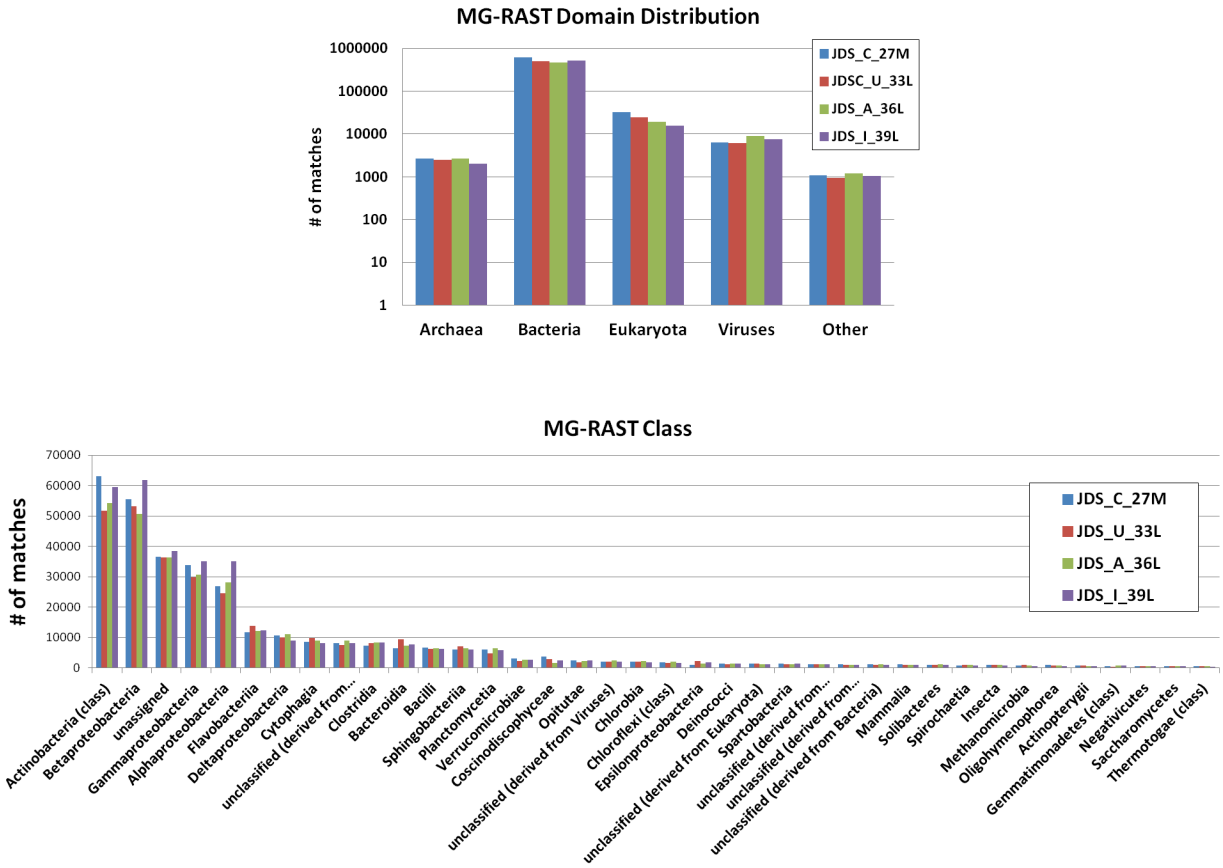

Figure 92: MG-RAST classification at the domain (top) and class (bottom) level with Y-axis representing abundance expressed as # of matches against the MG-RAST database

In order to quantify the small differences at the class level apparent in the raw MG-RAST results (Figure 92) statistical significance of the differences was evaluated between pairs of samples using Fisher's Exact Test. The analysis showed that, relative to the most upstream sample (JDS27M, Hercegszanto middle, low pollution site) there was a consistent and significant decrease of Actinobacteria in the other three samples (Fig. 93). Additional differences detected are a small (about 4%) increase of Alpha- and Betaproteobacteria between JDS27M and the "industrial pollution" site JDS39L. All of the remaining differences were in the order of 2% or less (Fig. 93). Pair-wise comparison between the other three samples JDS33L, JDS36L and JDS39L showed a small fluctuations of Alpha- and Betaproteobacteria between samples (data not shown). However, since all fluctuations observed are less than 10%, and therefore relatively small, these results will have to validated through replicate experiments.

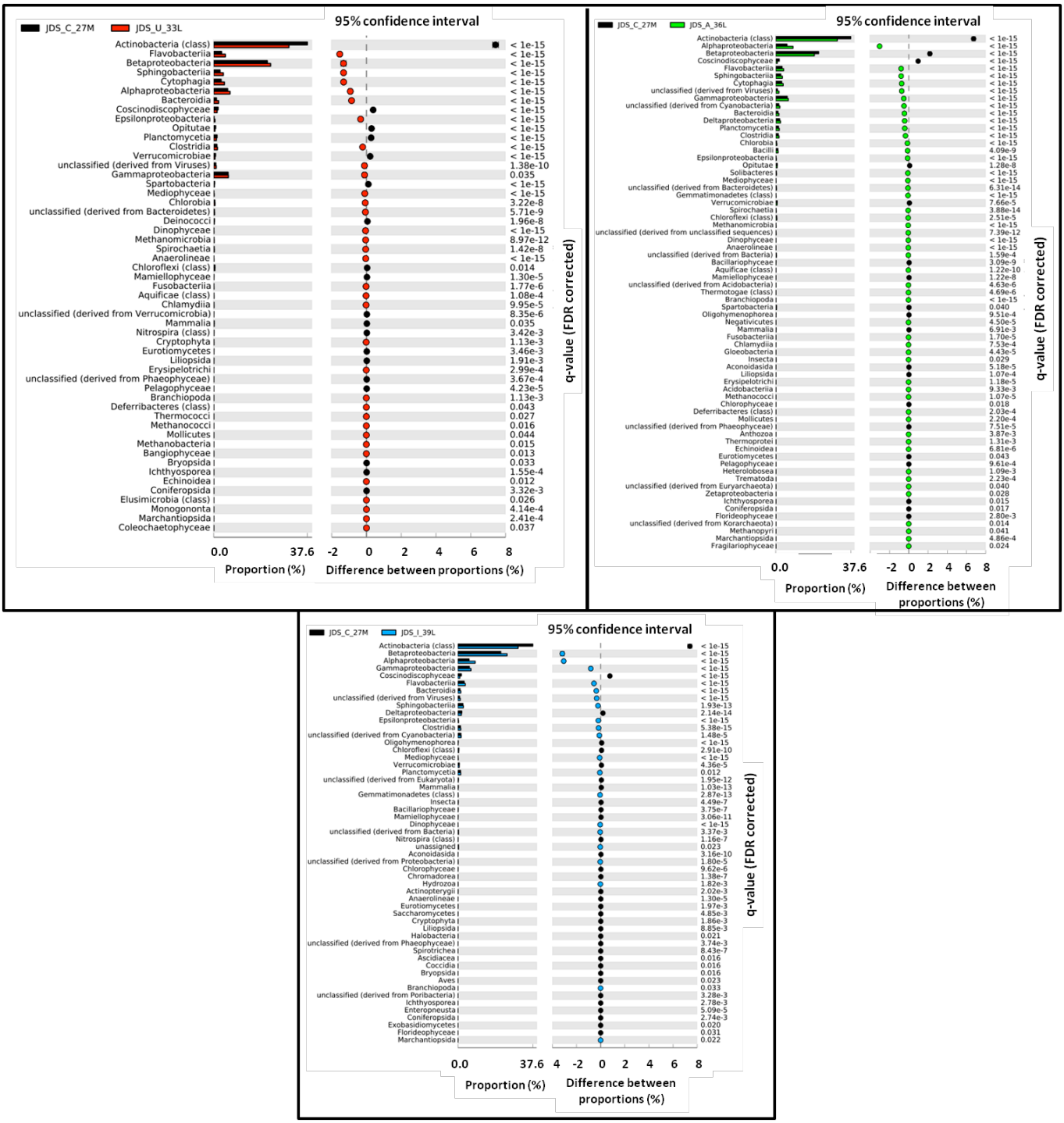

Figure 93: STAMP analysis of the MG-RAST classification results at the class level. Shown are the comparisons of sample JDS27M against JDS33L (top left), JDS36L (top right) and JDS39L (bottom). For each comparison the left side of the plot indicates the proportion of a particular class in the two samples while the right side illustrates the difference of proportions (%) and its statistical significance expressed as a False Discovery Rate (FDR) corrected q-value.

16.3.2.2 MYTAXA, an alternative method to MG-RAST

Also MYTAXA uses an alternative approach to analyze metagenomic data. Also in this case only small differences in taxonomy composition were observed between the samples. An example is shown in Fig. 94 representing the differences detected at the Genus levels (Fig. 94). On a first sight the values appear to indicate a relatively large change, for example for Arcobacter or Acineobacter between sample JDC27M and the other three samples. However, for a correct interpretation an analysis

equivalent to that performed for MG-RAST by the STAMP method is required, i.e. an analysis not relying simply on the absolute percent fraction (or the derived ratio between sample pairs) but considering also the absolute number of matches (against a particular organism group) relative to the overall total number of counts. The output format of the MYTAXA results did not however not allow to perform such an analysis.

An additional consideration that has to be taken into account is the following: it is well known in the literature that analysis of metagenomic data with different algorithms can give slightly different results, due to different methodologies and/or different genomic sequence reference databases that are used. Acinetobacter are organisms capable of degrading aromatic compounds which could explain why they appeared enriched at the "industrial" JDS39L site. Bacteroides represent normally the most substantial portion of the human gastrointestinal flora which could explain their higher abundance of Bacteroides mainly in the urban and industrial area (downstream Belgrade and Pancevo). However, like for MG-RAST, the observed differences have to be confirmed by additional experiments.

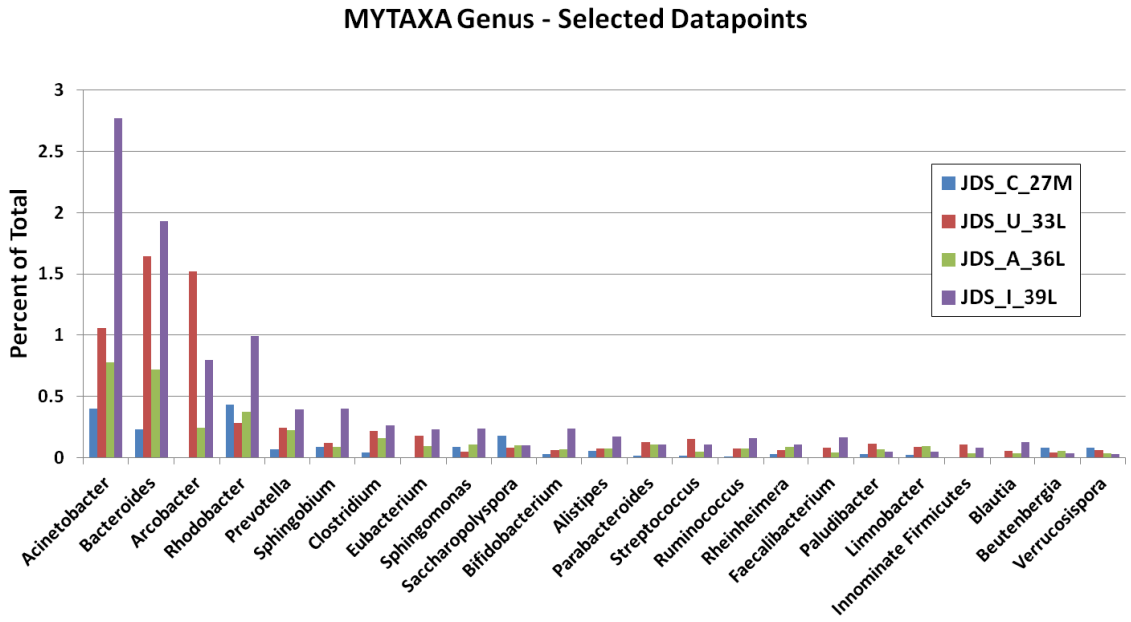

Figure 94: Selected MYTAXA differences between samples at the Genus level

16.4 Conclusions

Microbial Communities of four Danube areas were characterized by using a metagenomics approach. Only small differences were observed in the corresponding microbial composition. The expected close link between the microbial community and the existing environmental pressure like chemical pollutants therefore suggests that only small differences in anthropogenic pressures to be present among the sites, consistent with the available chemical data. A more in-depth analysis and validation of the results will only be possible once additional data from the same sites have been collected and analyzed. However, also small changes, once confirmed, might represent an indicator for a certain class of pollutants. It is presently unknown what changes should be expected due to the presence of an environmental pollutant and how different classes of chemical compounds might affect the microbial community composition. In this respect it is also important to point out the lack of adequate "clean reference samples" that could be used as a reference point. The preliminary results from the present preliminary taxonomical approach therefore need to be refined and complemented by the analysis of metabolic pathways and functional genes, since their modulation is also influenced by the environmental pressures (Kisand et al., 2012).

The present study should be extended including also other more distant sites in order to more completely monitor the microbial profile along the Danube river. A more comprehensive and global characterization of the microbial diversity will most likely reveal more substantial differences in microbial composition and detect the presence of many unknown and frequently unculturable microorganisms. Subsequently a selected subset of microorganisms and/or metabolic genes could be used as an ecological indicator complementing existing analysis focusing on chemical and additional stressors (Nardini, et al., 2010) and as indicators of a stress response and therefore of water quality.

To date, no "microbial-based" ecological indicators are included within the Water Framework Directive. At the same time they represent the link between ecological and chemical status of water quality. The current study suggests that additional efforts are needed in order to better characterize and understand these new indicators and make them as routine tool in monitoring water quality.

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16.6 Acknowledgments

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17 General Physico-Chemical Parameters and Nutrients

Carmen Hamchevici, Florentina Dumitrache, Fabrizio Sena, Gunther Umlauf, Carmen Postolache, Ion Udrea

17.1 Introduction

Natural background content of nutrients, especially Nitrogen (N) and Phosphorous (P) are essential to ecosystem biota, for balanced plant and microbial growth. However, excessive nutrients enrichment caused by anthropogenic activities negatively impacts the ecosystem, often resulting in eutrophication which impairs both the physical and biological health of an aquatic system. In the Danube Basin the nutrients issue has a long history in the monitoring activity, starting from the Bucharest Declaration (1985) up to the on-going Trans-National Monitoring Network (TNMN) operated under the ICPDR monitoring strategy. In addition, nutrients loads from the Danube Basin have a major role in protection of the Western Black Sea shelf from eutrophication, therefore several international projects at the basin wide level addressed the management of the nutrients in the Danube River Basin (daNUbs 2005; Kroiss et al. 2005; Schreiber et al. 2005). Starting with the entry into force of the Water Framework Directive (WFD) in 2000, Annex V of the WFD, in Section 1.1.1 (Rivers), requires three groups of quality elements to be used in the ecological status assessment, among which the third group refers to the "*chemical and physico-chemical elements supporting the biological elements*".

This chapter aims to present the longitudinal distribution of the selected physico-chemical parameters measured in the Danube River and major tributaries during JDS3 and to compare the obtained results with previous outcomes from both investigative and surveillance monitoring. Preliminary ecological indication given by these supportive elements is also assessed based on the ranges (minimum – maximum) of the environmental quality standards/guiding values reported by the Danube countries (where available) for *high/good* and *good/moderate* classes respectively; however, due to the lack of harmonisation of the class boundaries for these elements at the basin wide level, ecological indication obtained has a limited applicability.

17.2 Methods

Water samples were collected directly from the river with the help of the motor-boat used for the collection of biological samples. *In-situ* measurements (temperature, dissolved oxygen, pH and conductivity) were carried out by portable multiple-probe YSI – EXO2 instrument with dedicated probes, in three profiles of the river (left, middle and right), based on international standardised methods. This chapter takes into account the data recorded in the middle, since the differences among the three profiles are negligible (details in the extended report available on the attached CD). Nutrients forms and basic ions were analysed in water samples by selected laboratories according to EN ISO standardised methods based on molecular spectrophotometry (total forms of N and P) and ion chromatography (dissolved forms of N and P and major ions). The dissolved nutrients forms and major ions (sulphates, chlorides, sodium, potassium, calcium and magnesium) were analysed in 71 sites (apart from the 68 sites from the JDS3 Cruise Manual, three additional sampling sites were sampled: two on the Danube River – upstream *Olt* and upstream *Prut* and one tributary – *Olt*) by the *JRC – Water Research Unit* as in-kind contribution for this group of quality elements. The Summary Report briefly presents the spatial distribution of these parameters while the detailed analysis procedure and results are presented in the full report available on the attached CD.

17.3 Results

The longitudinal profiles of the measured concentrations of selected parameters are shown in Figures 95–98. Similarly to the previous surveys (JDS1, JDS2), the interpretation of the results was made according to the splitting of the Danube main course into three major sections (Joint Danube Survey, Final Report of the ICPDR, 2002; Joint Danube Survey 2, Final Scientific Report 2008): *the upper Danube* – from river km 2600 to river km 1880 (stations JDS1 – JDS12), the *middle Danube* – from river km 1868 to river km 1095 (stations JDS13 – JDS42) and the *lower Danube*: from river km 1077 to river km 0 (stations JDS43 to JDS68). One biological indicator (chlorophyll "a" – Chl. "a") and two physical parameters (suspended solids – SS and measured water discharge – Q) have also been considered in order to investigate the possible relationship among variables.

17.3.1 General physico-chemical determinands

17.3.1.1 Water temperature

Water temperature in the Danube River ranged between 17.8 ^{0}C at river km 132 (*Reni*) and 23.3 ^{0}C at river km 1586 (*Rackeve-Soroksar Danube Arm)*. In tributaries, the range was slightly larger, between 16.9 ^{0}C in the *Siret* and 24.0 ^{0}C in *Tisa* and *Sava* tributaries. Generally, the variation pattern followed the typical behaviour of this variable during the survey period (August – September) and the daily sampling time.

17.3.1.2 Conductivity

In the upper Danube stretch, conductivity significantly decreased from 566 µS.cm^{-1} at river km 2581 (*Böfinger Halde*) to 320 µS.cm^{-1} at river km 2007 (*Oberloiben*); the rapid change (from 497 to 377 µS.cm^{-1}) occurred at river km 2205 (*Jochenstein*), due to the increased water discharge (from 353.3 m^3.s^{-1} to 886 m^3.s^{-1}); this profile is determined by the influence of the *Inn* tributary, with low salt content and similar flow discharge (Laszlo 2002; Hamchevici and Craciun 2008). In the Middle and Lower Danube stretches, the conductivity remained relatively constant – around 400 µS.cm^{-1} – except for the values recorded in two side arms: *Moson Danube Arm end* – 456 µS.cm^{-1} and *Rackeve – Soroksar Danube Arm* – 358 µS.cm^{-1}. Four tributaries – *Sava, Velika Morava, Iskar* and *Jantra* had conductivity level very similar to the Danube River's, while eight tributaries recorded higher values than the main course, but no influence for the downstream stretch of the Danube was noticed. The maximum level (1122 µS.cm^{-1}) was measured in the *Timok* tributary and the minimum (295 µS.cm^{-1}) was measured in the *Drava*.

17.3.1.3 pH

The pH variation range was rather low in the Danube River (0.63 units), showing a good buffer capacity of the water. Nevertheless, the longitudinal profile showed several fluctuations (Figure 95): in the Upper Danube, pH value increased from 7.93 at river km 2285 (*Deggendorf*) to 8.45 at river km 1942 (*Klostemeuburg*); in the middle stretch, values slightly below 8.00 (7.85 and 7.95) were measured at river km 1586 (*Rackeve-Soroksar Danube Arm*) and downstream, at river km 1560 (*Dunafoldvar*). A significant decreasing profile appeared in the *Iron Gates reservoir area*, down to 7.82 at river km 1073 (*Banatska Palanka /Bazias*), due to the decomposition of organic matter in this slow flowing water stretch and consequently lowering the pH by the produced carbon dioxide. In the Lower Danube, most of the pH values were above 8.00. In tributaries, the variation range was higher than in the Danube itself (1.62 units), with the maximum value (8.35) recorded in the *Russenski Lom*; the minimum value (6.73) was measured in the *Timok* tributary, probably due to the effect of local mining activity.

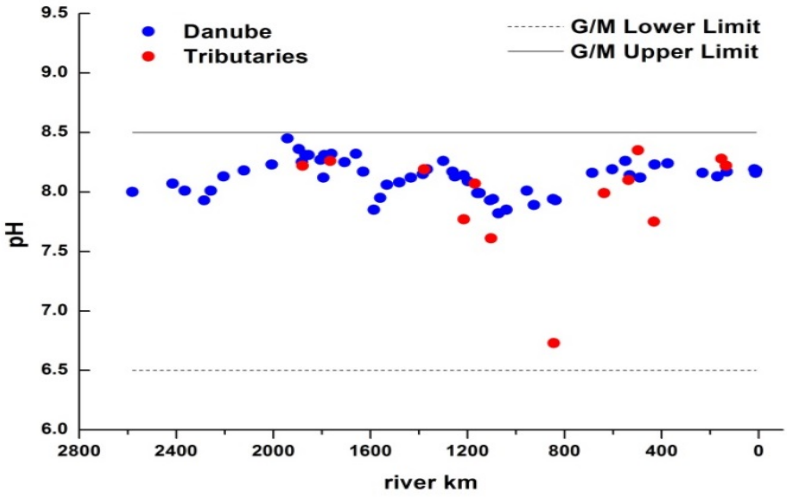

Figure 95: pH variation in water samples during JDS3 (the Danube River and selected tributaries)

17.3.1.4 Dissolved oxygen

The longitudinal profile of the dissolved oxygen content, concentration and saturation respectively (Figure 96 – a,b) is highly similar with the that observed for pH, showing a relatively good equilibrium between oxygen-consuming processes (decomposition of organic matter and respiration) and oxygen-releasing processes (production and physical transfer from the atmosphere), with most of the saturation levels situated between 80% and 110%. In the Danube River, except for two values, all sampling sites were characterised by concentrations above 6.0 $mg.L^{-1}$ with 80% saturation. Oxygen depletion (4.3 $mg.L^{-1}$ with 50.6%) appeared at river km 1586 (*Rackeve-Soroksar*), likely due to the high organic pollution in this dammed side arm. Particularly in the *Iron Gates* area, dissolved oxygen saturation fell below 80%, reaching 67.6% downstream the dam, at river km 926 (*Vrbica/Simijan*), this local fluctuation being caused by the increased biodegradation of organic matter. Two tributaries – *Tisa* and *Velika Morava* presented low dissolved oxygen content (5.2 $mg.L^{-1}$ with 61.5% and 4.3 $mg.L^{-1}$ with 48.4% respectively), but the rest of the tributaries showed a similar level compared to the main course of the Danube.

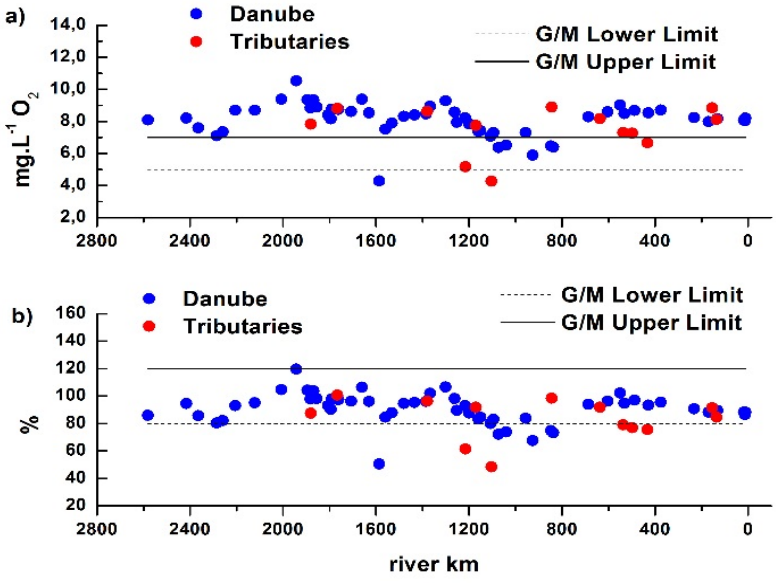

Figure 96: Dissolved Oxygen content – a) concentration and b) saturation – during JDS3 in the Danube River and selected tributaries

17.3.2 Nutrients

17.3.2.1 Total Nitrogen

Figure 97 shows the spatial pattern of the Total Nitrogen concentration in water samples, with a variation range of 2.62 mg.L^{-1} N in the Danube River and 5.08 mg.L^{-1} N in tributaries. A significant decreasing line from the Upper towards Middle and Lower Danube is noticed (N=55, r^2 = 0.6278, p<0.0001). The highest concentrations from the Danube (above 2.50 mg.L^{-1} N) were measured in the first five sampling sites, followed by a pronounced dropping after the river km 2285 (*Mühlau*). Along the middle stretch of the river, Total Nitrogen ranged between 1.40 and 2.04 mg.L^{-1} N, except for the low value (0.75 mg.L^{-1} N) recorded in the *Rackeve-Soroksar Danube Arm*, caused either by strong uptake during biological activity or by denitrification process in this slow-flow area. In the Lower Danube, the Total Nitrogen concentrations fell from 1.50 mg.L^{-1} N at rkm 1073 (*Banatska Palanka/Bazias*) to 1.06 mg.L^{-1} N at rkm 532 (*downstream Jantra*), the decreasing profile (mainly visible in the *Iron Gates reservoir* stretch) being a direct consequence of the denitrification process in this region. A slight increasing profile was present in the second part of the lower Danube, up to 1.39 mg.L^{-1} N in the *Sf. Gheorghe arm*, which shows that the Danube Delta has no influence in nitrogen retention or loss. All the tributaries sampled in the upper and in the middle stretch of the Danube had concentrations lower than in the Danube, except for the *Velika Morava*, with higher value. Very low concentrations (0.69 mg.L^{-1} N) were found in the *Tisa* and *Sava* tributaries. In the lower stretch, in four tributaries – *Iskar*, *Jantra*, *Siret* and *Prut* – Total Nitrogen concentrations were slightly higher than in the Danube, but in *Timok*, *Russenski Lom* and *Arges* elevated levels were measured: 3.77, 5.71 and 5.77 mg.L^{-1} N respectively, most likely caused by the insufficiently treated waste water discharge in these recipients.

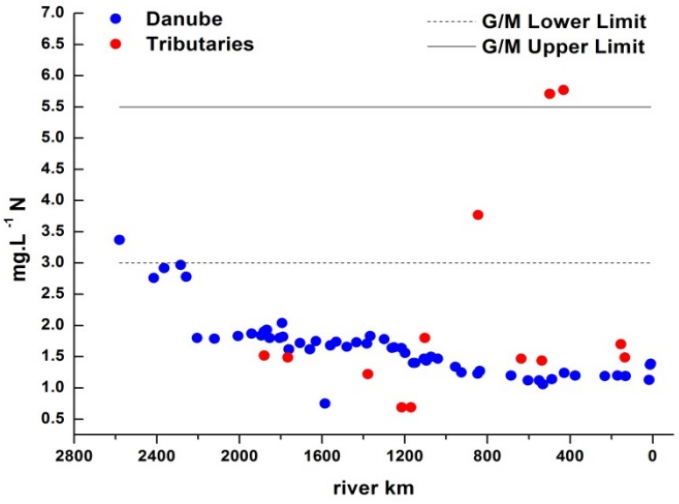

Figure 97: Total Nitrogen concentrations in water samples during JDS3 in the Danube River and selected tributaries

17.3.2.2 Total Phosphorous

The variation ranges of Total Phosphorous were 0.08 mg.L^{-1} P in the Danube River and 0.54 mg.L^{-1} P in tributaries (Figure 98). Unlike the Total Nitrogen, no systematic spatial trend was recorded along the Danube, but several local fluctuations were present: in the upper stretch, a decreasing line from 0.10 mg.L^{-1} P at rkm 2205 (*Jochenstein*) to 0.05 mg.L^{-1} P at rkm 1942 (*Klosterneuburg*). The concentration level of 0.10 mg.L^{-1} P was reached in the middle stretch in the side arms *Moson Danube* and *Rackeve-Soroksar* and at rkm 1384 (*upstream Drava*). The maximum value from the Danube River (0,11 mg.L^{-1} P) was measured at rkm 1367 (*downstream Drava (Erdut/Bogojevo)*), but not caused by the confluence with the *Drava* tributary, in which little content of Total Phosphorous was found (0.08 mg.L^{-1} P). In the lower stretch, a strong decreasing profile was noticed after the confluence with the *Tisa* tributary, from 0.08 mg.L^{-1} P at rkm 1199 (*downstream Tisa/Upstream Sava (Belegis)*) to 0.05 mg.L^{-1} P at rkm 1159 (*upstream Pancevo/downstream Sava*). The minimum

concentration (0.03 mg.L^{-1} P) was measured at rkm 847 (*upstream Timok (Rudujevac / Gruia)*). This significant declining profile in concentrations comes in good agreement with previous results according to which the *Iron Gates reservoir* and backwaters – as a net sedimentation area – act as a major retention sink for Total Phosphorous (daNUbs 2005). Along the rest of the lower stretch, Total Phosphorous concentrations gradually increased up to 0.09 mg.L^{-1} P in the *Sulina arm*, profile which is confirmed by similar previous findings which shows that the delta does not play a major role in Phosphorous retention (daNUbs 2005). The selected tributaries from the upper and middle stretches showed similar levels as the main course of the river. In the tributaries from the lower stretch concentrations varied from undetectable level (less than 0.03 mg.L^{-1} P) in *Timok* to the maximum level (0.55 mg.L^{-1} P) in *Arges*. Elevated concentrations were also measured in *Siret, Iskar*, *Prut, Jantra* and *Russenski Lom*.

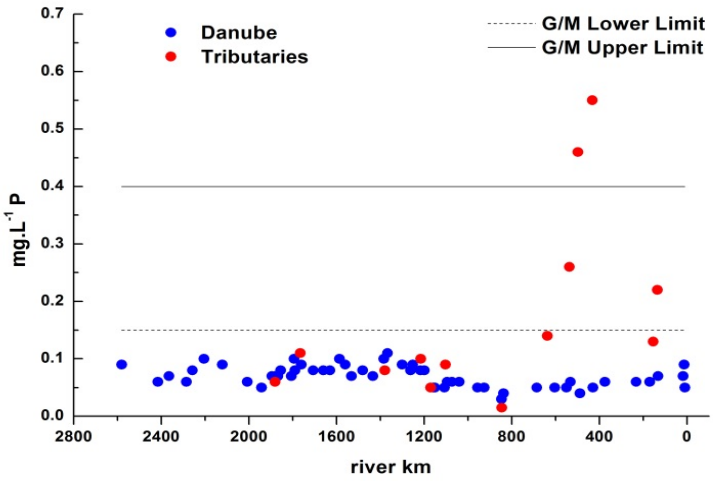

Figure 98: Total Phosphorous concentrations in water samples during JDS3 in the Danube River and selected tributaries

17.3.2.3 Dissolved nutrients forms: ammonium, nitrites, nitrates and ortophosphates

- Most of the sampling sites from the main course of the Danube River (50 out of 57) and nine tributaries presented N-ammonium concentrations below the limit of quantification (0.04 mg.L^{-1} N-NH_4); the maximum values (0.23 mg.L^{-1} N-NH_4 in the Danube and 3.71 mg.L^{-1} N-NH_4 in tributaries) were recorded at rkm 1586 (*Rackeve-Soroksar*) and in *Arges* tributary respectively, caused by the high organic pollution in these sites.
- All N-nitrites concentrations were below the limit of quantification (0.006 mg.L^{-1} N-NO_2) except for two values measured downstream the *Iron Gates reservoirs* area and at rkm 837 (*Pristol/Novo Selo Harbour*) and two values in tributaries (*Velika Morava* and *Jantra*).
- N-nitrates concentrations showed a decreasing longitudinal profiles from Upper to Middle and Lower Danube, starting from the maximum value of 3.20 mg.L^{-1} N-NO_3 at rkm 2581 (*Böfinger Halde*) to the level of 0.90 mg.L^{-1} N-NO_3 in the lower Danube. In tributaries, the highest concentrations (3.62 and 5.21 mg.L^{-1} N-NO_3) were found in *Timok* and *Russenski Lom* respectively.
- Ortho-phosphates concentrations presented a scattered spatial profile along the Danube, with a variation range of 0.107 mg.L^{-1} P-PO_4; in tributaries, values below the limit of quantification (0.0008 mg.L^{-1} P-PO_4) were measured in *Drava*, *Timok* and *Olt*, while the most elevated concentrations (0.258, 0.355 and 0.502 mg.L^{-1} P-PO_4) were found in *Morava*, *Russenski Lom* and *Arges*.

17.3.3 Major Ions

The major ions measured in the Danube River and tributaries ranged within the normal levels given by the local geological, climatic and geographical conditions. Rather high values of chlorides (139.0 $mg.L^{-1}$ Cl) and sulphates (533.4 $mg.L^{-1}$ SO_4) were measured in *Olt* and *Timok* tributaries respectively.

17.3.4 Correlation among variables

In order to have a more illustrative view of the interrelation of the selected parameters, the correlation matrix among variables was carried out for the data corresponding to the Danube River only (Table 28). Statistically significant coefficients ($p<0.05$) were found between variables showing causes (nutrients input), biological response ofz the primary production process (chlorophyll – a) and secondary effects (dissolved oxygen content and pH): positive correlations were obtained between Total Phosphorous and chlorophyll – a (0.489), between chlorophyll – a and both pH and dissolved oxygen saturation (0.516 and 0.634 respectively), while the highest coefficient was noticed between pH and dissolved oxygen saturation (0.906). Nevertheless, it turned out that the hydrological regime had a strong influence on the investigated elements: the dilution effect was highlighted by the negative significant coefficients between water discharge and conductivity (-0.585), more powerful in the case of Total Nitrogen (-0.720) than in the case of Total Phosphorous (-0.364), for the latter nutrient form the adsorption on the suspended solids being demonstrated by the significant positive coefficient (0.535) between these two variables.

Table 28: Correlation matrix for selected physico-chemical indicators and additional parameters (* marked correlations are significant at p < .05 for N=50 and case wise deletion of missing data)

parameter	w.t.	pH	Cond.	DO sat.	Total N	Total P	Chll. a	SS	Q
w.t.	1.000								
pH	**-0.335 ***	1.000							
Cond.	0.026	-0.261	1.000						
DO sat.	-0.110	**0.906 ***	-0.230	1.000					
Total N	0.151	-0.026	**0.700 ***	0.119	1.000				
Total P	-0.219	**0.294 ***	0.048	**0.344 ***	**0.323 ***	1.000			
Chll. a	0.254	**0.516 ***	**-0.312 ***	**0.634 ***	0.018	**0.489 ***	1.000		
SS	-0.190	**0.308 ***	-0.275	**0.299 ***	-0.087	**0.535 ***	**0.634 ***	1.000	
Q	0.254	-0.228	**-0.585 ***	-0.232	**-0.720 ***	**-0.364 ***	0.139	0.136	1.000

17.3.5 Comparison with previous outcomes

Among the specific objectives of the investigative monitoring surveys one refers to increasing the comparability between a homogenous data set produced by a single sampling procedure and laboratory analysis (JDS measurements) and data generated by long-term surveillance type of monitoring (TNMN data) carried out by the basin-wide network of National Reference Laboratories under the ICPDR Monitoring strategy. In order to have an optimal way of data comparison and given the survey timing of JDSs (August – September), the momentary results obtained during the three investigative surveys (JDS1 – 2001, JDS2 – 2007 and JDS3 – 2013) were compared with mean, median and 90-Percentiles of the TNMN data set from August – September during 2001 – 2011 (Yearbooks, 2001 – 2011). Data analysis was carried out for Total Nitrogen and Total Phosphorous, for the common sampling sites of TNMN and JDS located on the main course of the Danube and selected major tributaries (Laszlo 2002; Hamchevici and Craciun 2008). The box-plots shown in Figures 99 (a,b) – 100 (a,b) conclude the followings:

- **Total Nitrogen** (Figure 99 a, b): the general view shows high comparability of the three JDSs with TNMN data for the Danube River. A closer look indicates that the median value of concentrations measured during JDS3 (1.40 $mg.L^{-1}$ N) was lower than the ones from JDS1 and JDS2 (1.82 and 1.67 $mg.L^{-1}$ N) and lower than statistics of the TNMN (1.82, 1.71 and 2.29 $mg.L^{-1}$ N), which generally demonstrates an improvement in Total Nitrogen content in the main course of the river. The median value measured during JDS3 in tributaries (1.49 $mg.L^{-1}$ N) was lower than the one from JDS1 (2.14 $mg.L^{-1}$ N) and the TNMN statistics (2.02, 1.96 and 2.42 $mg.L^{-1}$ N), but slightly

higher than in JDS2 (1.26 mg.L^{-1} N). It is important to mention that the Total Nitrogen concentrations in JDS3 in the *Russenski Lom* and *Arges* tributaries were lower than the ones measured during the previous two surveys.

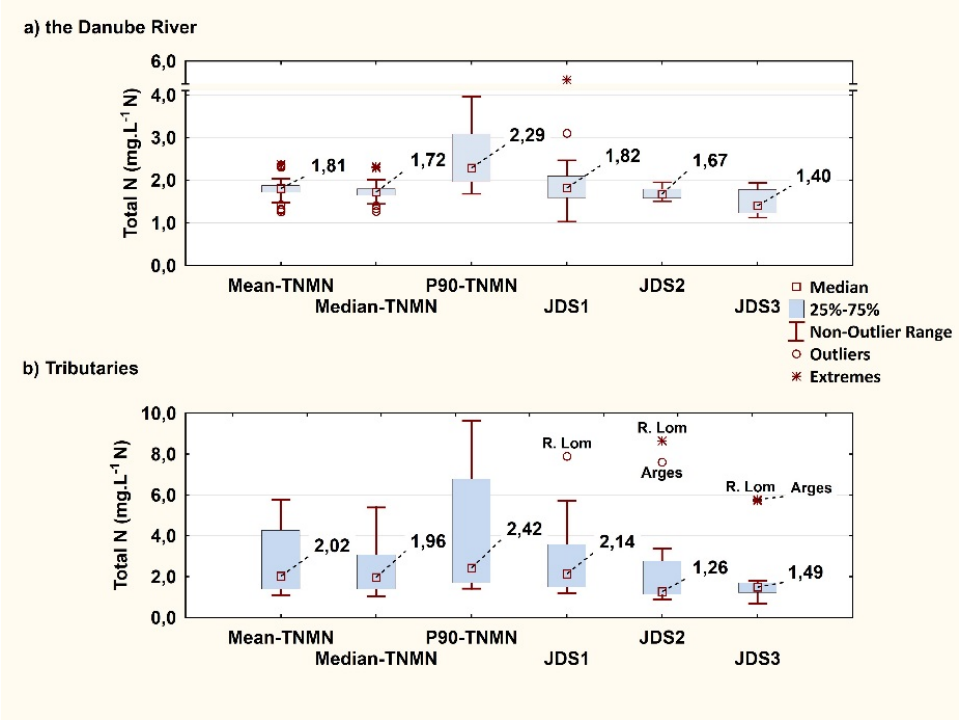

Figure 99: Comparative view of the data from Surveillance Monitoring TNMN (August – September during 2001 – 2011) and Investigative Monitoring (JDS1–2001, JDS2–2007 and JDS3–2013) for Total Nitrogen concentrations in a) the Danube River and b) selected tributaries

– **Total Phosphorous** (Figure 100 a, b): the same high comparability of the three JDSs with TNMN data for the Danube River is present also in the case of Total Phosphorous. In details, the median value of concentrations measured during JDS3 is the lowest from the three surveys and the TNMN statistics for the Danube River as well as for the selected tributaries. Unlike the Total Nitrogen, Total Phosphorous concentrations measured in JDS3 in the *Russenski Lom* and *Arges* tributaries were higher than the ones measured during JDS1 and JDS2, but still within the non-outlier range given by the 90-Percentiles of the TNMN data (this is valid for the *Arges* tributary only, for the *Russenski Lom* compilation was not carried out because of the data inconsistency).

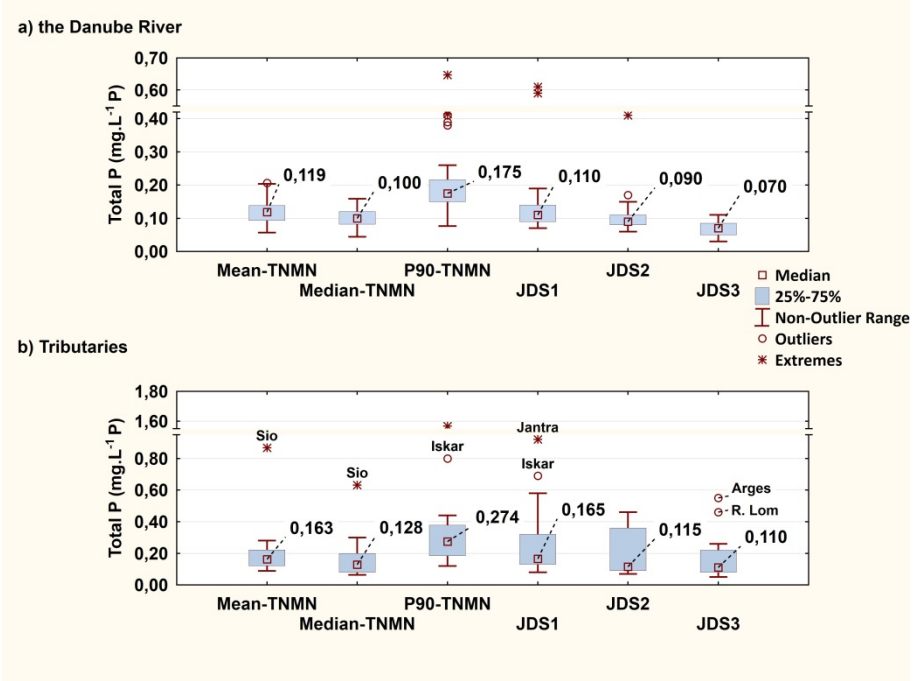

Figure 100: Comparative view of the data from Surveillance Monitoring TNMN (August – September during 2001 – 2011) and Investigative Monitoring (JDS1–2001, JDS2–2007 and JDS3–2013) for Total Phosphorous concentrations in a) the Danube River and b) selected tributaries

17.3.6 Compliance with Danube relevant environmental quality standards / guiding values

Despite the fact that one single measurement does not give fully reliable information on the ecological status as required by the WFD, a preliminary ecological indication described by the general physico-chemical elements is given below. Due to the lack of harmonisation of the environmental quality standards between ecological classes at the basin wide level for these quality elements, the compliance was made taking into account the ranges (lower – upper limits) of the values reported by the Danube countries (where available) for *high/good* and *good/moderate* classes respectively. From Figures 95-98 (in which only the critical *good/moderate* boundaries are drawn), the following information is obtained:

- all pH values are within the ”*good*” class for both Danube River and tributaries;
- the compliance results for dissolved oxygen content are different, depending on the quality standard involved: based on the saturation data, five sampling sites located in the *Iron Gates reservoirs* and downstream (JDS43, JDS44, JDS46, JDS47 and JDS49) fall into the ”*moderate*” class, while based on the concentration data, these five sites are still in ”*good*” class. Similar situation is shown in case of four tributaries: *Tisa*, *Jantra*, *Russenski Lom* and *Arges*: ”*moderate*” by saturation and ”*good*” by concentration. The *Rackeve-Soroksar arm* and *Velika Morava* are in ”*moderate*” class based on both concentration and saturation results. The rest of the sites from the main course of the Danube as well as the rest of selected tributaries are in ”*good”* or *”high”* classes based on both parameters;
- all Total Nitrogen and Total Phosphorous concentrations measured in the Danube and in most of the tributaries are characteristic to ”*good*” class, while the values from *Russenski Lom* and *Arges* fall in ”*moderate*” class; similarly, the dissolved nutrients forms put the Danube River and most of the selected tributaries in ”*high*” or ”*good*” class; ”*moderate*” class appears in three tributaries: *Morava* (P-orthophosphates), *Russenski Lom* (N-nitrates and P-orthophosphates) and *Arges* (N-ammonium and P-orthophosphates).

17.4 Conclusions

- Water temperature measured in the Danube River and in selected major tributaries followed the typical pattern for the timing of the survey (August – September), with larger variation range in tributaries than in the Danube.
- The longitudinal distribution of conductivity in the Danube River showed a strong decreasing line in the upper stretch, followed by a constant profile towards the middle and lower stretches. The dilution effect along the Danube was showed by the significant correlation coefficient of conductivity with water discharge values.
- pH and dissolved oxygen content (high positive correlation) demonstrated a general good balance between primary production and decomposition of organic matter, with most of the oxygen saturation levels situated around the equilibrium value. Several local depletions were found in specific areas (dammed *Rackeve-Soroksar* side arm, the *Iron Gates* reservoir) and two tributaries (*Tisa* and *Velika Morava*).
- Total Nitrogen presented a strong decreasing profile from upper to lower stretch of the Danube, significantly negatively correlated with water discharge. The typical lower profile was noticed in the *Iron Gates reservoir*, due to the denitrification process from this area. Most of the tributaries presented levels similar to those in the Danube, but elevated concentrations were found in the *Timok*, *Russenski Lom* and *Arges*.
- No systematic trend in Total Phosphorous concentrations along the Danube River was found; still, a slight decreasing line appeared in the lower stretch, more pronounced in the *Iron Gates reservoir* area, due to the retention of the suspended material on which this nutrient form is adsorbed. Six tributaries at their confluence to the Danube presented higher concentrations than the main course of the river, but no influence on the downstream stretch was noticed.
- The Total Nitrogen and Phosphorous levels measured in the three arms of the Danube Delta come in good agreement to previous findings which showed that the contribution of the Danube Delta in nutrients retention is negligible, because most of the Danube water passes directly to the Black Sea, almost not reaching the Delta itself.
- N-ammonium and N-nitrites showed levels below the limit of quantification in most of the sampling sites; N-nitrates showed a significant decreasing profile from upper to lower Danube, while no specific trend was noticed for P-orthophosphates; rather elevated values were detected in the mouth of *Morava* (P-orthophosphates), *Timok* (N-nitrates), *Jantra* (N-nitrites), *Russenski Lom* (N-nitrates and P-orthophosphates) and *Arges* (N-ammonium and P-orthophosphates).
- Major ions presented levels given by the local geological, climatic and geographical conditions. *Timok* and *Olt* tributaries showed elevated concentrations of sulphates and chlorides respectively.
- Compared with the JDS1 and JDS2 results, Total Nitrogen and Total Phosphorous concentrations measured in the Danube River during JDS3 were lower. For these two nutrients forms, high comparability was found between investigative monitoring data (JDS type) and corresponding data (August – September) from long-term surveillance monitoring (TNMN during 2001 – 2011). This outcome clearly demonstrates that one set of homogenous data produced by a single sampling procedure and laboratory analysis carried out by selected laboratory soundly confirms the on-going harmonisation and improvement of operational activity of the of National Reference Laboratories network and the effectiveness of the Analytical Quality Control (AQC) programme organised by the ICPDR at the basin wide level.
- The ecological indication given by the general physico-chemical quality elements was assessed based on the intervals for *high/good* and *good/moderate* ecological classes as resulted from the environmental quality standards/guiding values reported by the Danube countries. The general view is that most of the sampling sites located on the Danube River belongs to either ”*high*” or ”*good*” class, except for the dammed side arm *Rackeve-Soroksar* and the *Iron Gates reservoir area*, which fall in ”*moderate*” class due to the oxygen depletion. ”*Moderate*” class is also present in several tributaries (*Morava*, *Tisa, Velika Morava, Jantra, Russenski Lom and Arges*), caused by low oxygen saturation and dissolved nutrients forms.

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18 Quality and quantity of dissolved organic matter

Tz-Ching Yeh, Elisabeth Bondar-Kunze, Damir Tomas, Alexander Kirschner, Marija Marjanovic-Rajcic, Nina Welti, Thomas Hein

18.1 Introduction

Increasingly, scientists are taking advantages of the optical properties of dissolved organic material (DOM) for describing the pools in natural aquatic systems.Various studies used optical properties of DOM to elucidate the anthropogenic influence on aquatic systems, including sewage effluent (Reynolds and Ahmad 1997), urbanization (Westerhoff and Anning 2000), and landfill leachates (Baker and Curry 2004). Forty to sixty percent of total DOM present in natural systems is fluorescent, primarily consisting of protein and organic acids derived from decayed organisms within the catchment. Those fluorescent substances are called fluorophores which can be measured by scanning excitation and emission wavelengths simultaneously through a set path length to create a3-D contour plot which is called an Ex-Em matrix (EEM). The matrix is composed of peak intensities which are further related to the concentration of the fluorophore present in a water sample.

In large rivers such as the Danube which receive important inputs from human, terrestrial and in situ sources, the resulting DOM pools are a heterogeneous mixture of these carbon species (Massicotte and Frenette, 2011). Especially in some sections of the Danube the sources are subject to anthropogenic environmental heterogeneity (e.g., disconnection of side-arms, drying of wetlands, constructing dams). By using this simple and time efficient method we can interpret the evolution and movement of DOM species both temporally and spatially in the Danube River.

18.2 Methods

18.2.1 Sampling strategy

Three samples, “Left bank (L)”, “Right bank (R)” and “Middle (M)” were collected along the transect for each JDS station. L and R sites were 10 to15 meters away from the respective river banks; the sampling vessel was positioned in the middle of the river by GPS to collect “M” samples. Water was taken from 30 cm below the surface level and was filtered (pre-combusted Whatman GF/F, 2.5 h at 490°C) and stored in purged glass tubes (24 h in 10% HCl, pre-combusted 4 h at 490°C) at 4°C until analysis.

18.2.2 Optical properties of DOM

Fluorescence values were measured by a Hitachi Fluorescence Spectrophotometer F-7000. The scanning method follows Baker (2001) with minor modification, with excitation wavelength 200–450 nm at 5 nm steps and emission wavelengths between 250–600 nm at 2 nm steps. Blanks of Milli-Q water were run before and after each run and were used to standardize to a mean Raman peak.

As the fluorospectrometer scanned a defined wavelength range, fluorophores which exist in samples result as peaks with intensity in their corresponding excitation-emission matrix. Peak B (ex 225–275 nm/ em 300–325 nm) and T (ex 225–275 nm/ em 340–385 nm) represent protein-like substances (Tyrosine-like and Tryptophan-like, respectively) (Baker, 2001), whereas peak C (ex 300–370 nm/ em 400–500 nm) is related to humic-like substances.

For data interpretation, we used the relative relationship between each peak intensity to obtain well-defined indices addressing different aspects of DOM sources according to previous references (e.g., Coble 1996; Welti et al. 2012). Table 29 shows the calculation of each index and the respective indication present in this report.

Table 29: Name and representation of fluorescence indices

Indices	Peak intensity (int.) ratio	Representation	References
Fluorescence index (FI)	Int. at em 450 nm and 500 nm (ex 370)	Inversely related to the lignin content of DOM FI ≒ 1.2: terrestrial/higher plant source FI ≒ 1.8: dominant microbial source; from leachate of bacteria or algae	McKnight et al. 2001 Fellman et al. 2010
Freshness index (or β/α ratio,"BIX")	β(em 380 nm) /α (max. em at 420-435 nm) ex 310	Relative contribution of recently produced DOM to highly degraded DOM BIX > 1: autochthonous microbial origin BIX < 0.6: allochthonous terrestrial origin	Parlanti et al. 2000
Humification index (HIX)	Peak area under em spectra 435-480 nm/ 300-445 nm (ex 255)	Directly proportional to the humic content of DOM HIX > 16: Strong humic character/dominant terrigeneous contribution (high fulvic acid content) HIX 6~10: Dominant humic character and weak recent autochthonous component HIX 4~6: Weak humic character and important recent autochthonous component HIX< 4: of direct aquatic microbial or biotic origin (not humified)	Kalbitz et al. 1999 Zsolnay et al. 1999 Huguet et al. 2009
T280/C ratio	Peak T (ex 275 em 350)/ peak C (ex 320-340 em 410-430)	Tryptophan/fulvic-like fluorescence intensity	Baker 2001

18.2.3 DOC measurement

An additional water sample at each site was prepared in the same manner and was analyzed for its DOC concentration by Croatian Waters.

18.2.4 Data analysis and visualization

The mean value and standard deviation of L, M, R samples at each station were calculated for each fluorescence index, DOC concentration, and other DOM properties. Data was visualized by plotting values against river km to obtain an overview of spatial variation along the course of the Danube mainstream. Mean values for all stations were calculated for each value presented and were presented as dashed line in each graph. Tributaries are not shown.

18.3 Results

In this overview on the results longitudinal patterns of selected indices are presented.

18.3.1 DOC

High DOC concentration with large standard deviation was observed in the upper Danube especially in the first 3 stations and in the region around Vienna (Fig. 101). For the middle section, all stations showed values below overall average except for Hercegszanto. Some peaks were observed for the Lower Danube and the delta region, which might be due to the effect of tributary inputs (e.g. Olt and Siret).

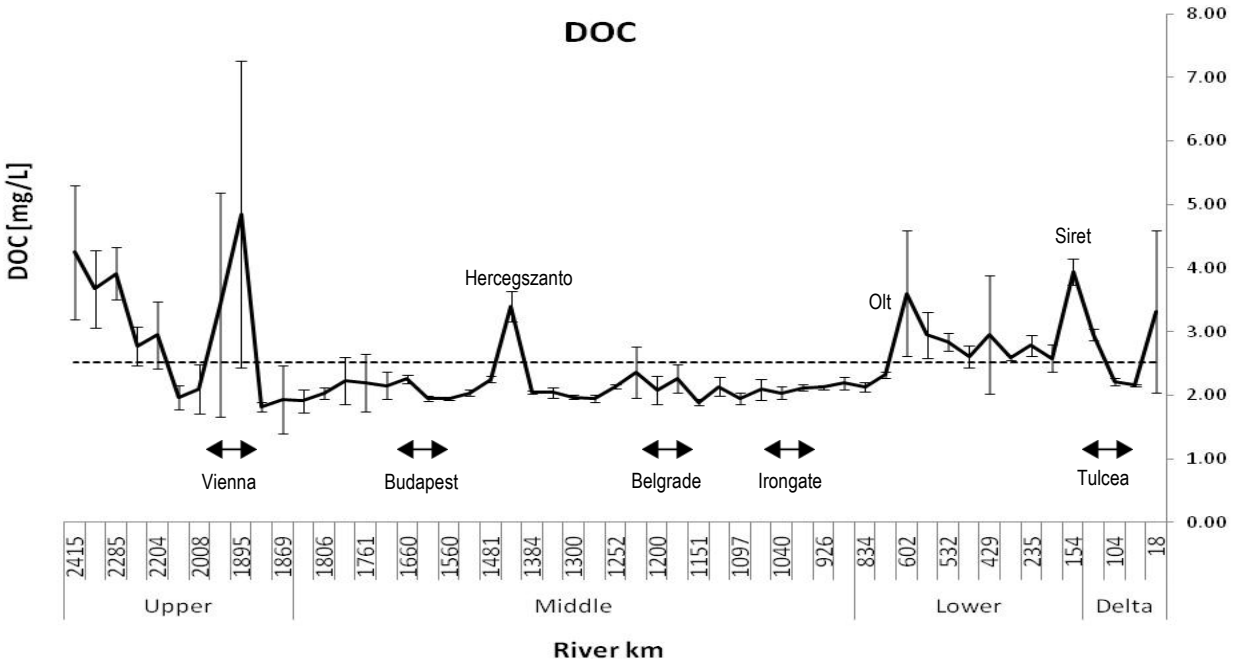

Figure 101: DOC concentration plotted along the Danube River (river km)

18.3.2 T/C ratio

Empirically, T/C ratio represents the intensity of microbial activity to substrate availability, and can be conceptualized as BOD/DOC ratio in inland waters (Coble et al., 2014). In the upper Danube section, especially Deggendorf, Jochenstein and the section between Klosterneuburg to Wildungsmauer (AT), generally higher T/C ratios were observed due to higher T280 values (Fig. 102), which indicates the biologically active organic matter is proportionally higher than more inactive ones (fulvic-like fluorophores) in these upper sections. The T/C ratio of the Middle and Lower Danube stations scatters around the overall mean line, which can be attributed to the downstream dilution effect and catchment inputs of allochthonous sources indicated by continuously increasing C values.

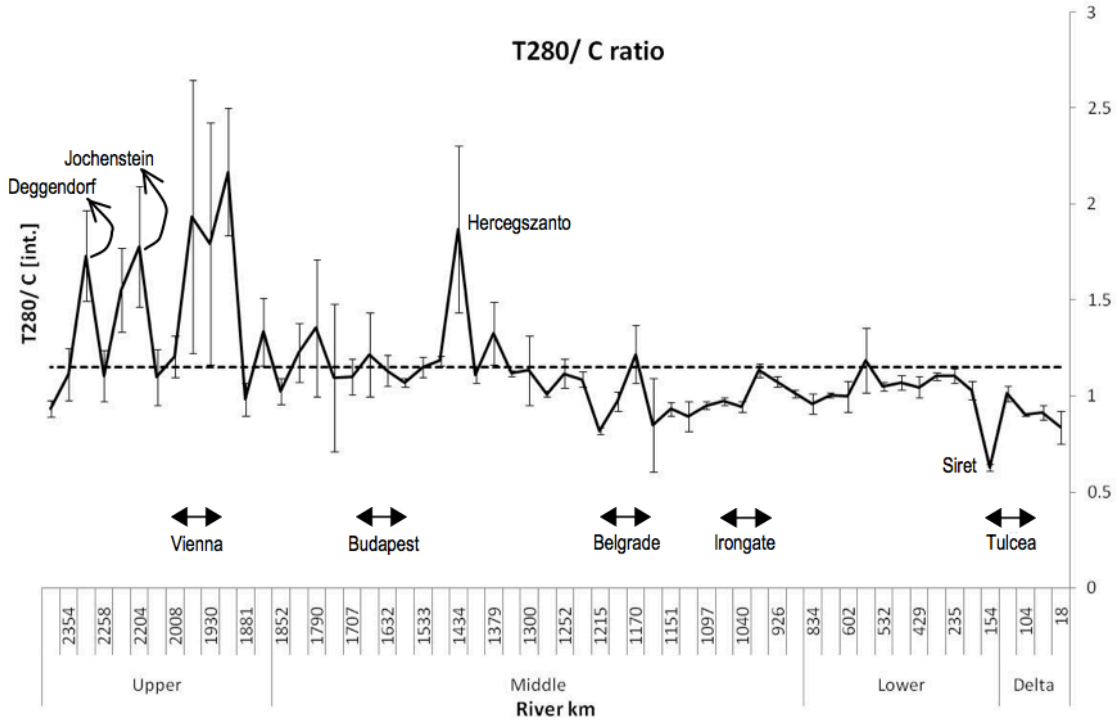

Figure 102: T/C ratio plotted along the Danube River (river km)

18.3.3 Humification index (HIX)

HIX values around 1-2 are associated with non-humified plant material and values >10 are commonly reported for fulvic acid extracts (Ohno, 2002). In the Deggendorf, Jochenstein, region around Vienna, and the Hercegszanto station the HIX peaks were lower than the mean value, while Siret has the highest HIX peak (Fig. 103). Kalbitz et al. (2003) found as biodegradation increases, the HIX value would decrease, which represents a smaller degree of humification. In the case of the Danube data, the T/C ratio corresponds well to HIX. For stations in Deggendorf, Jochenstein and the Vienna region, low HIX ratios may indicate the DOM is derived from algal primary producers and less from terrestrial plant material. In the lower Danube section, the highest HIX from Siret indicated DOM is rich in humified terrestrial substances; this could due to the mixing from tributary discharge, which would indicate releasing higher soil-derived carbon originating e.g. from higher runoff into the Danube River. However, all the HIX values observed are well below 4 indicating a low extent of humification, when compared to other inland water data (e.g., Huguet et al. 2009) suggesting that most of the DOM source is of likely autochthonous, aquatic origin and shows a low extent of degradation.

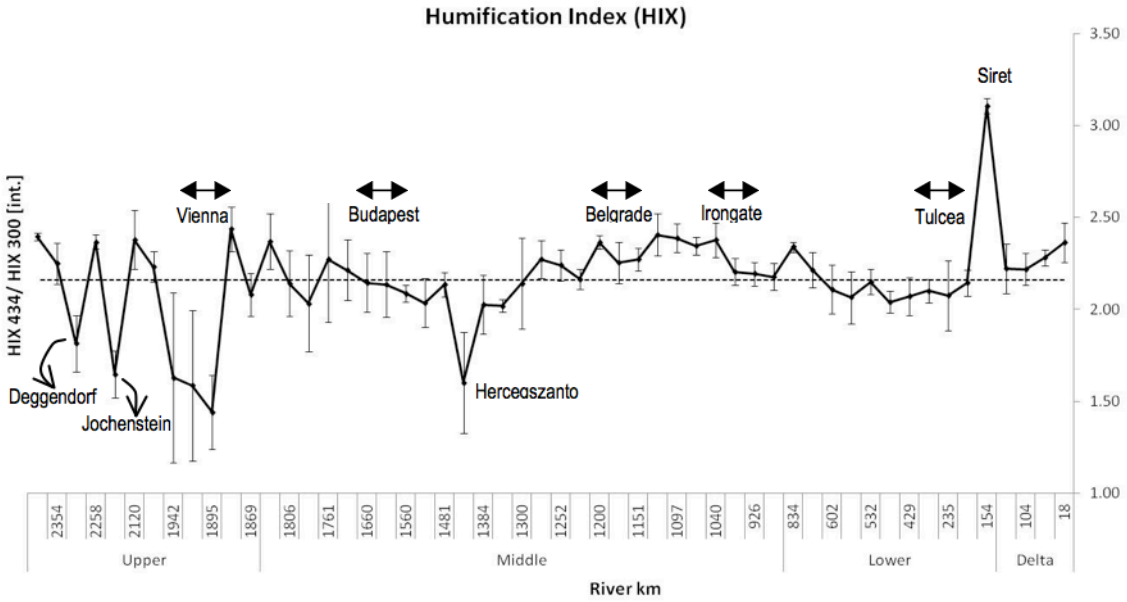

Figure 103: Humification index (HIX) plotted along the Danube River (river km)

18.3.4 Fluorescence index (FI)

The fluorescence index (FI) decreases significantly ($r^2 = 0.49$, $p < 0.05$) along the River from the Upper to the Lower Danube (Fig. 104). FI is inversely related to the lignin content, which indicates that DOM in the upper Danube was mainly originating from lignin-depleted sources such as algae, whereas lignin-rich source from terrestrial environments/higher plants was dominant in the downstream part of the river.

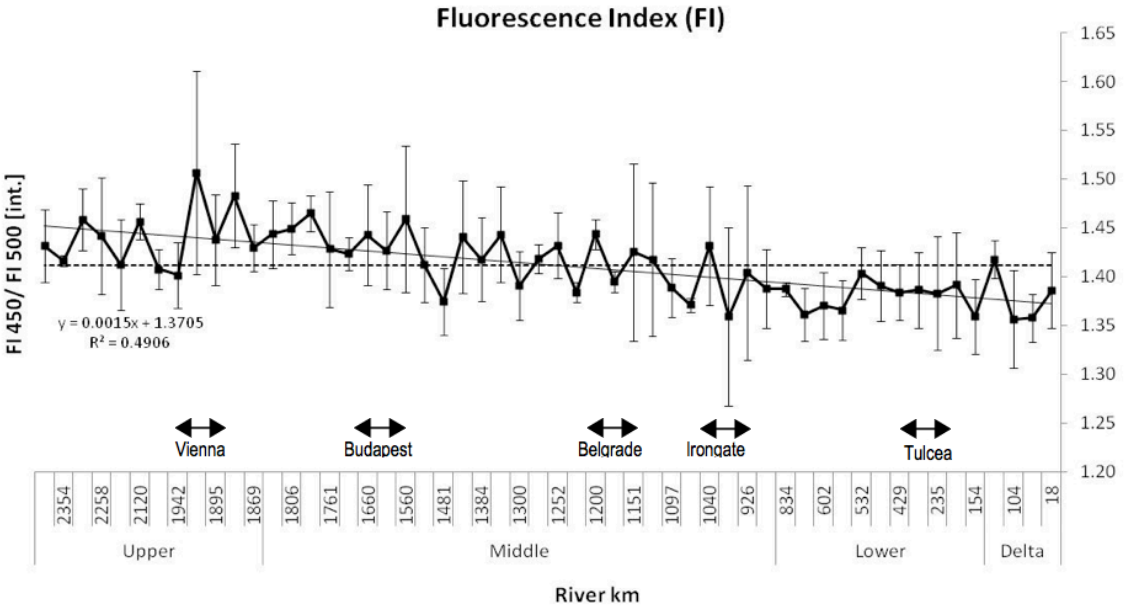

Figure 104: Fluorescence index (FI) plotted along the Danube River (rkm). Solid line indicates the trend line

18.4 Conclusions

- Optical properties of DOM and DOC concentrations showed clear longitudinal patterns and differences for some sections and indicate the dominance of different sources such as waste water, terrestrial sources from the catchment and in-river sources
- The DOC concentrations were in the lower range typical for large, intensely used rivers
- DOM properties reflect the importance of in-river processes versus less input from the catchment for the JDS samples. An indication of severe organic pollution was not found for the Danube River.
- There is evidence of all presented indices that besides terrestrial inputs, algal based sources significantly contribute to the overall carbon pool (as shown for the Upper Danube sections and this is in agreement with other findings as shown in the phytoplankton report).
- The results point to the low substrate availability and humic content in the upper section and more importance of terrestrial inputs in the lower parts. DOM of river water is influenced by site conditions and large scale patterns. Thus, these measurements are indicative for in-river processes (algal based sources) versus catchment effects and provide a link between water quality, catchment effects and biological components.

18.5 References

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19 Petroleum hydrocarbons

Peter Literathy

19.1 Introduction

Among the organic pollutants, petroleum hydrocarbons are considered as one of the most common and frequent organic pollutants, which are introduced from oil refineries, other industries, transportation, municipalities, and also from accidental releases. The oil pollutants, basically aliphatic, aromatic, cyclic and naphthenic hydrocarbons, hetero-compounds, have mainly hydrophobic properties, floating on the surface of the water, or dispersed/dissolved in the water column and associated with the suspended particulate matter (SPM), after settling in the bottom sediment. There is no single analytical method to characterize properly the oil pollution due to its complex nature, as mixture of chemical compounds.

During the Joint Danube Surveys (JDS1, JDS2 and JDS3), different analytical methods were used for characterizing/estimating the oil pollution in water, SPM and bottom sediment, including: GC-FID, UV absorption and fluorescence measurements. Determination of the Total Extractable Matter (TEM), and the Polyaromatic Hydrocarbons (PAHs), that are discussed also in Chapter 20, provided additional information on the petroleum hydrocarbon contamination (oil pollution), particularly in the SPM and bottom sediments.

Since the fluorescence measurements provided data for oil pollution during all three JDSs, the fluorescence fingerprints of the suspended and bottom sediment samples (water samples were analysed during JDS1 and JDS2 only), are used for comparative evaluation.

19.2 Methods

The fluorescence fingerprints of the cyclohexane extracts of water, suspended solids and bottom sediment samples were introduced for estimating petroleum hydrocarbons during JDS1 (ICPDR, 2002). Improved interpretation of the 3D fluorescence fingerprints was employed during JDS2 and JDS3. The degree of correlation between fingerprints of the samples and arbitrary standards was used for characterization of the type of dominating oil pollution (gasoline, diesel or crude oil), and the fluorescence intensity at specific Ex/Em wavelength was used for quantification of the contamination.

19.2.1 Extraction of the SPM and bottom sediment samples

After freeze-drying, 0.5 g SPM/sediment sample was mixed with around 1 g anhydrous sodium sulphate and extracted with 10 ml cyclohexane by sonication for 20 minutes. Aliquots of the extracts were used for recording fluorescence spectra, or GC/HRMS analysis of PAHs in selected samples.

19.2.2 Analysis of the samples

19.2.2.1 Fluorescence spectroscopy

Fluorescence spectra (fingerprints) of cyclohexane extracts of SPM and bottom sediment samples were recorded according to procedures described in detail elsewhere (Literathy, 2000). A Hitachi Model F-4500 (during JDS1 and JDS2), and Cary-Eclipse (during JDS3), fluorescence spectrophotometers were used to record the fluorescence spectra in the 220-450 nm excitation and

245-475 nm emission wavelength ranges. Figure 105 shows fluorescence fingerprints of the arbitrary standards (petroleum products) and PAH standard mixture.

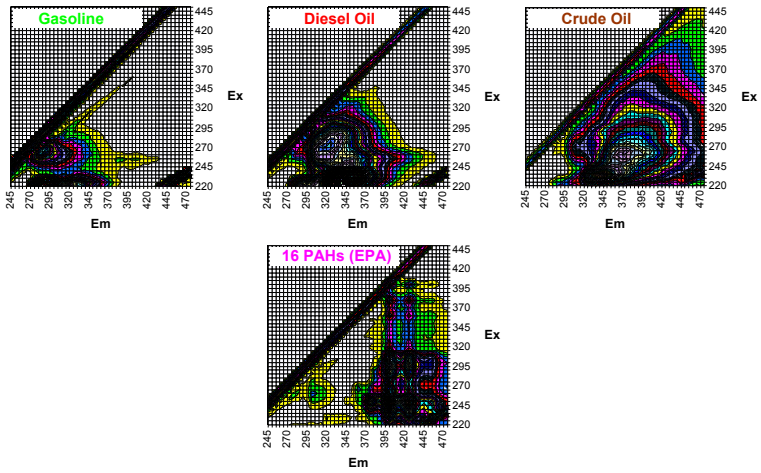

Figure 105: Fluorescence fingerprints (contour diagrams) of selected arbitrary standards (Gasoline, Diesel and Crude Oil, 1-1 µg/ml, and 16 PAHs, each 3 ng/ml, in cyclohexane)

19.2.2.2 Determination of the petroleum-related contamination

Determination of contamination type is based on the degree of correlation between the fingerprint of the arbitrary standards and the samples, and achieved by decomposing each fingerprint into 22 emission spectra (Rayleigh scattering removed) as follows:

Spectrum Number	Excitation Wavelength	Emission Range	Spectrum Number	Excitation Wavelength	Emission Range
Spectrum 1	220 nm	250-365 nm	…	…	…
Spectrum 2	225 nm	255-370 nm	Spectrum 20	315 nm	345-460 nm
Spectrum 3	230 nm	260-375 nm	Spectrum 21	320 nm	350-465 nm
…	…	…	Spectrum 22	325 nm	355-470 nm

These fluorescence emission spectra were then concatenated. Examples of the concatenated spectra are presented in Figure 106 for the candidate arbitrary standards and PAH standard mixture.

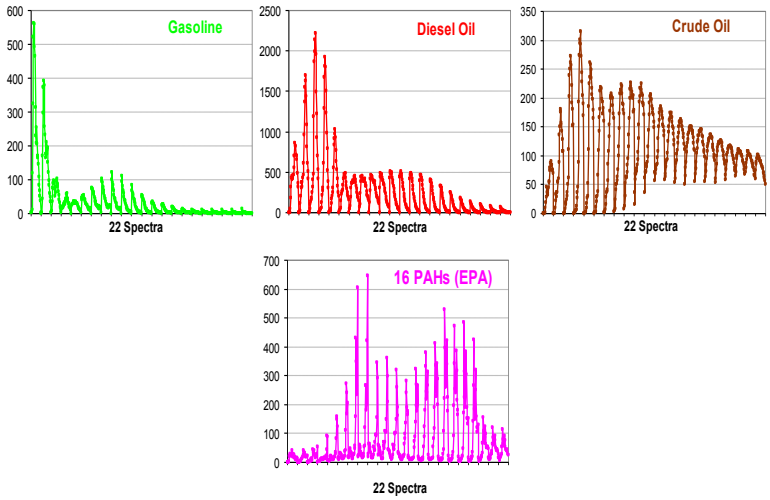

Figure 106: Concatenated fluorescence spectra of the arbitrary standards from Figure 105

After calculating the correlation between the samples and the arbitrary standards, the standard showing the highest correlation coefficient with the samples was used as calibration standard for estimating the concentration of the petroleum hydrocarbon contamination (Literathy, et al., 2006).

The fluorescence intensity at the excitation/emission (Ex/Em) wavelength, specified for each standard material was used for this estimation. As shown under the "Results," the highest correlation was observed with the crude oil in both the SPM and the bottom sediment samples. The specific Ex/Em wavelength in the case of crude oil was Ex/Em = 270/380 nm wavelength.

19.2.3 Determination of PAHs with GC/HRMS

Although PAHs are discussed in chapter 20, the cyclohexane extract of selected samples (showing high contamination on the basis of the fluorescence fingerprints), were analysed for PAHs using a GC/HRMS (Autospec) instrument; calibrated with the 16 PAHs (US-EPA) standard.

19.3 Results

Fluorescence fingerprinting of the cyclohexane extracts of the suspended particulate matter and bottom sediment samples provided an overall picture on the oil pollution during the three Joint Danube Surveys.

19.3.1 Characteristics of oil pollution in the SPM and bottom sediment

Figure 107 shows the correlation between the samples and the arbitrary standards for the SPM, and Figure 108 for the bottom sediment samples during JDS2 and JDS3.

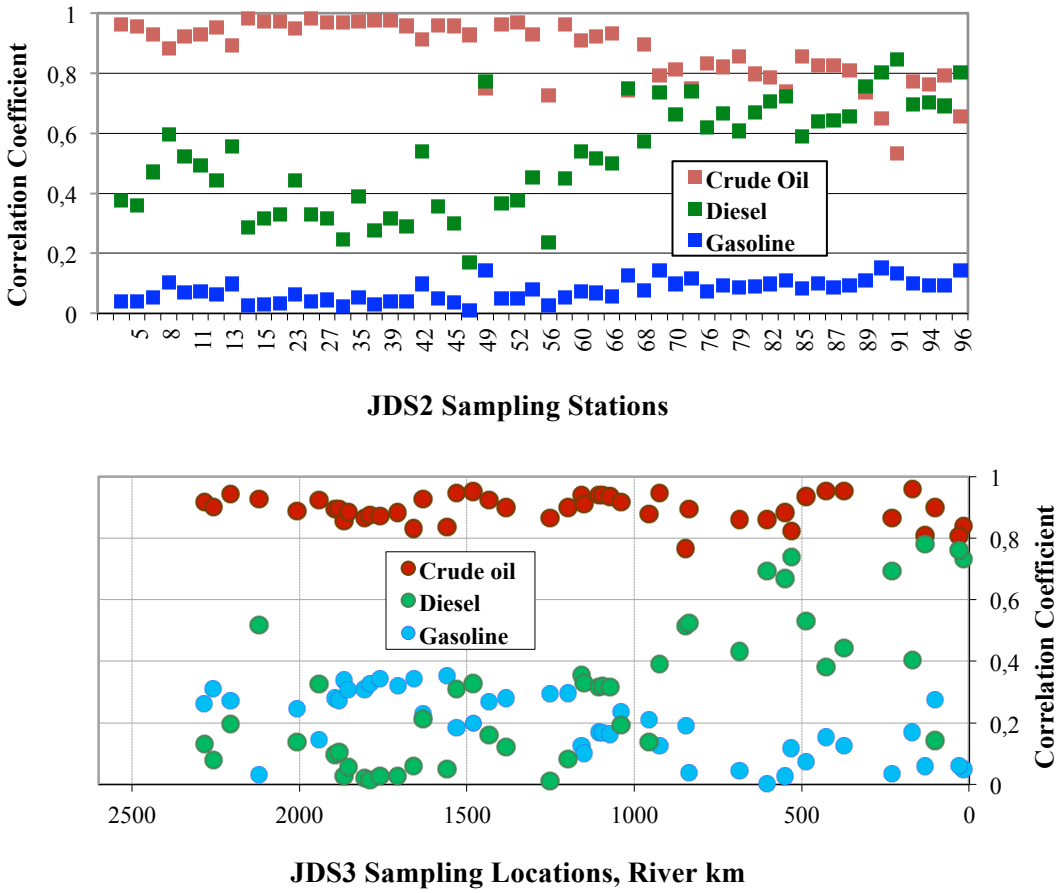

Figure 107: Suspended Particulate Matter: Correlation of the samples to the arbitrary standards

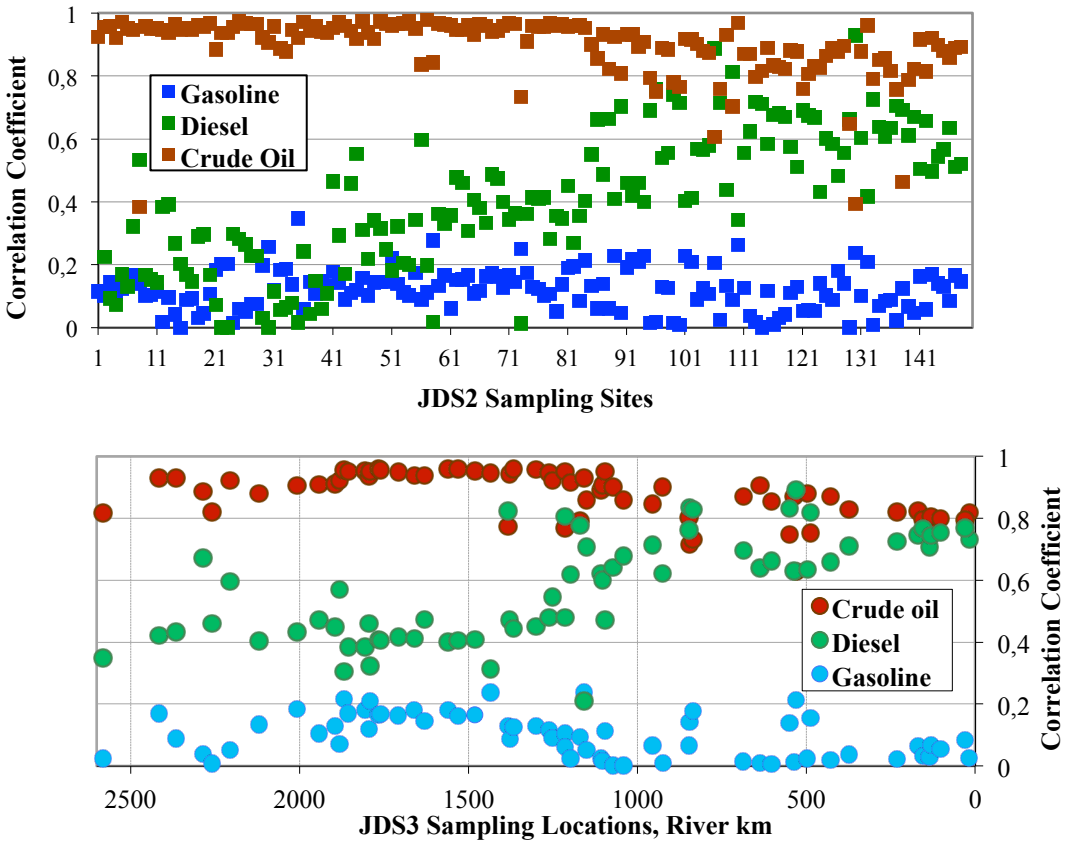

Figure 108: Bottom Sediment: Correlation of the samples' to the arbitrary standards

During both surveys and in both SPM and bottom sediment samples, the highest correlation was observed with the crude oil standard. This was particularly the case in the samples collected upstream of the Irongate reservoir. The significant difference between the correlation with the crude oil and the other two standards showed: (a) gasoline-type discharges evaporating relatively fast, BTEX compounds are more soluble in the water – this was demonstrated during JDS1, showing the highest correlation with the gasoline in the water samples, and show limited adsorption to the particulate matter, and (b) decreasing correlation with crude oil and increasing correlation with the diesel oil from the Iron gate reservoir to the Danube Delta indicates higher inputs from refined petroleum products (mainly diesel oil), and limited weathering of the pollutants. A few exceptions, i.e., higher correlation with the diesel oil compared to the crude oil, were also observed along the lower Danube reach.

19.3.2 Level of petroleum hydrocarbon contamination in the SPM and the bottom sediment

The fluorescence intensity values at the 270 nm excitation and 380 nm emission wavelength in the cyclohexane extract of the samples were used for quantitative estimation of the petroleum hydrocarbon contamination. The fluorescence intensity value, at the same Ex/Em wavelength, of the crude oil calibration standard was used for calculation of the level of contamination.

19.3.2.1 Petroluem hydrocarbons in the SPM

Figure 109 shows the variation in the petroleum hydrocarbon (TPH) contamination in the SPM along the Danube during the JDS1, JDS2 and JDS3 surveys.

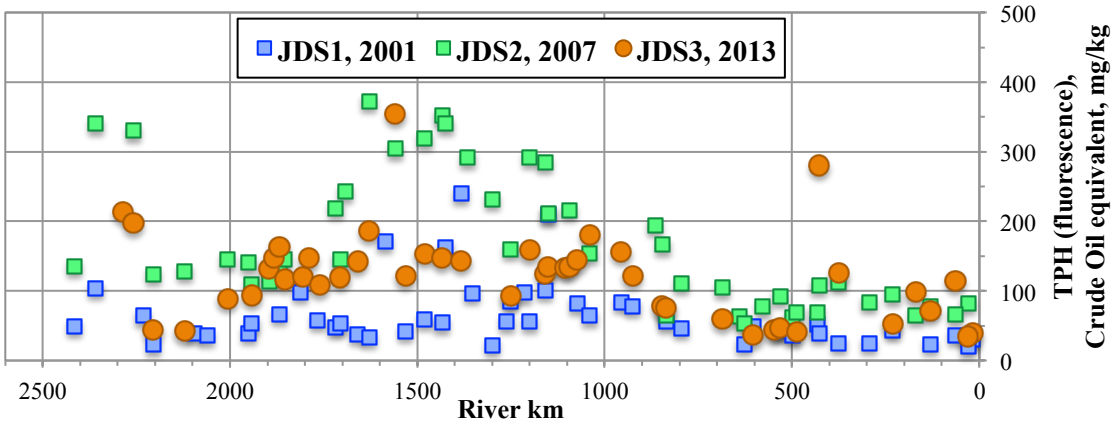

Figure 109: Variation in TPH concentrations in the suspended particulate matter along the Danube river during JDS1, JDS2 and JDS3

The surveys results distinguishing three characteristic sections along the Danube: (1) upstream of the Gabcikovo-reservoir, (2) section between the Gabcikovo and the Irongate dams, and (3) downstream of the Irongate reservoir. The most significant variation in the contamination levels was along the middle section, likely oil pollution inputs along the Slovakian-Hungarian Danube reach. The highest concentrations were observed at most of the sampling sites during JDS2, the lowest during JDS1. During JDS3 the contamination level was significantly higher downstream of Budapest (355 mg/kg at rkm 1,560) and downstream of the Arges confluence (280 mg/kg at rkm429), compared to the upstream stations. Unfortunately, SPM samples could not be collected from the tributaries.

19.3.2.2 Petroleum hydrocarbons in the bottom sediment

Figure 110 shows the variation in the petroleum hydrocarbon (TPH) contamination in the bottom sediment along the Danube during the JDS1, JDS2 and JDS3 surveys.

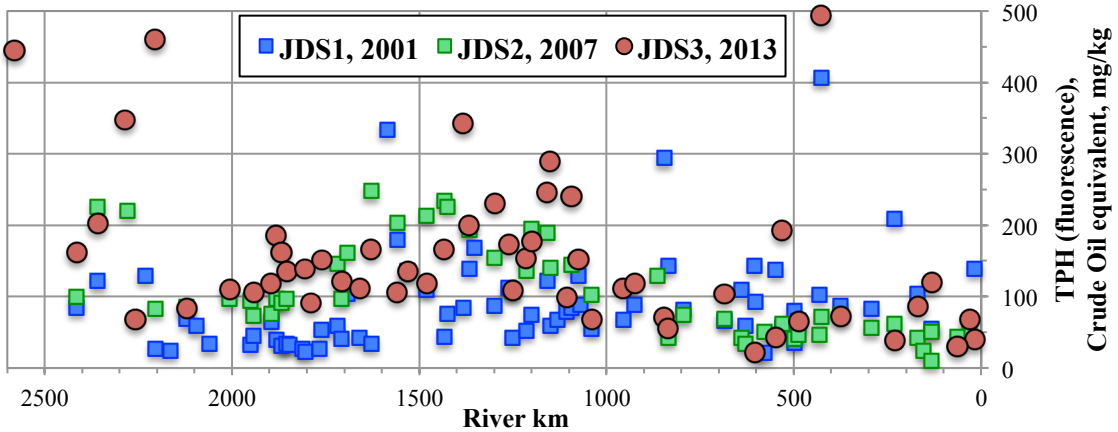

Figure 110: Variation in TPH concentrations in the bottom sediment along the Danube river during JDS1, JDS2 and JDS3

The three characteristic Danube sections can be distinguished also by the results obtained on the bottom sediment samples. The highest variation was observed along the middle section. It is likely that the contaminated SPM (observed in the period of JDS2), mainly settled to the bottom, resulting in increase in the oil-contamination in the bottom sediment from JDS1 through JDS2 to JDS3. To clarify the high TPH concentrations observed during JDS3, at three stations in the Upper Danube (in Germany) further monitoring would be needed. In the lower Danube reach, during all three surveys, an elevated concentration of TPH in the bottom sediment was observed downstream (at 429 rkm) of the Arges confluence (particularly during JDS3 and JDS1). This phenomenon is similar to that observed for the concentrations measured in the SPM.

19.3.3 PAHs in the sediments samples

PAH compounds can be the major contributors to the fluorescence in the organic solvent (cyclohexane) extracts of environmental samples. Although the PAHs discussed in chapter 20, the cyclohexane extracts of some selected samples during JDS3 were analysed for PAHs. The particular reason was to compare the concentration of selected PAHs to the results of the fluorescence fingerprints. Table 30 shows the results for comparison.

Table 30: Concentration of selected PAHs in selected bottom sediments during JDS3

Parameter	Unit	High TPH Samples	Low TPH Samples	Min-Max during JDS2
Fluoranthene	µg/kg	215 to 265	21 to45	15 and 853
Benzo[a]pyrene	µg/kg	104 to 114	41 to 52	10 and 115
Benzo[a]anthracene	µg/kg	66 to 71	26 to 32	
Benzo[b]fluoranthene	µg/kg	183 to 214	35 to 56	
TPH (fluorescence)	mg/kg	444 to 550	56 to 90	11 and 248

The results in Table 30 demonstrate that the higher TPH concentrations correspond to higher concentration of the PAHs. Unfortunately, the recent Directive 2013/39/EU shows EQS for water and biota only. However, considering the Canadian Sediment Quality Guidelines (CCME, 2001), even the maximum concentration of the selected PAHs are far below the PELs = probable effect limits, such as 2,355, 782 and 385 µg/kg for Fluoranthene, Benzo[a]pyrene and Benzo[a]anthracene, respectively.

19.4 Conclusions

Evaluation of fluorescence fingerprint of cyclohexane extracts of environmental samples proved to provide information on the characteristics and level of the petroleum hydrocarbon contamination. The results of the analysis of SPM and bottom sediment samples during the three JDS concluded:

- Petroleum hydrocarbons contamination, in both the SPM and the bottom sediment, was characterised with the fluorescence of crude oil, that was used for estimation of the TPH concentration, expressed in crude oil equivalents;
- The TPH contamination expressed in the SPM was lowest during JDS1 and highest during JDS2;
- The TPH contamination in the bottom sediment showed slowly increasing trends during the three surveys, likely increasing accumulation caused by settling of the contaminated SPM;
- Characteristics in TPH contamination divided the Danube into three sections: upstream of the Gabcikovo-reservoir, section between the Gabcikovo and the Irongate dams, and downstream of the Irongate reservoir. The most significant variation in the contamination levels was along the middle section, likely oil pollution inputs along the Slovakian-Hungarian Danube reach; and
- The PAH compounds determined in selected sediment extracts showed comparable level of contamination to the TPH, and even the highest concentrations were far below the PELs (i.e., Probable Effect Limits), in the Canadian Sediment Quality Guidelines.

19.5 References

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20 Priority and other organic substances

Karin Deutsch, Manfred Sengl

20.1 Introduction

20.1.1 Regulatory situation

For reducing chemical pollution of water by individual pollutants or groups of pollutants presenting a significant risk to or via the aquatic environment a strategy is set out in Art.16 of the Water Framework Directive 2000/60/EC (WFD). Based on the described strategy for a list of 33 substances (including 4 heavy metals) of priority concern environmental quality standards (EQS) in the field of water policy have been adopted with Directive 2008/105/EC. It includes EQS for whole water samples (filtered samples in the case of heavy metals) as well as EQS for three substances in biota. EQS are given for different types of waters as "annual average" and "maximum allowable concentration". An additional obligation is the arrangement of a long-term trend analysis for those priority substances, which tend to accumulate in sediment and/or biota.

In 2013 an amendment of the Directives 2000/60/EC and 2008/105/EC has been adopted with Directive 2013/39/EU. It includes additional EQS for 12 new substances, updating some surface water EQS and adding EQS in biota for 8 substances.

The challenge of JDS3 regarding priority substances was on one hand to describe possible changes of these substances, where the foregoing JDS2 indicated possible problems, and on the other hand to get a first overview of the occurrence of the "new" priority substances.

It has to be stressed that AA-EQS in water for priority substances are defined for an average value of 12 measurements within one year. JDS3 provided a single sample from August/September, which is certainly not representative for the time period of one year. It is not according to WFD rules to assess the chemical status from one single measurement.

20.1.2 Selection of priority substances for analysis

The selection of priority substances for analysis was based on the following lists and requirements:

- the present list of priority pollutants according to Directive 2008/105/EU
- new EQS for "old" priority substances according to Directive 2013/39/EC
- the selection of new priority substances according to Directive 2013/39/EC
- results from JDS1, JDS2 and TNMN

In a first step a thorough review of existing data was done to select the priority substances without need for analysis. The selection was based on imission data from JDS1/2 and regular TNMN well below the existing EQS. Thus the following priority substances were skipped from the agenda of JDS3:

- polar pesticides: atrazine, simazine, alachlor, trifluralin;
- volatile organic compounds (VOC);
- alkylphenoles: 4-iso-nonylphenole, 4-t-octylphenole;

– organochlorine compounds: aldrine, dieldrine, endrine, isodrine, DDT, hexachlorocyclohexanes, trichlorobenzene, pentachlorobenzene, pentachlorophenol.

Depending on physico-chemical properties as well as availability of laboratory capacities relevant priority pollutants were analysed in whole water, suspended particular matter (SPM), sediments and fish muscle. Data for accumulating compounds in SPM/sediments and/or fish can be used for trend analysis. Table 31 gives an overview of the priority pollutants investigated during JDS3.

Table 31: Overview of priority substances analysed in different sample types

Priority substance	Whole water	SPM	Sediment	fish
"old" priority substances according to directive 2008/105/EC				
anthracene	x	x	x	
brominated diphenylethers		x (JRC only BDE-209)		x (JRC only BDE- 209)
C_{10}-C_{13} chloroalkanes	x	x		
di(2-ethylhexyl)phthalate	x	x	x	
diuron	x			
fluoranthene	x	x	x	
isoproturon	x			
naphthalene	x			
benzo(a)pyrene	x	x	x	
benzo(b)fluoranthene	x	x	x	
benzo(k)fluoranthene	x	x	x	
benzo(g,h,i)perylene	x	x	x	
indeno(1,2,3-c,d)pyrene	x	x	x	
tributyltin compounds	x	x	x	
"new" priority substances according to directive 2013/39/EC				
dicofol		x	x	x
perfluorooctansulfonic acid (PFOS)	x (JRC)	x (JRC)		x (JRC)
dioxins and dioxin-like compounds		x (JRC)	x	x (JRC)
quinoxyfen	x			
aclonifen	x			
bifenox	x			
cybutryne	x			
cypermethrin		x	x	x
dichlorvos	x			
hexabromocyclododecane				x
heptachlor and heptachlor epoxide		x	x	x
terbutryn	x			

("x(JRC)" = analysis by Joint Research Centre, Ispra – not all sampling sites analysed)

By the use of multi-component methods also data for other compounds from the groups of polar pesticides and pesticide metabolites as well as for the pharmaceutical diclofenac and the biocide triclosan were gathered. Results will be discussed shortly in chapter 20.3.10.

20.2 Methods

The analysis of priority substances in whole water, SPM, sediments and fish was done by international standardised methods whenever available. For some determinants variations of international standards or in-house-methods were used.

Methods for extraction of water samples comprised liquid-liquid-extraction, stirbar sorptive extraction, online solid-phase extraction or direct injection for LC-MS/MS-analysis. SPM and sediments were prepared using ultrasonic extraction or accelerated solvent extraction (ASE). Fish samples were extracted using the QUECHERS-method used in food analysis.

While PAH and DEHP were analysed with classical HPLC with fluorescence or UV-detection, most polar pesticides and hexabromocyclododecane were analysed with HPLC-MS/MS. For tinorganic compounds and other halogenated pesticides GC-MS was applied.

In comparison to analytical methods used during JDS2 lower limits of quantification could be achieved as a consequence of the improved sensitivity of analytical equipment. Nevertheless all laboratories involved reported uncertainties of measurement ≤50% (k = 2).

20.3 Results

20.3.1 Di(2-ethylhexyl)phthalate (DEHP)

DEHP is used worldwide as plasticiser in huge amounts since many years and can be found in all environmental compartments.

During JDS3 DEHP was analysed in water, SPM and sediments (see Figure 111– Figure 113). Only half of the sampling sites showed DEHP water concentrations above the LOQ of 0,2 µg/l. The maximum at JDS32 site (upstream Novi-Sad) with a concentration of 0,84 µg/l was well below the EQS of 1,3 µg/l. The concentrations of DEHP were significantly lower compared to JDS2, when in 42 out of 96 water samples (43,8%) the EQS of 1,3 µg/l was exceeded and DEHP was therefore identified as the most problematic priority pollutant.

On the other hand DEHP is again present in most of the SPM and sediment samples showing maximum concentrations of 5,55 mg/kg for SPM at sampling site 38 (upstream Pancevo/downstream Sava) and 26 mg/kg for sediments at sampling site JDS9 (Klosterneuburg). Also the sediment downstream Velika Morava has a very high DEHP contamination with 16,7 mg/kg.

For protection of benthic organisms the Priority Substance data sheet for DEHP from 2005 (see https://circabc.europa.eu/datasheet DEHP) provides a proposal for specific quality standards in sediment with 100 mg DEHP/kg sediment. All JDS sites show concentrations far below this specific quality standard.

Average concentrations of DEHP in SPM are higher in comparison to JDS2 survey, in SPM and sediments showing higher values again in the middle part of the Danube. SPM representing the actual contamination status of rivers show very low DEHP concentration in the upper Danube whereas DEHP is still present in the sediments in concentrations of more than 4 mg/kg.

Table 32: Comparison of DEHP-concentrations of JDS3 and JDS2 survey

DEHP-concentrations	JDS3 – median	JDS2 – median	JDS3 – maximum/site	JDS2 – maximum/site
whole water (µg/l)	<LOQ (0,2)	1,11	0,84 (JDS32, upstream Novi-Sad)	4,53 (Wildungsmauer)
SPM (mg/kg dry matter)	2,52	1,21	26 (JDS9, Klosterneuburg)	9,32 (Tisa river)
sediment, fraction <63 µm (mg/kg dry matter)	1,64	0,53	5,55 (JDS38, upstream Pancevo)	16 (downstream Arges)

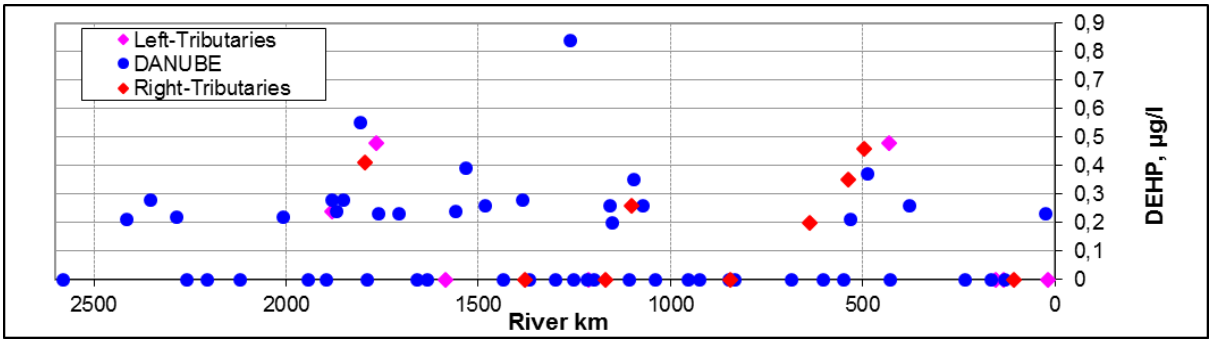


Figure 111: DEHP concentration in water

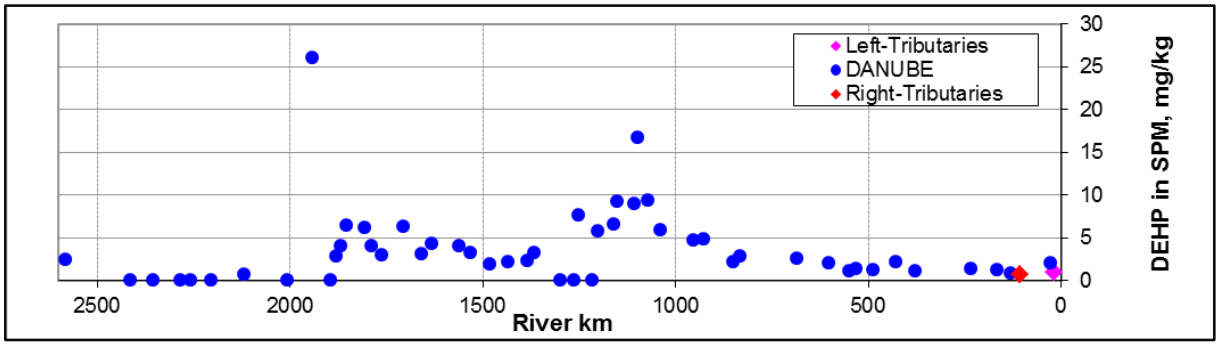


Figure 112: DEHP in suspended particular matter

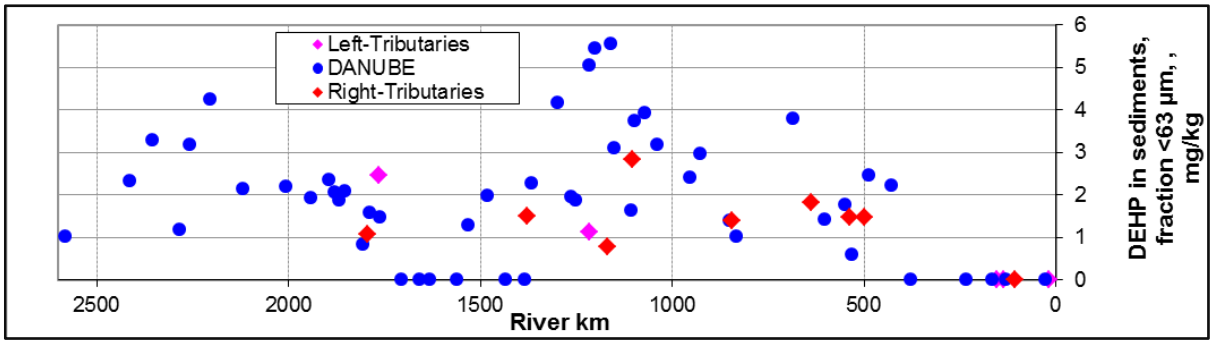


Figure 113: DEHP in sediments

The overall results in SPM and sediments are not consistent with the clearly decreasing values in the whole water phase in comparison to JDS2. Comparing JDS2 and JDS3 the distribution of DEHP in SPM and sediments along the Danube is also rather inhomogeneous. This may be due to analytical insecurities and also reflects the ubiquitous immission characteristics of DEHP stemming from point- and non-point-sources.

20.3.2 Polyaromatic hydrocarbons (PAH)

As PAHs are by-products of the combustion of any carbon-based combustory material they are ubiquitous contaminants in the environment.

Eight substances from the group of the polyaromatic hydrocarbons were previously defined as priority pollutants. Directive 2013/39/EC changed the basis for risk assessment, as benzo(a)pyrene is now regarded as a marker for the sum of benzo(b)fluoranthen, benzo(k)fluoranthene, benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene instead of defining individual EQS.

Table 33 gives an overview of PAH-maxima in water samples in comparison to the relevant AA-EQS.

Table 33: PAH-concentrations in water samples

substance	EQS in µg/l (2008/105/EC)	EQS in µg/l (2013/39/ EC)	LOQ µg/l	number of JDS3-samples > LOQ	maximum concentration in µg/l	sampling site with maximum
naphthaline	2	2	0,002	67	0,0401	JDS24
anthracene	0,1	0,1	0,002	17	0,0098	JDS24
fluoranthene	0,1	0,0063	0,002	59	0,0204	JDS24
benzo(a)pyrene	0,05	0,00017	0,002	3	0,0024	JDS7
benzo(b)fluoranthene	Σ = 0,03		0,002	5	0,0027	JDS24
benzo(k)fluoranthene			0,002	1	0,0022	JDS7
benzo(g,h,i)perylene	Σ = 0,002		0,0005	66	0,029	JDS32
indeno(1,2,3-cd)pyrene			0,0005	15	0,0049	JDS28

The limit of quantification in water samples was 0,002 µg/l for each PAH substance except for benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene for which a LOQ of 0,0005 µg/l was reached. Thus only the new EQS for benzo(a)pyrene – representing 4 other PAH substances – could not be assessed.

The maximum concentrations of some PAH show that EQS are exceeded in various samples all along the Danube. According to Directive 2013/39/EC the group of PAH substances represented by benzo(a)pyrene are "substances behaving like ubiquitous PBTs (persistent, bioaccumulative and toxic substances)" and can be reported in separate maps describing the chemical status of water bodies. Most of the highest individual PAH values were found at sampling site 24 (Dunafoldvar).

PAH concentrations in SPM were analysed on 50 sampling sites. Table 34 gives an overview of PAH-maximum concentrations in SPM. Most of the compounds were found at more than 50% of the sites, the maximum values lay between 21 – 191 µg/kg. For most of the compounds the maximum concentrations were found at site JDS1 (Böfinger Halde). Only for anthracene most SPM concentrations showed values below the LOQ of 20 µg/kg, the highest concentrations were found in the upper and the middle stretch of the Danube. A comparison with the results of JDS2 showed comparable concentrations.

Table 34: PAH-concentrations in SPM

substance	LOQ µg/kg	number of JDS3-samples > LOQ	maximum concentration in µg/kg	sampling site with maximum
anthracene	20	2	21	JDS47
fluoranthene	20	48	191	JDS1
benzo(a)pyrene	20	35	110	JDS1
benzo(b)fluoranthene	20	39	122	JDS1
benzo(k)fluoranthene	20	25	55	JDS1
benzo(g,h,i)perylene	20	33	75	JDS1
indeno(1,2,3-cd)pyrene	20	14	62	JDS1

PAH concentrations in sediment (< 63µm) were analysed on 65 sampling sites. Table 35 gives an overview of PAH-maxima concentrations in sediment. Most of the compounds were found on more than 50% of the sites, the maximum values lay between 57- 489 µg/kg.

Table 35: PAH-concentrations in sediment (< 63 µm)

substance	specific quality standards (µg/kg); see data sheet PS*	LOQ µg/kg	number of JDS3-samples > LOQ	maximum concentration in µg/kg	sampling site with maximum
anthracene	24	20	3	57	JDS6
fluoranthene	2000	20	55	690	JDS6
benzo(a)pyrene	91,5	20	41	370	JDS1
benzo(b)fluoranthene	70,7	20	49	489	JDS6
benzo(k)fluoranthene	67,5	20	16	259	JDS6
benzo(g,h,i)perylene	42	20	33	328	JDS1
indeno(1,2,3-cd)pyrene	-	20	9	179	JDS6

* see Priority Substance data sheets from 2011 (https://circabc.europa.eu/PAH datasheet 2011)

For protection of the benthic community the Priority Substance data sheets from 2011 provide proposals for specific quality standards in sediment. Most JDS sites (about 90%) show concentrations below these specific quality standards. An exceedance of these values can be observed mostly in the upper part of the Danube and the tributaries Vah and Iskar On the other hand other sources like the Canadian Sediment Quality Guidelines (CCME, 2001) define higher "probable effect limits" for PAH in sediments, which are not exceeded by JDS3 results.
For further results discussing the sources of PAH contamination see also chapter 19.

20.3.3 Polar pesticides and biocides in water

The pesticides under investigation have a different status of admission in the Danube countries. In general most of the polar pesticides except isoproturon are applied in agriculture during the main growing season from April-July. Thus the analysed concentrations in JDS-samples taken in August/September are not representative as was also shown during the last surveys. On the other hand biocides are emitted on a regular basis. Terbutryn might have a double use as pesticide and biocide in some countries leading to a higher and continuous contamination of surface waters.

Table 36: Polar pesticides and biocides in water

substance	LOQ or LOD* in µg/l	AA-EQS in µg/l (2013/39/EC)	number of JDS3-samples > LOQ	maximum concentration in µg/l	sampling site with maximum
diuron	0,001	0,2	65	0,037	JDS56
isoproturon	0,0004	0,3	55	0,09	JDS56
quinoxyfen	0,025*	0,015	0	-	-
aclonifen	0,025*	0,12	0	-	-
bifenox	0,025*	0,012	0	-	-
dichlorvos	0,025*	0,0004	0	-	-
terbutryn	0,0004	0,065	64	0,0045	JDS4
cybutryne	0,0004	0,0025	0	-	-

In most of the water samples diuron, isoproturon and terbutryn can be found in the low ng/l-range with maximum concentrations of 0,037, 0,09 and 0,0045 µg/l, respectively (see Table 36). These substances are not relevant as these concentrations are far below the respective EQS.

For the first time a complete data set for the biocide cybutryne is available. The LOQ of 0,0004 µg/l reached by LfU/Augsburg is below the EQS of 0,0025 µg/l and was not exceeded in any JDS water sample. A second data set from JRC reaching a lower LOQ of 0,00018 µg/l showed 17 positive results for cybutryne in a concentration range from 0,00020 – 0,00083 µg/l and thus also below the EQS of 0,0025 µg/l. All positive results were found in the Danube from Germany to Hungary with the maximum at sampling site 23 (Rackeve-Soroksari).

Also quinoxyfen, aclonifen, bifenox and dichlorvos were not found in concentrations above the LOD of 0,025 µg/l. But in case of quinoxyfen, bifenox and dichlorvos the required LOD for checking the EQS could not be reached. The semiquantitative determination of quinoxyfen by LC-HRMS showed only few positive results with a rough estimation of 0,1 µg/l as a maximum concentration.

Data for atrazine, simazine and chlorfenvinphos were gathered with multi-component LC-MS-methods applied by Croatian Waters and UFZ/Leipzig (see chapters 27 and 30). While simazine was only detected in ca. one third of all water samples with a maximum concentration of 0,011 µg/l, atrazine can be found in nearly all samples with a maximum in the Arges with 0,07 µg/l. These maximum values are far below the relevant EQS and support the fact that these pesticides are banned in many Danube countries. Also chlorfenvinphos was not detected in water samples (UFZ/Leipzig, LOD 1 ng/l).

20.3.4 Pesticides in SPM, sediment and biota

The pesticides dicofol, heptachlor and cypermethrin are “new” priority substances according to directive 2013/39/EC. They have in common very low EQS in water, which cannot be analysed in the concentration level required, and a high tendency to accumulate on suspended particles (log K_{ow} > 4). Dicofol and heptachlor have also EQS in biota and have to be included in long term trend analysis.

For getting a first picture about the abundance of these substances in the Danube, they were analysed in biota (bream filet), suspended matter (SPM) and sediment (see Table 37).

Table 37: Pesticides in biota, sediment and SPM

substance	analysed fraction	LOQ in µg/kg	AA-EQS (2013/39/EC) in µg/kg	number of JDS3-samples > LOQ	maximum concentration in µg/kg	sampling site with maximum
dicofol	biota	2,5	33 µg/kg ww	0	< LOQ	
	sediment (< 2mm)	10		0	< LOQ	
	SPM	10		1	10	JDS59
cypermethrin	biota	2,5		0	< LOQ	
	sediment (< 2mm)	10		0	< LOQ	
	SPM	10		0	< LOQ	
heptachlor and heptachlor epoxide	biota	0,4 µg/kg ww	0,0067 µg/kg ww	0	< LOQ	
	sediment (< 63 µm)	1		0	< LOQ	
	SPM	1		2	1,1	JDS2

Dicofol, heptachlor and cypermethrin were analysed in biota (bream filet) at 7 sampling sites. All sites show values below LOD and below the existing EQS in biota, with the restriction that in case of heptachlor and heptachlorepoxide the required LOQ could not be reached.

Dicofol and cypermethrin were analysed in SPM and sediment at all 68 JDS sampling sites, heptachlor in SPM at 47 JDS-sites and sediment at 65 JDS sampling sites. The majority of the sites show values below LOQ. Only dicofol and heptachlor in SPM show single (1-2) sites with detectable concentration, but the maximum values are in the range of the LOQ.

Additional data of cypermethrin in water on 22 sites by UFZ/Leipzig support these findings as all values lay below the LOQ of 0,068 µg/l.

20.3.5 C10-C13-Chloroalkanes

C10-C13-chloroalkanes are widely used as plasticisers, additives in lubricants, cutting fluids and flame retardants. They consist of C10-C13 n-alkanes with a chlorination range between 40 and 70% by weight.

With JDS3 full data sets for C10-C13-chloroalkanes for water and SPM are available. An LOQ of 0,2 µg/l enables a sound assessment of the EQS of 0,4 µg/l. All water samples showed concentrations below LOQ.

C10-C13-chloroalkanes can accumulate in SPM or sediments but results from European rivers are very scarce so far. In order to check current pressures, SPM were analysed. The results are rather homogeneous with an average value of 23 µg/kg and a median of 27 µg/kg. The highest value was found at sampling site JDS59 (downstream Arges) with 79 µg/kg (see Figure 114).

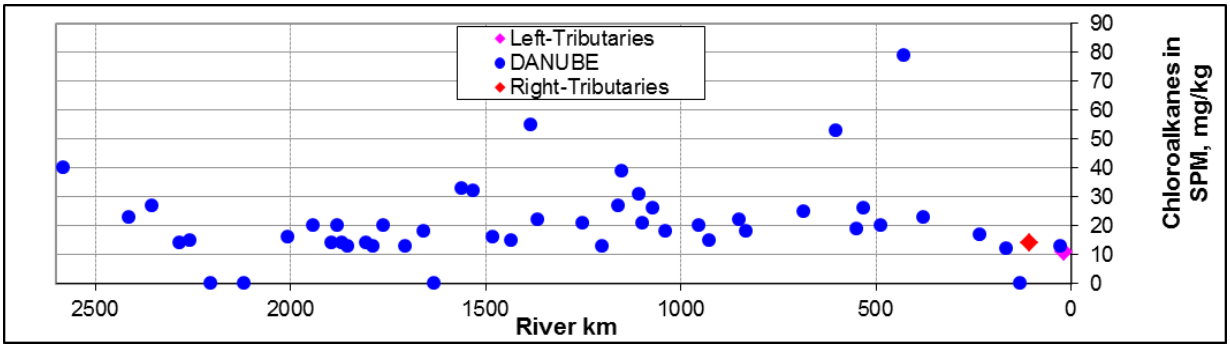


Figure 114: C10-C13-Chloroalkanes in SPM

20.3.6 Organotin compounds

Organotin compounds are used as biocides and in PVC manufacturing. Concerns over toxicity of some of the compounds have led to a range of restrictions in use. Directive 2008/105/EC has set EQS for tributyltin compounds (including tributyltin cation) in water and identified it as a priority hazardous substance. Dibutyltin is regulated in some countries as national relevant specific pollutant. All regulations are related to water, in one country there are EQS for dibutyltin in sediment/suspended solids as well.

During JDS3 up to 7 different organotin compounds were analysed in water, SPM and sediments (see Table 38 – Table 40).

Table 38: Organotin compounds in water samples

substance	LOQ/LOD in ng/l	AA-EQS (2008/105/EC) in ng/l	AA-EQS (national) in ng/l	number of JDS3-samples > LOQ	maximum concentration in ng/l	sampling site with maximum
dibutyltin	0,2/0,1		10-20	37	0,86	JDS18
tributyltin	0,2/0,1	0,2		7	0,69	JDS7
tetrabutyltin	0,2/0,1			2	0,25	JDS1
dioctyltin	0,2/0,1			3	0,28	JDS68
diphenyltin	0,2/0,1			1	0,34	JDS12
triphenyltin	0,2/0,1			1	0,21	JDS12

6 organotin compounds were analysed in water at all 68 JDS sampling sites. Dibutyltin was found at more than 50% of the sites (number of samples > LOQ), but the highest concentration of 0,86 ng/l in river Vah lay well below the national EQS of 10 ng/l. Tributyltin was found at 7 from 68 sites with values above the LOQ of 0,2 ng/l, which is also the AA-EQS for this substance. All sites showing positive results were in the upper/middle part of the Danube. The highest concentrations were found in JDS7 (Upstream Abwinden Asten) and in the tributary Morava (JDS12). For the other 4 analysed compounds only 1 to 3 sites showed concentrations with values above the LOQ.

A comparison of the water-concentrations with the results of JDS2 showed lower maximum-values for dibutyltin and tributyltin in 2013 which reflects the restrictions for the use of this substance. In JDS2 (2007) the observed maximum concentration for tributyltin was 14 ng/l.

Table 39: Organotin compounds in SPM

substance	LOQ/LOD in µg/kg	EQS in µg/kg national	number of JDS3-samples > LOQ	maximum concentration in µg/kg	sampling site with maximum
monobutyltin	2/1		34	17	JDS25
dibutyltin	2/1	100	7	4,1	JDS22
tributyltin	2/1		10	9,3	JDS15
tetrabutyltin	2/1		0	0	
dioctyltin	2/1		7	5,5	JDS 4
diphenyltin	2/1		22	8,1	JDS15
triphenyltin	2/1		23	19	JDS21

7 organotin compounds were analysed in SPM at 50 JDS sampling sites. Except from tetrabutyltin, all analysed compounds were detected with concentrations above LOQ at 7 or more sites. Monobutyltin was found at more than 65% of the sites (number of samples > LOQ). The highest concentration of 19µg/kg was found for triphenyltin upstream Budapest (JDS21). For dibutyltin the highest concentration of 4,1 µg/kg (JDS22, downstream Budapest) lay well below the national EQS of 100 µg/kg.

A comparison of the SPM-concentrations with the results of JDS2 showed lower maximum values for monobutyltin, dibutyltin and tributyltin concentrations than 2007. In JDS2 the observed maximum concentration for tributyltin in SPM was 230 µg/kg. The reduction by a factor of 20 is in line with the decline in the observed water concentrations.

Table 40: Organotin compounds in sediment (< 2mm)

substance	LOQ/LOD in µg/kg	EQS in µg/kg national	number of JDS3-samples > LOQ	maximum concentration in µg/kg	sampling site with maximum
monobutyltin	2/1		46	27	JDS 9
dibutyltin	2/1	100	33	19	JDS18
tributyltin	2/1		16	13	JDS28
tetrabutyltin	2/1		0	0	
dioctyltin	2/1		2	2,5	JDS21
diphenyltin	2/1		17	8,3	JDS22
triphenyltin	2/1		30	28	JDS22

7 organotin compounds were analysed in sediments (< 2 mm) at 65 JDS sampling sites. Monobutyltin, dibutyltin and triphenyltin were the most abundant compounds. The highest concentration of 28 µg/kg was found for triphenyltin downstream Budapest (JDS22). For dibutyltin the highest concentration of 19 µg/kg was found in river Vah and lay well below the national EQS of 100 µg/kg.

In difference to the water and SPM data a comparison of the sediment concentrations with the results of JDS2 showed comparable results. In JDS2 (2007) the observed maximum concentration for tributyltin was 12 µg/kg, the results from JDS3 showed maximum values of 13 µg/kg.

20.3.7 Dioxins and dioxin-like compounds in sediment

Dioxins and dioxin-like compounds are "new" priority substances according to directive 2013/39/EC. They are by-products of poor combustion involving organic matter and chlorine and a variety of chemical processes.

The assessment refers to a sum of 7 polychlorinated dibenzo-p-dioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs) and 12 dioxin-like polychlorinated biphenyls (PCB-DL). Due to their similar toxicological behaviour but different potencies, the concentration of the different compounds/congeners are converted into toxic equivalents (according to WHO 2005 Toxic Equivalence Factors -TEF) and summed up. The sum of PCDD+PCDF+PCB-DL has only an EQS in biota (EQS 0,0065 µg/kg TEQ wet weight) and has to be included in long term trend analysis.

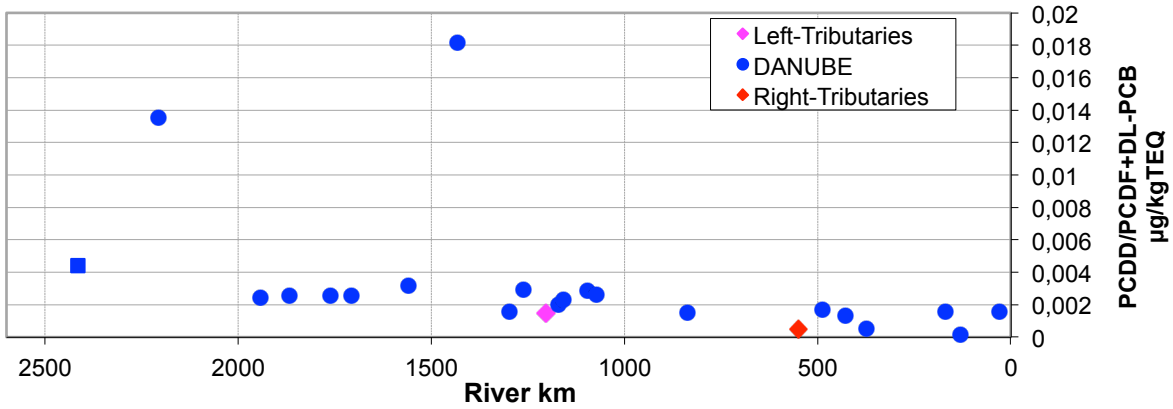


Figure 115: Dioxins and dioxin-like compounds in sediment

All relevant dioxins and dioxin-like compounds are analysed in sediment (< 2mm) at 23 JDS sampling sites (see Figure 115). Most of the PCDDs and PCDFs were quantified in all samples, PCB-DL only partly. The average patterns of PCDD/Fs were dominated by OCDD.

The summary results (expressed as µg/kg TEQ) show very similar concentration at all sampling sites, the average value is 0,003 µg/kg TEQ. There are only two sampling sites (JDS6/Jochenstein and JDS27/Hercegszanto) which show higher values between 0,014 – 0,018 µg/kg TEQ. The percentage of PCB-DL versus PCDD/F (in TEQ) is varying between 1 – 40% with a decreasing tendency in the lower Danube.

A comparison of the sediment concentrations with the results of JDS2 (2007) shows very similar results. The average values were about 0,0034 µg/kg TEQ (WHO-TEQ 1998), the maximum values were about 0,022 µg/kg TEQ (WHO-TEQ 1998). The higher concentration in Hercegszanto was also observed in 2007.

Additional analysis in SPM and biota were done by JRC/Ispra **(**see chapter 23**).** They found comparable concentration levels of PCDD/F and PCB-DL (in TEQ) in SPM. Biota analysis of bream filet showed no exceedance of the new EQS in biota (EQS 0,0065 µg/kg TEQ wet weight).

20.3.8 Perfluorooctansulfonic acid (PFOS)

PFOS was found in 94% of the water samples in concentrations above the limit of quantification of 0,001 µg/l by JRC/Ispra. The AA-EQS for PFOS and PFOS-derivatives of 0,00065 µg/l is exceeded throughout the Danube. For details see chapter 22.

20.3.9 Hexabromocyclododecane (HBCDD) and polybrominated diphenylethers (PBDE)

Hexabromocyclododecane (HBCDD) is one of the "new" priority substances according to directive 2013/39/EC. It is used as a flame retardant, mainly by the polymer and textile industry. One major application is in polystyrene insulation panels in building constructions.

Table 41: HBCDD in biota

substance	LOQ in µg/kg dw	AA-EQS in µg/kg wt (2013/39/EC)	number of JDS3-samples > LOQ	maximum concentration in µg/l
hexabromocyclododecane (HBCDD)	100	167	0	< LOD

Hexabromocyclododecane (HBCDD) was analysed in biota (bream filet) on 7 sampling sites (see Table 41). The achieved LOQ is low enough to assess the biota EQS. All sampling sites show values below the LOD and therefore below the EQS.

The brominated diphenylethers (PBDE) were analysed in SPM and biota by JRC/Ispra. Based on the fact that BDE-209 dominated by far the PBDEs detected in SPM during JDS2, the current study is limited to BDE-209 (details see chapter 23). As directive 2013/39/EC refers the EQS to the sum of the concentrations of congener numbers 28, 47, 99, 100, 153 and 154 a comparison of the results of BDE-209 with the EQS of directive 2013/39/EC is not possible.

20.3.10 Other organic substances

20.3.10.1 Bisphenol A

Bisphenol A is used in the chemical industry as plasticiser in the production of polycarbonate and epoxy resin and as antioxidant in cosmetics. Several countries have regulated bisphenol A as a national relevant specific pollutant. All regulations are related to water.

Bisphenol A was analysed at 68 JDS sites in water, at 65 sites in sediment and at 50 sites in SPM (see Table 42).

Table 42: Bisphenol A in water, sediment and SPM

fraction	LOQ	AA-EQS national	number of JDS3-samples > LOQ	maximum concentration	sampling site with maximum
water	0,1 µg/l	1,6-10 µg/l	4	1,94 µg/l	JDS 52
Sediment (<63µm)	10 µg/kg		0	< LOQ	
SPM	10 µg/kg		0	< LOQ	

Most of the analysed samples were below LOQ, only the water analysis shows detectable concentrations at 4 sites. Just one site (JDS52/Downstream Olt) has an exceedance of the most stringent national EQS.

20.3.10.2 Other polar pesticides, pesticide metabolites, diclofenac and biocides

The multi-method used by UBA Vienna (direct-injection of filtered sample, LC-MS/MS) gave information on additional polar pesticides, some important pesticide metabolites, diclofenac as well as the biocides triclosan and DEET. As these substances are not tending to accumulate on SPM the results for the dissolved fraction stands for the whole water sample.

For the following substances all 68 samples showed concentrations below the limit of determination of 0,025 µg/l:

- polar pesticides: chloridazon, chlortoluron, flurtamone, metazachlor, metolachlor, propiconazol;
- pesticide metabolites: metazachlor-oxanilic acid, metolachlor-oxanilic acid, desethylterbutylazine;
- biocides: triclosan.

For an overview of the positive results see Table 43.

Table 43: Other organic substances with positive results

substance	LOD in µg/l	AA-EQS national	number of samples > LOD	maximum concentration in µg/l	Sampling site with maximum
Pesticides					
terbutylazine	0,025	0,5	1	0,065	JDS12
bentazone	0,025	0,1	1	0,093	JDS58
glyphosate	0,030	15-20	5	0,073	JDS3
pesticide metabolites					
chloridazon-desphenyl	0,025		44	0,25	JDS12
chloridazon-methyl-desphenyl	0,025		1	0,055	JDS12
metolachlor ethane sulfonic acid (ESA)	0,025		13	0,087	JDS12
metazachlor ethane sulfonic acid (ESA)	0,025		3	0,33	JDS12
aminomethylphosphonic acid (AMPA)	0,030		66	0,96	JDS56
pharmaceutical, biocide					
DEET	0,025		3	0,12	JDS12
diclofenac	0,025		1	0,24	JDS58

The results show that (stable) metabolites of pesticides applied in the field reach the surface waters. Chloridazon was used in the cultivation of beets for decades and its metabolites can also be found in many groundwaters. Glyphosate is currently the most abundant herbicide in use worldwide. It is applied in different cultures and reaches the surface waters also via sewage treatment plant effluents. Glyphosate is readily degraded to aminomethylphosphonic acid (AMPA) and could only be found in five Danube samples. In JDS3 the highest concentrations for AMPA – which is detected in 66 out of 68 water samples – were found in several tributaries, while in the Danube itself an unusual stable concentration level around 0,25 µg/l in all sections can be seen (see Figure 116). Sulfonic acid metabolites of metolachlor and metazachlor play a minor role but were found in concentrations up to 0,33 µg/l.

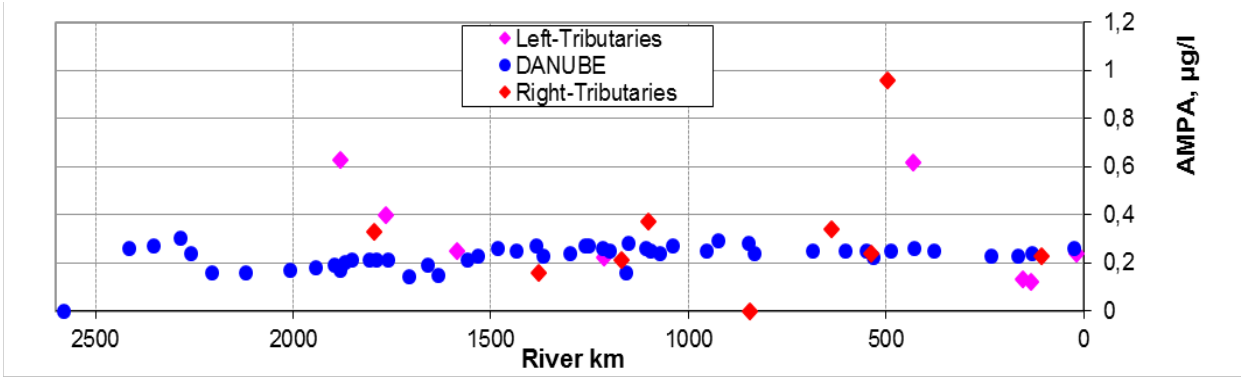


Figure 116: Concentration of AMPA (aminomethylphosphonic acid) in water

Diclofenac is an important anti-inflammatory drug and was on the candidate list for priority pollutants with a proposed EQS of 0,1 µg/l. The decision to include also pharmaceuticals in the list of priority pollutant was postponed by the EU commission as it was decided to elaborate a new strategy for pharmaceuticals in the environment on the European level until 2015. Based on directive 2013/39/EU diclofenac shall be included in the first watch list, in order to gather EU-wide monitoring data. UBA/Vienna reported only one positive result in Arges (0,24 µg/l) exceeding the proposed EQS. This result in Arges was confirmed by other laboratories (JRC/Ispra: 0,25 µg/l and UFZ/Leipzig: 0,32 µg/l). JRC/Ispra could detect diclofenac in many water samples in concentrations <0,020 µg/l (see also chapter 22) as they reached a very low limit of detection of 0,00068 µg/l.

20.4 Conclusions

During JDS3 “old” and “new” priority substances could be analysed in the relevant matrices and concentration ranges. Only for a few substances the limits of quantification did not meet the requirements. For some substances more than one laboratory provided data so cross-checking of these results is possible. The main findings are as follows:

- DEHP in water was present in all samples significantly below the AA-EQS of 1,3 µg/l whereas during JDS2 in 44% of the water samples DEHP concentrations were above the AA-EQS. In comparison to JDS2 DEHP was found in higher concentrations in SPM and sediments showing an accumulation of this ubiquitious pollutant, but all concentrations lay far below the specific quality standard derived for the protection of benthic organisms (see Priority Substance data sheet for DEHP from 2005)
- For the first time C10-C13-chloroalkanes could be analysed in water. All measured concentrations were below 0,2 µg/l and thus below the AA-EQS of 0,4 µg/l. C10-C13-chloroalkanes were found in SPM in concentrations up to 79 µg/kg dry mass.
- Concentrations of PFOS exceed the AA-EQS of 0,00065 µg/l at 94% of the sampling sites.
- For PAH and tributyl-tin again few scattered results indicate an exceedance of AA-EQS at a few sampling sites. On the other hand the analytical methods applied are not able to reach the new EQS for benzo(a)pyrene of 0,00017 µg/l, so more positive results are to be expected when applying more sensitive analytical methods.
- As the months of sampling were August/September are not the main season for pesticide application only low concentrations were detected. Positive data for terbutryn hint at the fact that its predominant use is as a biocide. More time integrating analysis of 3 pesticides in biota showed no detectable concentration. The limits of quantification for the pesticides quinoxyfen, bifenox, heptachlor and dichlorvos have to be lowered in future as they are above the AA-EQS.
- The biocide cybutryne was analysed in all samples for the first time detecting only very low concentrations well below the AA-EQS. The summary results of dioxin and DL-compounds in sediment and SPM show very similar concentration levels as the results of JDS2 (2007). Biota analysis of bream filet showed no exceedance of the new EQS in biota (EQS 0,0065 µg/kg TEQ wet weight).
- For HBCDD all biota sampling sites showed values below the new EQS in biota.
- AMPA, as a degradation product of glyphosate, was detected in most water samples with an unusual stable concentration level around 0,25 µg/l in all sections of the Danube itself. The up to 5 times higher concentration levels in some tributaries did not appreciable influence the concentration in the Danube itself.

21 Metals

Dietmar Krämer, Bernd Gawlik

21.1 Introduction

Metals are elementary substances and natural parts of our environment. Heavy metals and metalloids may occur as major parts in minerals or as accompanying elements in rocks and soils, amounts varying according to geological composition.

As a result of wide spread human use of metals they are also present in industrial and municipal waste waters as well as in run-offs of sealed and agricultural areas and also in atmospheric deposition. Exceedances of the tolerance levels in water as well as in sediment are likely to cause adverse effects in the aquatic ecosystem. They also can limit drinking water supplies, affect livestock or disturb irrigation purposes. Accumulative properties of some metals enhance them to enrich in the food chain leading to environmental or public health risks.

As affinity to particle surfaces is high for most metals, solubility in water is limited. Therefore metal concentration was not only measured in water, but metal content was estimated also of suspended particulate matter (SPM) and bottom sediment. The latter is of special interest, because changes in redox conditions, particularly in the case of anaerobic conditions within the settled layers might cause increased mobility and bioavailability and therefore increase the risk of adverse effects.

21.2 Methods

21.2.1 Methods used and Laboratories involved

Metal concentrations in 68 filtrated water samples were measured by Croatian Waters; Zagreb (HR). Filtration was done on board via 0,45µm borosilicate glass filters. Samples were stored in PE-bottles after acidification with nitric acid to a pH <2. Mercury samples were stabilized with potassium dichromate and stored in amber glass bottles. Cadmium (Cd), Lead (Pb), Nickel (Ni), Copper (Cu), Zinc (Zn) and Arsenic (As) were determined by use of ICP-MS according to ISO 17294-2 for. Chromium (Cr) was measured with ET-AAS following ISO 15586. Mercury (Hg) was analysed by cold vapour generation according to EPA method 245.7.

A number of 12 metals were determined in suspended particulate matter collected by continuous flow centrifugation at 50 sites. In addition to the above-mentioned elements investigations were made on Bismuth (Bi), Cobalt (Co), Molybdenum (Mo) and Manganese (Mn). Analysis of the freeze dried samples were done by the Bavarian LfU – Landesamt für Umwelt; Augsburg (DE) by use of ICP-MS according to ISO 17294-2 after aqua regia digestion following EN 13346-A with exception of Mercury, which was measured off-line without sample preparation by HG-AAS according to EPA method 7473. At a few sampling sites Mercury was also measured by JRC – Joint Research Centre; Ispra (IT).

Sediment samples taken at 49 sites were classified to core size <0,63µm on board, freeze dried and analysed after digestion with use of ICP-OES. Determinations were made on 7 metals (As, Cd, Cr, Cu, Ni, Pb, Zn). Analyses were carried out by the Institute for Biological Research “Sinisa Stankovic”; Belgrade (RS) following EPA method 3052.

At a small number of Danube sites Hg was determined also in biota samples (fish) by JRC.

Table 44 gives an overview of methods applied and limits of quantification (LOQ) achieved.

Table 44: Analytical methods and corresponding LOQs in alphabetical order

Element	Water Method / Standard / LOQ [µg/l]	SPM Method / Standard / LOQ [mg/kg dry weight]	Sediment Method / Standard / LOQ [mg/kg dry weight]
As	ICP-MS / ISO 17294-2 / 0,030	ICP-MS / EN 13346-A / ISO 17294-2../ 1,2	ICP-OES / EPA 3052 / 0,0630
Bi		ICP-MS / EN 13346-A / ISO 17294-2 / 0,06	
Cd	ICP-MS / ISO 17294-2 / 0,010	ICP-MS / EN 13346-A / ISO 17294-2 / 0,12	ICP-OES / EPA 3052 / 0,0010
Co		ICP-MS / EN 13346-A / ISO 17294-2 / 0,12	
Cr	ET-AAS / ISO 15586 / 0,100	ICP-MS / EN 13346-A / ISO 17294-2 / 12	ICP-OES / EPA 3052 / 0,0167
Cu	ICP-MS / ISO 17294-2 / 0,027	ICP-MS / EN 13346-A / ISO 17294-2 / 12	ICP-OES / EPA 3052 / 0,0136
Hg	CV-AAS / EPA 245.7 / 0,002	HG-AAS / EPA 7574 / EPA 7473 / 0,005	
Ni	ICP-MS / ISO 17294-2 / 0,040	ICP-MS / EN 13346-A / ISO 17294-2 / 6	ICP-OES / EPA 3052 / 0,0092
Mn		ICP-MS / EN 13346-A / ISO 17294-2 / 1,5	
Mo		ICP-MS / EN 13346-A / ISO 17294-2 / 0,3	
Pb	ICP-MS / ISO 17294-2 / 0,009	ICP-MS / EN 13346-A / ISO 17294-2 / 18	ICP-OES / EPA 3052 / 0,0285
Zn	ICP-MS / ISO 17294-2 / 0,040	ICP-MS / EN 13346-A / ISO 17294-2 / 30	ICP-OES / EPA 3052 / 0,0054

ICP-MS Inductively Coupled Plasma Mass Spectrometry
ET-AAS Electrothermal Atomic Absorption Spectrometry (Graphite Furnace)
CV-AAS Cold Vapour Atomic Absorption Spectrometry
HG-AAS Hydride Generation Atomic Absorption Spectrometry
ICP-OES Optical Emission Detected Inductively Coupled Plasma Spectrometry

21.2.2 Regulation – EU wide and National EQS

The investigated elements were categorised into two groups as follows:

- Group 1: Heavy metals included in the Priority List of the Water Framework Directive (WFD): Cadmium (Cd), Mercury (Hg) Nickel (Ni) and Lead (Pb);
- Group 2: Other heavy metals and metalloids: Arsenic (As), Bismuth (Bi), Cobalt (Co), Chromium (Cr), Copper (Cu), Manganese (Mn), Molybdenum (Mo) and Zinc (Zn).

For group 1 metals regulation is given by the EC. Environmental Quality Standards EQS presently in force are stipulated in Directive 2008/105/EC. These EQS had to be established as national law by EU member states until July 13th, 2010 at the latest. EQS to be effective in future have been laid down in Directive 2013/39/EU, which have to be transferred to national law not later than Sep. 14th, 2015.

For some of the group 2 elements there are national regulations in some countries. These of course are not uniform, and sometimes they may be dependent on other parameters (like total hardness as for Cd in group 1) or on hydrogeological conditions, bioregions etc.

Environmental Quality Standards are either given as Annual Average (AA-EQS) or as Maximum Allowable Concentration (MAC-EQS) or both and are usually valid for the dissolved fraction. An overview of currently valid (2008/105) and future effective (2013/39) EQS for Priority Substances and ranges of EQS for group 2 elements being in force at the time in national legislations is given in Table 45.

Table 45: Current valid and future effective EQS for in the course of JDS3 determined parameters and compartments relevant for the Danube and its tributaries (given EQS valid for dissolved fraction unless otherwise noted)

Element	AA-EQS		MAC-EQS		Compartment
EU regulated Priority Substances (Group 1)					
Cd	2008/105 and 2013/39:		2008/105 and 2013/39:		
	Hardness dep.		Hardness dep.		
	≥100 – <200mg $CaCO_3$/l:	0,15µg/l	≥100 – <200mg $CaCO_3$/l:	0,9µg/l	water
	≥200mg $CaCO_3$/l:	0,25µg/l	≥200mg $CaCO_3$/l:	1,5µg/l	water
Hg	2008/105:		2008/105:		
		0,05µg/l		0,07µg/l	water
		20µg/kg fresh weight			biota
	2013/39:		2013/39:		
		n.a.		0,07µg/l	water
		20µg/kg fresh weight			biota (fish)
Ni	2008/105:		2008/105:		
		20µg/l		n.a.	water
	2013/39:		2013/39:		
	Bioavailable concentration	4µg/l		34µg/l	water
Pb	2008/105:		2008/105:		
		7,2µg/l		n.a.	water
	2013/39:		2013/39:		
	Bioavailable concentration	1,2µg/l		14µg/l	water
Substances with national regulation (Group 2)					
As	AT:	24µg/l	AT:	n.a.	water
	SK/HR:	7,5µg/l	SK/HR:	n.a.	water
	HU:		HU: total As / C-90	20µg/l	water
	SI*): total As	7µg/l	SI:	n.a.	water
	RS:	50µg/l	RS:	n.a.	water
	BH:	20µg/l	BH:	n.a.	water
	BG: total As	10µg/l	BG: total As	25µg/l	water
	RO:	49µg/l	RO:	n.a.	water
	DE:	40mg/kg dry weight	DE:	n.a.	SPM/sediment
Cr	AT: + add. background conc.	8,5+0,5µg/l	AT:	n.a.	water
	SK/HR:	9µg/l	SK/HR:	n.a.	water
	HU:		HU: C-90	20µg/l	water
	SI*): total Cr	12µg/l	SI:	n.a.	water
	RS:		RS:		
	Cr^{III}	100µg/l	Cr^{III}	n.a.	water
	Cr^{VI}	100µg/l	Cr^{VI}	n.a.	water
	BH:	15µg/l	BH:	n.a.	water
	BG: total Cr		BG: total Cr	l	
	Cr^{III}	4,7µg/l	Cr^{III}	32µg/l	water
	Cr^{VI}	3,4µg/l	Cr^{VI}	8µg/l	water
	RO:	8,8µg/l	RO:	n.a.	water
	DE:	640mg/kg dry weight	DE:	n.a.	SPM/sediment
Cu	AT: hardness dep. + add. background conc.		AT:		
	>100mg $CaCO_3$/l:	8,8+0,5µg/l		n.a.	water
	SK/HR: hardness dep.		SK/HR:		
	>100mg $CaCO_3$/l:	8,8µg/l		n.a.	water
	HU:		HU: C-90	10µg/l	water
	SI*): total Cu	8,2µg/l	SI:	n.a.	water
	RS:	100µg/l	RS:	n.a.	water
	BH:	15µg/l	BH:	n.a.	water
	BG: total Cu, hardness dep.		BG:		
	>100 – 250mg $CaCO_3$/l:	10µg/l		n.a.	water
	>250mg $CaCO_3$/l:	22µg/l		n.a.	water
	RO: hardness dep.		RO:		
	>100mg $CaCO_3$/l:	10µg/l		n.a.	water
	DE:	160mg/kg dry weight	DE:	n.a.	SPM/sediment

Table 44 continued					
Zn	AT: hardness dep. + add. background conc.		AT:	n.a.	
	>100mg $CaCO_3$/l:	52,0+1,0µg/l			water
	SK/HR: hardness dep.		SK/HR:		
	>100mg $CaCO_3$/l:	52µg/l		n.a.	water
	HU:		HU: C-90	75µg/l	water
	SI*): total Zn	100µg/l	SI:	n.a.	water
	RS:	200µg/l	RS:	n.a.	water
	BH:	15µg/l	BH:	n.a.	water
	BG: total Zn, hardness dep.		BG:		
	>100 – 250mg $CaCO_3$/l:	75µg/l		n.a.	water
	>250mg $CaCO_3$/l:	100µg/l		n.a.	water
	RO: hardness dep.		RO:		
	>100mg $CaCO_3$/l:	73,0µg/l		n.a.	water
	DE:	800mg/kg dry weight	DE:	n.a.	SPM/sediment

*) given EQS for Slovenia may change by regarding natural background concentration, hardness, pH value etc.

21.3 Results

21.3.1 Metals in water

The results of the determination of dissolved heavy metals and metalloids in 68 surface water samples, i.e. 53 samples of the Danube and 15 samples of tributaries, are summarised in Table 46.

Table 46: Minimum and maximum concentration of dissolved heavy metals and Arsenic in water samples of the Danube River and its tributaries and accompanying hardness in equivalents of $CaCO_3$

	Danube		Tributaries	
Element	**Minimum [µg/l]**	**Maximum [µg/l]**	**Minimum [µg/l]**	**Maximum [µg/l]**
As	1,09	2,46	1,49	5,33
Cd	<0,01	0,145	0,011	1,050
Cr	0,29	6,73	0,23	67,13
Cu	1,06	9,93	0,74	282,54
Hg	<0,002	0,0070	<0,002	0,0063
Ni	0,78	24,63	0,76	230,08
Pb	0,20	8,08	0,23	2,64
Zn	1,13	12,95	1,03	60,73
Hardness	142mg $CaCO_3$/l	421mg $CaCO_3$/l	155mg $CaCO_3$/l	637mg $CaCO_3$/l

As Table 46 shows, Cd and Hg concentration were sometimes below the LOQs. For Cd this was the case in only 4 samples from the Upper Danube; for Hg concentrations lower than the LOQ occurred in 13 samples from the Danube as well as in 3 tributaries. As total hardness is relatively high in all samples, corresponding EQS are higher as well and therefore LOQs are low enough to allow evaluation for all sites.

In Table 47 the highest concentrations found are summarized.

Table 47: Highest concentrations of dissolved heavy metals and Arsenic in water samples found in the course of the survey (3 each)

Element	JDS3 site code	Sampling site	Concentration [µg/l]
As	JDS 51	/Iskar (rkm 0.3)	5,33
	JDS 41	/Velika Morava	4,97
	JDS35	/Tisa (rkm 1.0)	4,34
Cd	JDS 48	/Timok (rkm 0.2)	1,050
	JDS 41	/Velika Morava	0,301
	JDS19	Iza/Szony	0,145
Cr	JDS 41	/Velika Morava	67,13
	JDS11	Hainburg upstream Morava	6,73
	JDS 09	Klosterneuburg	5,30
Cu	JDS 48	/Timok (rkm 0.2)	282,54
	JDS 51	/Iskar (rkm 0.3)	12,80
	JDS 49	Pristol/Novo Selo Harbour	9,93
Hg	JDS 47	upstream Timok (Rudujevac/Gruia)	0,0070
	JDS 66	Vilkova – Chilia arm/Kilia arm	0,0064
	JDS 56	/Russenski Lom	0,0063
Ni	JDS 41	/Velika Morava	230,08
	JDS 48	/Timok (rkm 0.2)	35,81
	JDS11	Hainburg upstream Morava	24,63
Pb	JDS19	Iza/Szony	8,08
	JDS20	Szob	4,18
	JDS 41	/Velika Morava	2,64
Zn	JDS 48	/Timok (rkm 0.2)	60,73
	JDS35	/Tisa (rkm 1.0)	13,34
	JDS 03	Geisling power plant upstream dam	12,95

Table 46 as well as Table 47 show that some concentrations measured during the survey were extremely high. Of course higher concentrations are possible at every time, nevertheless in some cases contamination of the sample cannot entirely be excluded, particularly when high values occur in the Danube, which cannot be recovered some kilometres downstream a short time after.

21.3.1.1 Comparison of metal concentration in water with results from JDS1 and JDS2

Table 48 gives information on ranges of metal and arsenic concentrations in water during JDS3 and previous Danube surveys. Only sites investigated during JDS3 were taken into consideration, therefore given values may differ from values published in former reports.

Table 48: Range of element concentrations in the water samples of the Danube River and some of its tributaries during JDS1, JDS2 and JDS3

	Concentration [µg/l]					
	Danube			Tributaries		
Element	JDS1	JDS2	JDS3	JDS1	JDS2	JDS3
As	<1,0 – 4,6	<1,0 – 3,6	1,1 – 2,5	1,5 – 44,8	0,9 – 5,7	1,5 – 5,3
Cd	<0,2 – 0,5	<0,2 – <0,2	<0,01 – 0,15	<0,2 – <0,2	<0,2 – <0,2	0,01 – 1,05
Cr	<1 – 1	<0,25 – <0,25	0,3 – 6,7	<1 – 1	<0,25 – 1,3	0,2 – 67,1
Cu	2 – 5	<1,0 – 14,6	1,1 – 9,9	2 – 16	<1,0 – 34,5	0,7 – 282,5
Hg	<0,2 – <0,2	<0,05 – 0,07	<0,002 – 0,007	<0,2 – <0,2	<0,05 – <0,05	<0,002 – 0,006
Ni	<1 – 3	<1,0 – 12,2	0,8 – 24,6	<1 – 6	<1,0 – 33,3	0,8 – 230,1
Pb	<1,0 – 1,4	<2 – <2	0,2 – 8,1	<1,0 – 1,3	<2 – <2	0,2 – 2,6
Zn	<1,0 – 10,5	<2,5 – 7,7	1,1 – 13,0	3,8 – 15,6	<2,5 – 9,3	1,0 – 60,7

Looking at Table 48 it is obvious that analytical methods now achieve much lower LOQs especially regarding Cd and Hg. Ranges of element concentrations seem to have not only shifted to lower LOQs but also to higher concentrations especially in the tributaries but also in the Danube. This impression is due to a few abnormally high values, which were found during JDS3. Looking at the whole data sets it

can be seen, that concentration levels are very similar. Deviations in the results mainly arise from different conditions during the surveys (e.g. discharge, weather, seasonal influences).

21.3.1.2 Longitudinal profiles for results of water analysis

Arsenic showed slowly but stable increasing concentrations in the Danube as far as the mouth of Sava (from 1,0µg/l to approx. 2,2µg/l). From there on Arsenic concentration was constant unto the Delta. Some tributaries had higher concentrations (Morava (JDS12), Moson Danube Arm (JDS16), Vah (JDS18), Soroksar Danube Arm (JDS23), Tisa (JDS35), Velika Morava (JDS41), Iskar (JDS51) and Russenski Lom (JDS56)), a few (Timok (JDS48), Jantra (JDS54), Siret (JDS63) and Prut (JDS64)) had lower concentrations than the Danube.

Cadmium (Figure 117a) also exhibited increasing concentrations up to Sava confluence (starting with contents lower than the quantification limit reaching approx. 0,04µg/l) and a more or less constant concentration level from there on downstream. In contrast to As several sites showed concentrations lower (JDS8, JDS14) as well as higher (JDS7, JDS9-13, JDS19-20, JDS38, JDS43) than the neighbouring stations. Tributaries were usually in the same range as the Danube results, those with higher concentrations were Morava (JDS12), Moson Danube Arm (JDS16), Iskar (JDS51), and especially Velika Morava (JDS41) and Timok (JDS48), whereas Soroksar Danube Arm (JDS23), Russenski Lom (JDS56), Arges (JDS58), Siret (JDS63) and Prut (JDS64) had a lower Cd-contents.

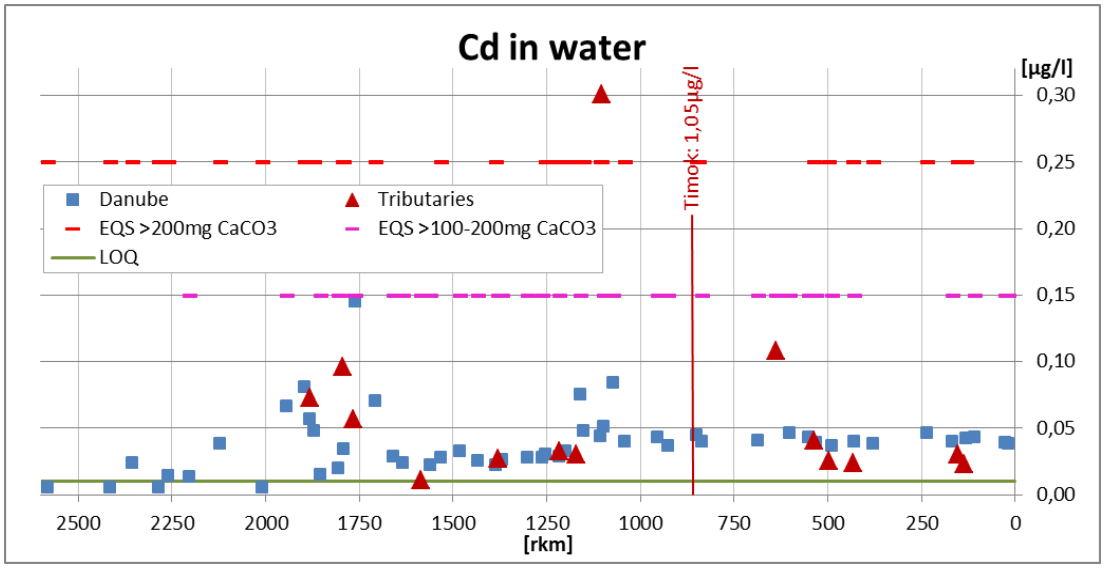
Chromium showed relatively homogenous results along the Danube fluctuating between 0,5 and 0,8µg/l. Several sites had higher concentrations (JDS9, JDS11, JDS21, JDS31, JDS43, JDS50, JDS60) which similarly were observed also for Nickel for most of these stations (see there). Remarkably high were concentrations at Danube sites JDS9 (5,3µg/l) and JDS11 (6,7µg/l). Except Siret (JDS63), which showed a decreased concentration and Velika Morava (JDS41), in which 67,1µg/l were measured as well as Iskar (JDS51) with 1,9µg/l and Russenski Lom (JDS56) with 1,5µg/l, the tributaries were in the same concentration range as the Danube.

Copper showed a very homogenous concentration profile along the Danube. Starting at concentrations of approx. 1,4µg/l down to Sava confluence, then at the level of about 2,0µg/l down to JDS47. Downstream Timok confluence the Copper concentration in the Danube raise up to 10µg/l (JDS49) due to the impact by the tributary, then downstream Iskar went down again to concentrations around 3,0µg/l. Timok (JDS48) flew in with an extremely high concentration of about 280µg/l. Iskar (JDS51) had a concentration of 13µg/l and Arges (JDS58) of nearly 6µg/l. All other tributaries showed the same concentrations as the Danube in the related stretches.

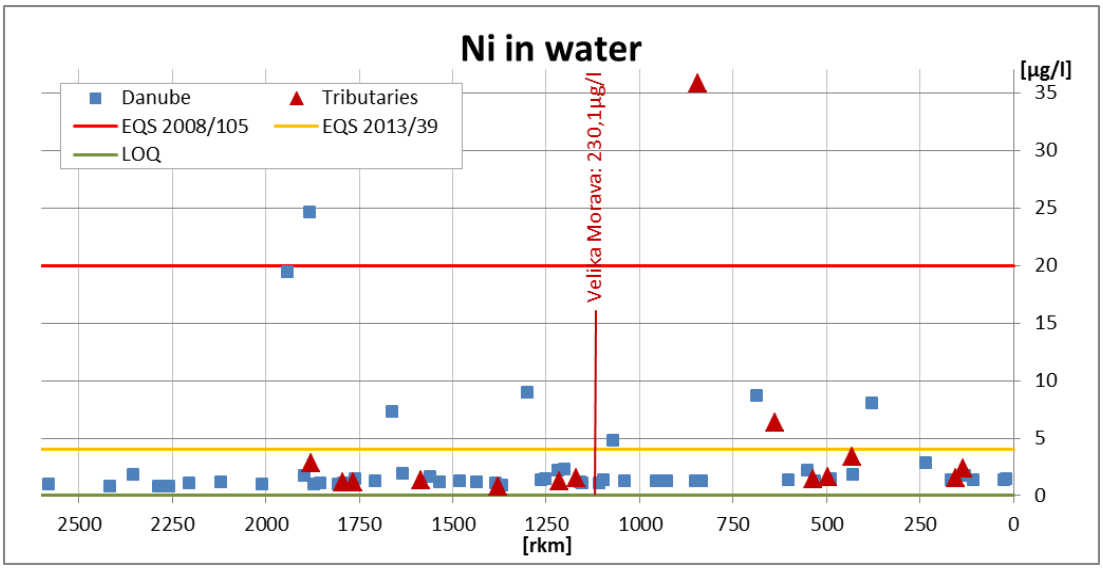
Mercury was detected in a narrow concentration range too. Starting with concentration below the quantification limit the Danube reached a level of 0,004-0,006µg/l downstream the Moson Danube Arm. Tributaries in general were in the range of the Danube, Soroksar Danube Arm (JDS23), Sava (JDS37), Timok (JDS48), Siret (JDS63) and especially Velika Morava (JDS41) had lower contents, the latter below LOQ.

Nickel (Figure 117b) in general appeared in concentrations between 1,0 and 1,8µg/l in the Danube. Some sites showed slightly higher concentrations. At seven Danube sites concentrations above 4,0µg/l were observed (JDS9, JDS11, JDS21, JDS31, JDS43, JDS50, JDS60). This pattern is almost the same as that found for Chromium. The most elevated concentrations in the Danube were found at JDS9 (19,4µg/l) and JDS11 (24,6µg/l) . The tributaries showed concentrations similar to the Danube, only Drava (JDS29) and Tisa (JDS35) were a bit lower in concentration, while Morava (JDS12), Iskar (JSD51), Arges (JDS58) and Prut (JDS64) and especially Velika Morava (JDS41) and Timok (JDS48) had higher Nickel contents, (Ni-concentration in Velika Morava reached 230,1µg/l).

a)



b)



c)

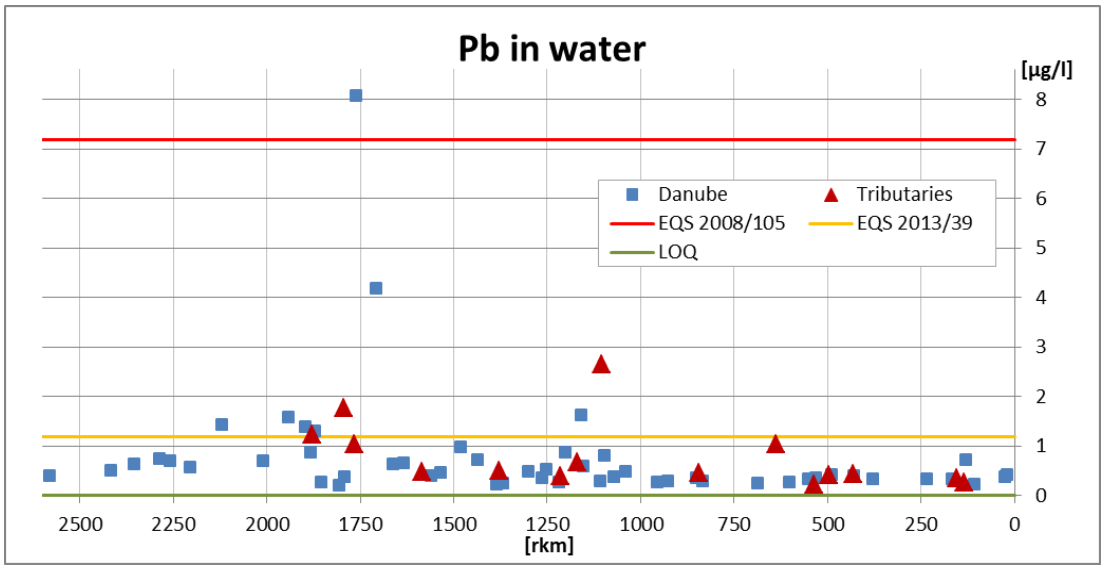


Figure 117a-c: Distribution of Cd, Ni and Pb in water samples in the Danube and its tributaries

Lead (Figure 117c) showed a relatively flat concentration distribution in the Danube varying around 0,5µg/l. Higher concentrations were found at JDS7, JDS9-10, JDS13 JDS19-20 and JDS38. Extremely high levels were detected at stations JDS19 (8,1µg/l) and JDS20 (4,2µg/l). Tributaries exhibited Pb-concentrations in the same order as the Danube, only Moson Danube Arm (JDS16), Velika Morava (JDS41) and Iskar (JDS51) held higher concentrations.

Zinc gave results between 5 and 8µg/l in the upper Danube as far as Sava confluence. From there downstream to the Delta concentrations were in most cases down to 2 to 3µg/l. In some tributaries (Morava (JDS12), Soroksar Danube Arm (JDS23) and Tisa (JDS35) and especially Timok (JDS48)) higher Zn-concentrations as in the related Danube stretches were observed. The other tributaries were very much alike the Danube.

21.3.2 Metals in suspended particulate matter

The results of the determination of heavy metals and metalloids in suspended particulate matter (SPM) are summarised in Table 49. Altogether 50 Danube samples have been analysed by LfU (and 23 Hg-samples by JRC resp.).

Table 49: Minimum and maximum contents of metals and metalloids in SPM-samples of the Danube River

Element	Minimum [mg/kg dry weight]	Maximum [mg/kg dry weight]
As (LfU)	8,4	27,6
Bi (LfU)	0,27	0,65
Cd (LfU)	0,26	1,16
Co (LfU)	8,3	23,8
Cr (LfU)	37,0	76,1
Cu (LfU)	26,8	86,7
Hg (LfU)	0,12	0,38
Hg (JRC)	0,08	0,62
Mn (LfU)	592	2854
Mo (LfU)	0,51	1,95
Ni (LfU)	29,0	69,3
Pb (LfU)	<18,0	48,7
Zn (LfU)	99,5	245,6

With exception of JDS13 “Bratislava”, in which lead was below the LOQ of 18mg/kg dry weight all metals were present in measureable amounts and showed very homogenous distribution patterns without any extremes. The maximum concentrations were from 2,1 to 4,8 times higher than the minimum concentrations. With exception of two single values (comp. Table 50) results for Hg delivered by JRC match very well with results of LfU.

In Table 50 the highest concentrations found are summarized.

In exception of the generally homogenous distribution for As, Bi, Co, Cr, Cu, Mo and Ni at the site JDS06 “Jochenstein” values were observed, which were slightly higher than those at the neighbouring stations. Elevated concentrations for most of these elements were also found on the next 2 downstream stations JDS7 “Abwinden-Asten upstream dam” and JDS8 “Oberloiben”. The influence of the Inn was not investigated in this survey but possible impacts should not be neglected.

Table 50: Highest contents of metals and metalloids in SPM samples found in the course of the survey (2 each)

Element	JDS3 site code	Sampling site	Concentration [mg/kg dry weight]
As	JDS6	Jochenstein	27,6
	JDS46	Vrbica/Simijan	18,9
Bi	JDS6	Jochenstein	0,65
	JDS36	Belegis downstream Tisa / upstream Sava	0,48
Cd	JDS46	Vrbica/Simijan	1,16
	JDS45	Irongate reservoir (Tekija/Orsova)	1,10
Co	JDS6	Jochenstein	23,8
	JDS7	Abwinden-Asten upstream dam	17,5
Cr	JDS68	Sf.Gheorghe – Sf.Gheorghe arm	76,1
	JDS6	Jochenstein	68,3
Cu	JDS47	upstream Timok (Rudujevac/Gruia)	86,7
	JDS49	Pristol/Novo Selo Harbour	70,0
Hg (LfU)	JDS42	downstream Velika Morava	0,38
	JDS24	Dunafoldvar	0,31
Hg (JRC)	JDS36	Belegis downstream Tisa / upstream Sava	0,47
	JDS60	Chiciu/Silistra	0,62
Mn	JDS46	Vrbica/Simijan	2854
	JDS49	Pristol/Novo Selo Harbour	2253
Mo	JDS6	Jochenstein	1,95
	JDS7	Abwinden-Asten upstream dam	1,89
Ni	JDS68	Sf.Gheorghe – Sf.Gheorghe arm	69,3
	JDS43	Banatska Palanka/Bazias	66,3
Pb	JDS46	Vrbica/Simijan	48,7
	JDS44	Irongate reservoir (Golubac/Koronin)	45,3
Zn	JDS3	Geisling power plant upstream dam	245,6
	JDS46	Vrbica/Simijan	227,4

21.3.2.1 Comparison of metal and metalloid contents in SPM with results from JDS1 and JDS2

Table 51 gives information on ranges of metal and metalloid contents in suspended particulate matter during JDS3 and former Danube surveys. Only sites investigated during JDS3 were taken into consideration, therefore given values may differ from values published in former reports.

Table 51: Range of element concentrations in the SPM samples of the Danube River during JDS1, JDS2 and JDS3

	Concentration [mg/kg dry weight]		
Element	JDS1	JDS2	JDS3
As	9,4 – 31,2	9,5 – 18,7	8,4 – 27,6
Bi	not observed	0,33 – 0,67	0,27 – 0,65
Cd	0,10 – 7,60	0,29 – 2,23	0,26 – 1,16
Co	not observed	10,1 – 18,8	8,3 – 23,8
Cr	33,0 – 107,5	40,8 – 83,9	37,0 – 76,1
Cu	28,3 – 193,7	38,1 – 110,8	26,8 – 86,7
Hg	0,10 – 0,55	0,10 – 0,39	0,08 – 0,62
Mn	565 – 4028	770 – 2808	592 – 2854
Mo	not observed	0,59 – 2,17	0,51 – 1,95
Ni	21,5 – 89,8	31,6 – 85,0	29,0 – 69,3
Pb	18,9 – 85,0	25,3 – 62,6	<18,0 – 48,7
Zn	109 – 398	130,6 – 325,2	99,5 – 245,6

Ranges given in Table 51 indicate a very homogenous and stable situation of metal contents in SPM. Not only that ranges themselves seem to be narrow, but also comparison of results from one survey with another does not show big differences. For some metals a decrease in contents seems to have taken place, for instance for Cd, Cr, Cu, Ni, Pb and Zn.

21.3.2.2 Longitudinal profiles for results of SPM analysis

For some elements the contents are very similar all along the Danube. In a few cases this pattern is interrupted by single results that show somewhat higher contents, but without extremely high values.

As, Bi and Co are in one level for all samples except JDS6, the same for Cu but for this element higher results were found at JDS1, JDS47 and JDS49.

For Cd (Figure 118a), Mn and Pb (Figure 118c) the flat distribution along the Danube is interrupted by higher results in the region of the Iron Gate reservoir (from JDS43 to JDS49), the same pattern is obvious for Zn but with higher values for JDS1 and JDS3.

Slightly increased contents of metal in SPM samples from JDS43 down to and including the delta were observed for Cr and Ni (Figure 118b). For both also a higher value at JDS6 was determined.

Mo showed also a flat distribution of results all over the Danube with exception of the region from the German border to the Gabcikovo reservoir (JDS6 – JDS14).

Lower contents of Hg were observed in the lowest Danube reach (down from JDS61). Only the result of JDS42 was a bit higher than the other values. Mercury results of JRC done for 23 sites confirmed the results of LfU. Only for JDS36 and JDS60 values of JRC did not match well with LfU-results (concentrations for these sites found by JRC were remarkably higher than those measured by LfU – twice and three times resp.). In addition 15 samples taken in the course of JDS2 in 2007 were analysed by JRC in the course of this survey. All of them have similar concentrations as JDS3 samples.

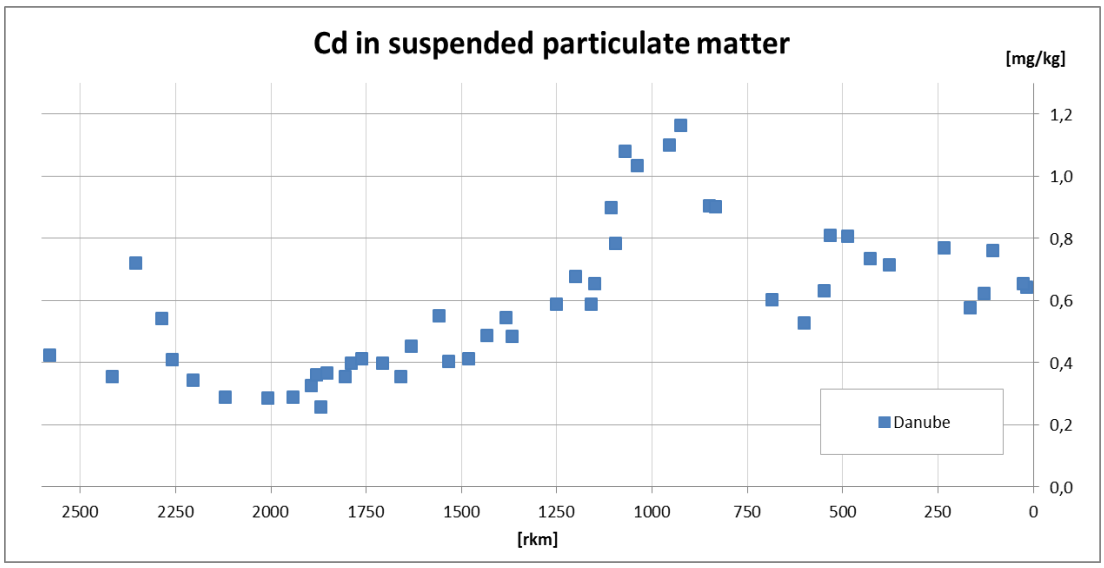
21.3.3 Metals in bottom sediment

The results of the determination heavy metals and metalloids in bottom sediment are summarised in Table 52. Altogether 49 samples have been analysed, 45 of the Danube and 4 of tributaries (Timok, Iskar, Arges and Siret).

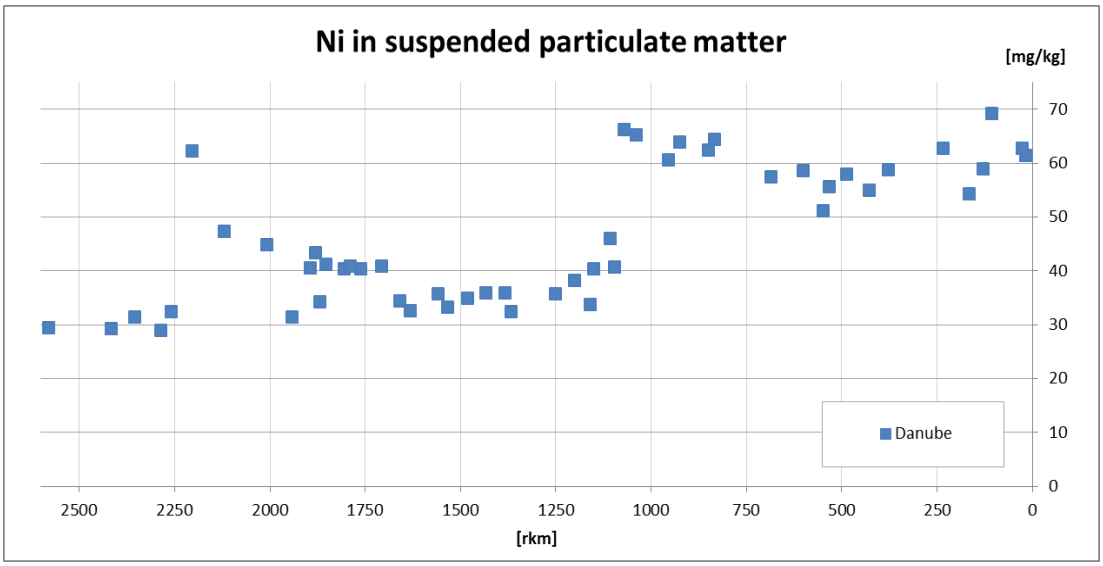
Table 52: Minimum and maximum contents of metals and arsenic in bottom sediment samples of the Danube River and its tributaries

	Danube		Tributaries	
Element	Minimum [mg/kg dry weight]	Maximum [mg/kg dry weight]	Minimum [mg/kg dry weight]	Maximum [mg/kg dry weight]
As	<LOQ	28,5	<LOQ	17,1
Cd	0,72	1,95	0,80	1,86
Cr	9,8	101,1	30,7	59,3
Cu	<LOQ	105,0	12,0	526,5
Ni	5,1	69,2	22,4	41,5
Pb	13,7	85,0	35,5	70,9
Zn	23	198	43	199

a)



b)



c)

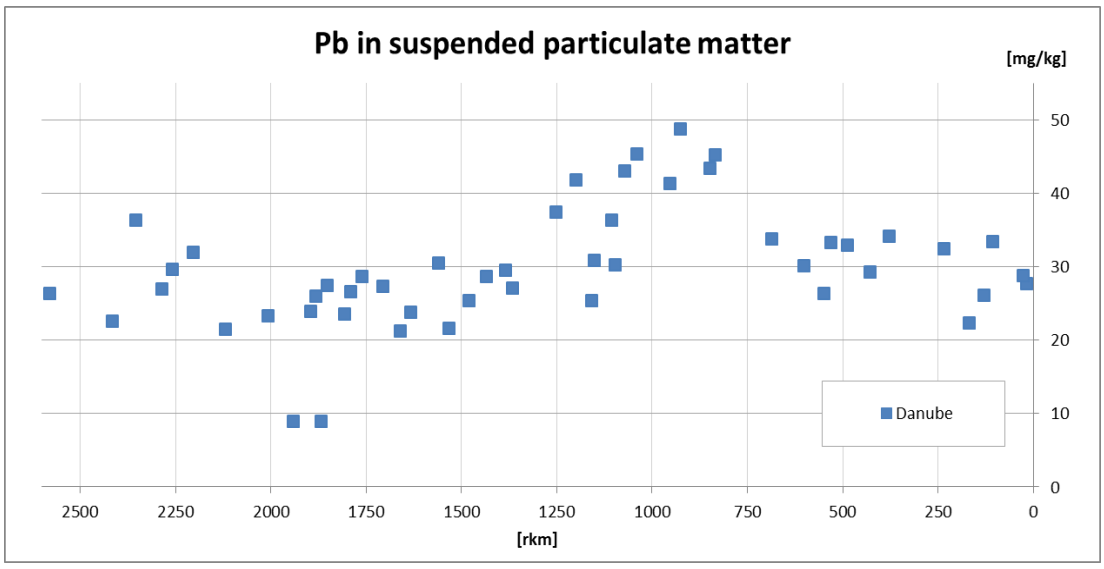


Figure 118a-c: Distribution of Cd, Ni and Pb in SPM samples in the Danube

Arsenic could be quantified only in 13 samples (i.e. 26,5%). Copper content was below LOQ in two Danube samples. The other metals were present in measureable amounts in all samples.

The metal content in bottom sediment was somewhat lower in the German Danube stretch (for all metals) showing slightly increasing amounts downstream to Austria there reaching a level that was more or less constant for the whole Danube down to the Black Sea then.

Here and there significant higher concentrations were found reaching twice or three times the median value of all results, a few also showing extremely high contents as in case of Copper (the 29 fold).

In Table 53 the highest contents found are summarized.

Table 53: Highest contents of metals and arsenic in bottom sediment samples found in the course of the survey (2 each)

Element	JDS3 site code	Sampling site	Concentration [mg/kg dry weight]
As	JDS44	Irongate reservoir (Golubac/Koronin)	28,5
	JDS50	downstream Kozloduy	23,6
Cd	JDS27	Hercegszanto	1,95
	JDS36, JDS48	Belegis downstream Tisa / upstream Sava; /Timok (rkm 0.2)	1,86
Cr	JDS43	Banatska Palanka/Bazias	101,1
	JDS39	downstream Pancevo	66,5
Cu	JDS48	/Timok (rkm 0.2)	526,5
	JDS49	Pristol/Novo Selo Harbour	105,0
Ni	JDS39	downstream Pancevo	69,2
	JDS38	downstream Sava / upstream Pancevo	67,3
Pb	JDS60	Chiciu/Silistra	85,0
	JDS39	downstream Pancevo	74,4
Zn	JDS58	/Arges	199
	JDS39	downstream Pancevo	198

Sampling sites showing very high metal contents or moderately higher contents for more than one element are JDS27 and JDS36 (Cd, Zn), JDS38 and JDS39 (Cd, Cr, Ni, Pb, Zn), JDS43 (Cr), JDS48 (Cd, Cu, Ni, Zn), JDS49 (Cd, Cu, Zn), JDS58 (Cr, Zn) and JDS60 (Cd, Cu, Pb).

Sites with the highest metal concentrations in sediment seem to be JDS38 “downstream Sava / upstream Pancevo”, JDS39 “downstream Pancevo”, JDS60 “Chiciu/Silistra” and JDS48 Timok. A comparison of the results for SPM and bottom sediment generally shows a good comparability, but not for every single site. One reason for this may be that bottom sediment usually reflects past time contaminations, whereas suspended matter mainly gives information on current conditions.

21.3.3.1 Comparison of metal and arsenic contents in bottom sediment with results from JDS1 and JDS2

Table 54 gives information on ranges of metal and arsenic contents in bottom sediment during JDS3 and former Danube surveys. Only sites investigated during JDS3 were taken into consideration, therefore given values may differ from values published in former reports.

Comparing the ranges for JDS3 given in Table 54 with those for the former Danube surveys it can be seen, that most of them are narrower, some of them even significantly. This is due to some extraordinary high maximum concentrations detected in the past (such as for Cu, Ni, Pb and Zn). This decrease however shall not be necessarily interpreted as a general decrease of metal loads as it might also be a consequence of an increased experience in sampling and improvements in analytical practice.

Table 54: Range of element concentrations in the bottom sediment samples of the Danube River and some of its tributaries during JDS1, JDS2 and JDS3

	Concentration [mg/kg dry weight]					
	Danube			Tributaries		
Element	JDS1	JDS2	JDS3	JDS1	JDS2	JDS3
As	9,7 – 33,0	39,2 – 432,3	<LOQ – 28,5	9,9 – 388,0	58,7 – 96,2	<LOQ – 17,1
Cd	0,20 – 14,05	0,31 – 3,12	0,72 – 1,95	1,10 – 32,90	0,40 – 8,40	0,80 – 1,86
Cr	35,6 – 124,6	43,6 – 204,8	9,8 – 101,1	48,8 – 209,9	67,5 – 345,7	30,7 – 59,3
Cu	32,7 – 371,1	54,3 – 3005,8	<LOQ – 105,0	40,0 – 8088,0	50,7 – 11431,5	12,0 – 526,5
Hg	0,10 – 0,64	0,10 – 0,70		0,10 – 0,65	0,10 – 0,40	
Ni	28,3 – 98,4	39,5 – 234,8	5,1 – 69,2	45,7 – 86,1	121,2 – 324,5	22,4 – 41,5
Pb	24,2 – 90,6	49,0 – 1637,2	13,7 – 85,0	20,5 – 541,8	436,7 – 2111,3	35,5 – 70,9
Zn	105,5 – 379,5	125,4 – 491,5	22,8 – 197,8	98,0 – 2010,0	106,8 – 923,8	43,2 – 198,9

In general there is a very good comparability of sediment monitoring with results from SPM samples.

Looking at the longitudinal concentration profiles of metals in sediments (see Chapter 21.3.3.2) a decline for most metals compared to previous surveys (especially JDS2) can be seen (e.g. Cu, Ni, Zn).

21.3.3.2 Longitudinal profiles for results of bottom sediment analysis

Arsenic contents were mostly below the limit of quantification both for the Danube and for its tributaries. Some results for the Danube as well as for Iskar showed concentrations up to 10mg/kg dry weight. Only single Danube sites (JDS44, JDS50, JDS60) and Timok (JDS48) had higher As-contents with maximum concentration of approx. 30mg/kg.

Cadmium results (Figure 119a) showed a very homogenous distribution along the Danube as well as in its tributaries with Cd-contents between approx. 0,8 and 1,1mg/kg dry weight. Only a few Danube sites (JDS27, JDS36, JDS38-39, JDS46-47, JDS49, JDS60) and Timok (JDS48) had higher sediment concentrations up to a maximum of approx. 2,0mg/kg.

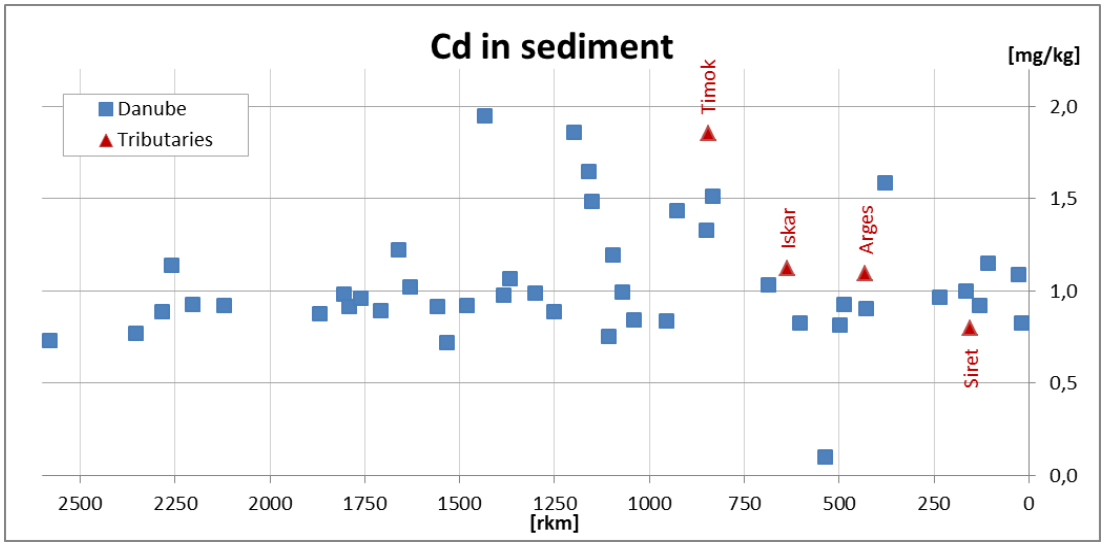
Chromium contents in sediment went up and down along the Danube in a range of approx. 15 to 50mg/kg dry weight. Also the samples of the tributaries were in same order. Single sites had higher concentrations (Danube sites JDS38-39 and JDS43 as well as Arges JDS58) which reached up to 100mg/kg.

Copper contents ranged from 10 to 25mg/kg dry weight, sometimes the concentrations were below the limit of quantification, in few cases higher Cu-concentrations were found in the Danube (JDS49 "Pristol/Novo Selo Harbour" downstream Timok and JDS60 "Chiciu/Silistra") and also some tributaries (Iskar, Arges and especially Timok) showed an elevated Cu-concentration. The latter showed the maximum concentration of 527mg/kg, which is the more than 15fold mean value.

Nickel contents (Figure 119b) at the upper Danube started with approx. 5-10mg/kg dry weight in the German stretch, raised up to about 20mg/kg in Austria and stood stable at this level down to the Iron Gate (JDS45) with only two exceptions (JDS38-39) which gave maximum results of nearly 70mg/kg. From the Iron Gate on down to the delta Ni-contents climbed up to approx. 30mg/kg. Higher values, but not more than approx. 40mg/kg, were observed at some Danube stations (JDS49, JDS60, JDS62) and in the Timok (JDS48).

Lead (Figure 119c) similarly to Chromium showed an unsteady distribution. The concentrations generally ranged from 15 to 50mg/kg dry weight. A bit higher contents up to 85mg/kg dry weight were observed at some Danube-sites (JDS21, JDS38-39, JDS49, JDS60, JDS62) and the tributaries Timok, Iskar and Arges. The maximum concentration of 85mg/kg was estimated at JDS60.

a)



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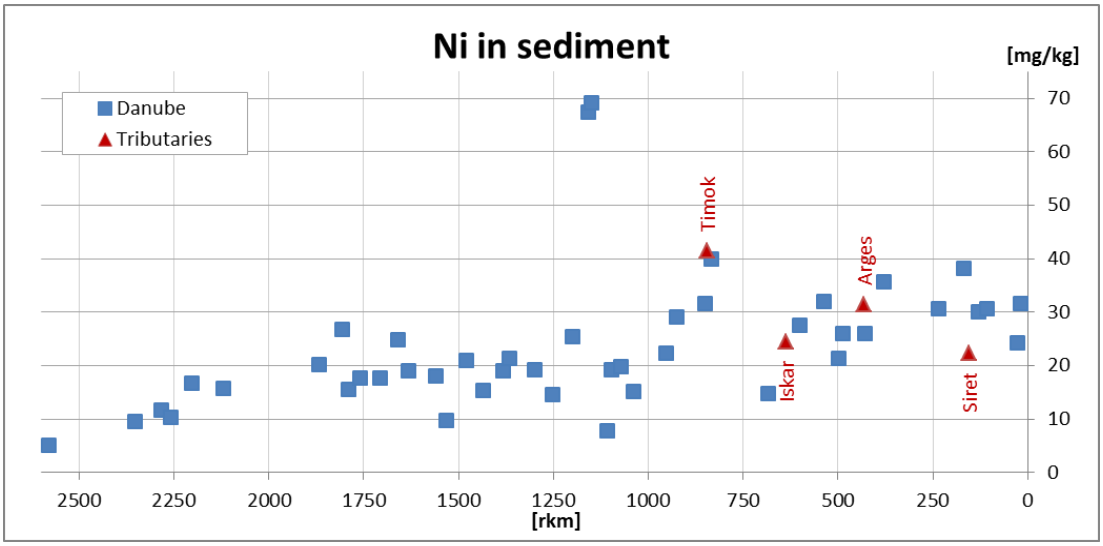

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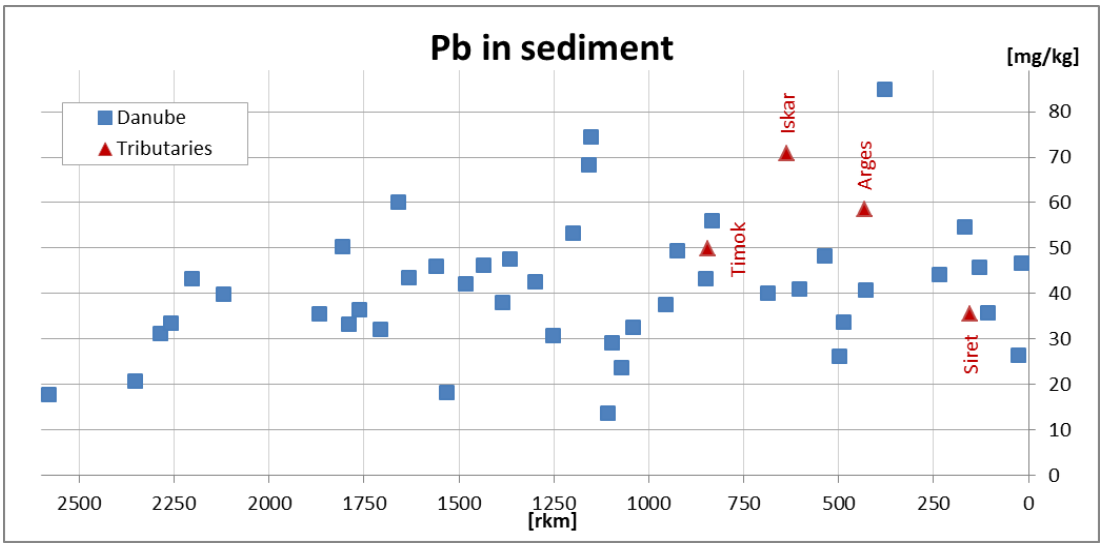

Figure 119a-c: Distribution of Cd, Ni and Pb in bottom sediment samples in the Danube and its tributaries

Zinc like Chromium and Lead had alternating concentrations in sediment samples along the Danube varying between approx. 20 and 120mg/kg dry weight. A number of Danube stations had higher Zn-concentrations up to 200mg/kg (JDS21, JDS27, JDS36, JDS38-39, JDS49, JDS60) and also the Timok and the Arges showed higher contents of about 170mg/kg and 200mg/kg resp., whereas the Zn-content of Siret went below the concentration of the neighbouring Danube sites (which was also shown in the case of the other elements).

21.3.4 Mercury in biota

At six sites Mercury was also determined in fish samples by JRC. For analysis dried fish tissue (muscle) of *Abramis brama* was used. Results ranged between 0,46 and 2,29mg/kg dry weight. Calculated for fresh weight this means mercury contents from 0,11 to 0,35mg/kg wet weight.

In addition 3 fish samples taken in 2007 were also analysed in the course of this survey. Results were of good comparability with those of 2013 giving Hg contents of 0,98 – 1,86mg/kg dry weight according to 0,21 – 0,44mg/kg wet weight.

21.4 Conclusions

In general, the concentrations of heavy metals and arsenic in water, and the contents of metals and metalloids in suspended particulate matter and bottom sediments estimated during JDS3 were similar to those observed in the JDS1 and JDS2 samples. Sediment bound metal contents seem to be slightly declining.

21.4.1 Evaluation

Results of the survey were compared with Environmental Quality Standards EQS for those heavy metals included in the Priority List of the Water Framework Directive (Cd, Hg, Ni and Pb) both using presently valid EQS (given in 2008/105/EC) as well as future EQS (set in 2013/39/EU). For the other elements investigated during JDS3 (As, Bi, Co, Cr, Cu, Mn, Mo and Zn) evaluation was done by comparing the results with the national regulation values (comp. Chapter 21.2.2)

Environmental Quality Standards are either given as Annual Average (AA-EQS) or as Maximum Allowable Concentration (MAC-EQS) or both. An overview of currently valid (2008/105) and future effective (2013/39) EQS for Priority Substances and ranges of EQS for group 2 elements being in force at the time in national legislations is given in Table 45.

21.4.1.1 Evaluation for results of water analysis

According to the WFD for evaluation a collective of at least 12 samples equally distributed over a year´s time is necessary. Nevertheless comparison with given environmental quality standards is possible and might give an idea of existing contaminations and risks of exceeding EQS and probably not achieving good chemical or ecological status in the Danube or one or some of its tributaries. Table 55 gives the JDS numbers for sites, at which EQS were exceeded. In case of the Group 2 metals and metalloids there is also given information, national standards of which countries has not been met (in brackets).

Having a look on Table 55 one can see, that there are not too much non conformity situations. As mentioned above some of these are due to extremely high results, not all of the latter likely to be reproducible.

Table 55: Results of water analysis exceeding given EQS

	Danube		Tributaries	
Element	Number of exceeding values	JDS3 site codes	Number of exceeding values	JDS3 site codes
EU regulated Priority Substances (Group 1)				
Cd	0	-	2 AA	41, 48
Hg	0	-	0	-
Ni	2008/105:		2008/105:	
	1 AA	11	2 AA	41, 48
	2013/39		2013/39	
	7 AA	9, 11, 21, 31, 43, 50, 60	3 AA	41, 48, 51
	0 MAC		2 MAC	41, 48
Pb	2008/105:		2008/105:	
	1 AA	19	0	-
	2013/39		2013/39	
	7 AA	7, 9, 10, 13, 19, 20, 38	3 AA	12, 16, 41
	0 MAC	-	0 MAC	-
Substances with national regulation (Group 2)				
As	0	-	0	-
Cr	2 AA (BG*)	9, 11	1 AA (all)	41
	0 MAC	-	1 MAC (HU, BG*)	41
Cu	1 AA (AT, HR, SK, SI)	49	2 AA (nearly all)	48, 51
	0 MAC		2 MAC (HU)	48, 51
Zn	0 AA		1 AA (AT, BH, HR, SK)	48
	0 MAC	-	0 MAC	-

* Quality standard given for Cr^{III} and Cr^{VI} separately

For Mercury and Arsenic there were no violations of limits at all.

Only on two Danube sites and on three tributaries EQS were exceeded for more than one element. These are the Danube sites JDS9 "Klosterneuburg" and (Ni, Pb, Cr), JDS11 "Hainburg upstream Morava" (Ni, Cr) as well as tributaries Velika Morava JDS41 (Cd, Ni, Pb, Cr), Timok JDS48 (Cd, Ni, Cu, Zn) and Iskar JDS51 (Ni, Cu).

In general Table 55 also gives a hint that future problems may be expected with the low AA-EQS for Nickel (4µg/l) and Lead (1,2µg/l). Concerning this a clear interpretation is necessary, what precisely the "bioavailable concentration" of the metal content is.

21.4.1.2 Evaluation for results of SPM analysis

As shown in Table 45 there are almost no EQS values for metals in SPM, except a national regulation of Germany for As, Cr, Cu and Zn. For former surveys quality target values given in Table 56 were used.

Table 56: Quality targets for metals and metalloids in SPM and bottom sediment

Target values [mg/kg dry weight]	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
German EQS (comp. Table 45)	40	-	640	160	-	-	-	800
JDS quality target values	20	1,2	100	60	0,8	50	100	200

German targets were met by SPM results at all sites for all elements.

The JDS quality target values were not exceeded for Cd, Cr, Hg and Pb. The target value for As was not met at 1 site, for Cu at 3 sites, for Ni at 20 sites and for Zn at 7 sites. JDS sites, at which target levels were exceeded, are listed in Table 57.

Table 57: SPM results for metals and arsenic exceeding JDS quality target values (cf. Table 56)

	Danube	
Element	Number of exceeding values	JDS3 site codes
EU regulated Priority Substances (Group 1)		
Cd	0	-
Hg	0	-
Ni	20	06, 43, 44, 45, 46, 47, 49, 50, 52, 53, 55, 57, 59, 60, 61, 62, 65, 66, 67, 68
Pb	0	-
Substances with national regulation (Group 2)		
As	1	06
Cr	0	-
Cu	3	01, 47,49
Zn	7	01, 03, 44, 45, 46, 47,49

21.4.1.3 Evaluation for results of bottom sediment analysis

As stated in Chapter 21.3.3.1 there are almost no EQS values for metals in sediment. For evaluation of the bottom sediment results values from Table 56 were used too.

German targets were with one exception met by all sediment results of all sites for all elements. Only Copper at JDS48 exceeded the quality target value of 160mg/kg by a factor of 3,3.

JDS limits were not exceeded for Pb and Zn. The target value for As was not met at 2 sites, for Cd at 10 sites, for Cr at 1 site, for Cu at 3 sites and for Ni at 2 sites. JDS sites at which target levels were exceeded, are listed in Table 58.

Table 58: Bottom sediment results for metals and arsenic exceeding JDS quality target values (cf. Table 56)

	Danube		Tributaries	
Element	Number of exceeding values	JDS3 site codes	Number of exceeding values	JDS3 site codes
EU regulated Priority Substances (Group 1)				
Cd	9	21, 27, 36, 38, 39, 46, 47, 49, 60	1	48
Ni	2	38, 39	0	-
Pb	0	-	0	-
Substances with national regulation (Group 2)				
As	2	44, 50	0	-
Cr	1	43	0	-
Cu	2	49, 60	1	48
Zn	0	-	0	-

21.4.1.4 Evaluation for results of Mercury in biota

As mercury is highly toxic and the EQS derived for water is that low, that it cannot be met by standard analytical methods, for this element an EQS in biota was set (see 2008/105/EC and 2013/39/EU resp.). This EQS is referred to fish and is 0,020mg/kg wet weight.

Results obtained by JRC are all above the EQS exceeding it by factors between 5 and 18. Also stored fish samples from 2007 (JDS2) were above this limit.

The observed exceedance in biota has been reported also from many other European countries. Therefore this is not a Danube specific problem. The fact, that an exceedance can also be observed in pristine areas indicates a high ubiquitous portion where long-range transport plays an important role. Future results will show whether the international efforts in reducing the emissions will lead to a decline.

22 Target analysis of emerging polar organic substances in water, fish and SPM using solid-phase extraction followed by UHPLC-MS-MS analysis

Robert Loos, Simona Tavazzi, Bruno Paracchini, Jerker Fick

22.1 Introduction

The analysed polar organic compounds which are shown in Table 59 were identified by the ICPDR as the emerging substances which require further investigation.

Cybutryne, terbutryn, and PFOS are new priority substances under the WFD. Diclofenac, a non-steroidal anti-inflammatory drug, was proposed as a new priority substance, and will be monitored by the newly introduced WFD "watch list" mechanism. The benzotriazoles are high production volume chemicals (corrosion inhibitors) related to industrial and urban emissions. 2,4-D, MCPA, and metolachlor are important herbicides. Carbamazepine, a mood-stabilizing drug, is one of the pharmaceuticals most often analysed in the environment; 10,11-dihydro-10,11-dihydroxy-carbamazepine is an important degradation product of carbamazepine for which only little monitoring data is available. DEET is an important insect repellent, and sulfamethoxazole an antibiotic substance often detected in the environment. Perfluorooctanoic acid (PFOA) was one of the most important contaminants in JDS2, coming mostly from a fluoropolymer production plant located in Germany on the Inn River tributary. In addition, other relevant perfluoroalkyl substances were analysed.

Table 59: Target polar organic substances analysed by SPE-UHPLC-MS-MS

Analyte	CAS No.
1-H-Benzotriazole	95-14-7
Methylbenzotriazoles	136-85-6
2,4-D (2,4-Dichlorophenoxyacetic acid)	94-75-7
Carbamazepine (CBZ)	298-46-4
10,11-Dihydro-10,11-dihydroxy-carbamazepine (CBZ-metabolite)	58955-93-4
Cybutryne	28159-98-0
DEET (N,N-Diethyl-m-toluamide)	134-62-3
Diclofenac	15307-86-5
MCPA (2-Methyl-4-chlorophenoxyacetic acid)	94-74-6
Metolachlor	51218-45-2
PFBS (Perfluorobutane sulfonic acid)	375-73-5
PFHxA (Perfluorohexanoic acid)	307-24-4
PFHpA (Perfluoroheptanoic acid)	375-85-9
PFOA (Perfluorooctanoic acid)	335-67-1
PFOS (Perfluorooctane sulfonic acid)	1763-23-1
PFNA (Perfluorononanoic acid)	375-95-1
Sulfamethoxazole	723-46-6
Terbutryn	886-50-0

Moreover, the benzodiazepine anxiolytic drugs shown in Table 60 were analysed by Umeå University, Department of Chemistry, Sweden. It was recently shown that environmental levels of oxazepam changes fish behaviour (increased activity, reduced sociality, and increased feeding rate in exposed perch) (Brodin et al. 2014).

Table 60: Benzodiazepines analysed by online-SPE-UHPLC-MS-MS

Analyte	CAS No.
Alprazolam	28981-97-7
Bromazepam	1812-30-2
Chlordiazepoxide	58-25-3
Clobazam	22316-47-8
Clonazepam	1622-61-3
Diazepam	439-14-5
Flunitrazepam	1622-62-4
Halazepam	23092-17-3
Lorazepam	846-49-1
Midazolam	59467-64-0
Oxazepam	604-75-1
Prazepam	2955-38-6
Temazepam	846-50-4

22.2 Methods

22.2.1 Multi-compound SPE-UHPLC-MS-MS (JRC)

Analyses were performed by automated solid-phase extraction (SPE) followed by ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC-MS-MS). SPE of water samples was performed with a *Dionex Autotrace AT280* automated SPE system (*Thermo Scientific*, Waltham, MA, USA) using 200 mg *Oasis HLB* cartridges (*Waters*); UHPLC-MS-MS was performed with an *Acquity* UHPLC system (*Waters Corporation*, Milford, MA, USA) coupled to a hybrid triple-quadrupole linear ion trap mass spectrometer *5500 QTRAP* with a turbo ion spray source from *AB SCIEX* (Foster City, CA, USA). The analytical column used was an *Acquity UPLC BEH* C18, 1.7 µm, 50 × 2.1 mm (*Waters*); the flow rate was 600 µl/min and injection volume 5 µl. Mobile phases used were: A: water – methanol (95:5%, v/v), 0.1% acetic acid, and B: acetonitrile – methanol (50:50%, v/v), 0.1% actic acid. The chromatography was performed in gradient mode, starting with 90% A which was hold for 1 min, gradient rise to 90% B in 6 min, and re-equilibration, giving a total analysis time of 10 min.

The water sample volume extracted was 1 l. After drying of the cartridges with nitrogen, the sample was eluted with 10 ml ethyl acetate. Half of the received extract (i.e. about 5 ml) was kept for polar compounds analysis. The remaining aliquot (i.e. about 5 ml) was evaporated to 50-100 µl under nitrogen flow for GC-MS determination of organophosphate ester flame retardants.

The internal surrogate standards used for "isotope dilution" quantification are shown in Table 61.

Table 61: Internal surrogate standards used for isotope dilution analysis

Internal surrogate standards
1-H-Benzotriazole d_4
2,4-D (2,4-Dichlorophenoxyacetic acid) d_6
Carbamazepine d_{10}
Cybutryne d_9
DEET (N,N-Diethyl-m-toluamide) d_6
$^{13}C_6$-Diclofenac
MCPA (2-Methyl-4-chlorophenoxyacetic acid) d_3
Metolachlor d_6
$^{13}C_2$-PFHxA (Perfluorohexanoic acid)
$^{18}O_2$-PFHxS (Perfluorohexane sulfonic acid)
$^{13}C_4$-PFOA (Perfluorooctanoic acid)
$^{13}C_4$-PFOS (Perfluorooctane sulfonic acid)
$^{13}C_5$-PFNA (Perfluorononanoic acid)
$^{13}C_6$-Sulfamethoxazole
Terbutryn d_5

22.2.2 Extraction of fish and SPM for PFOS analyses

Extraction of freezed-dried fish liver and SPM samples (ca. 1 g) was performed after addition of $^{13}C_4$-PFOS internal standard by (repeated) ultrasonic extraction with methanol followed by ENVI-Carb sorbent clean-up as described in Roland et al. (2014). Fish and SPM data from 2007 (JDS2) were obtained from frozen samples stored at the JRC and analysed together with the samples from 2013 (JDS3).

22.2.3 On-line SPE-UHPLC-MS-MS (Umeå University)

Water samples (10 ml) were filtered using 0.45 µm syringe filters and the internal standard oxazepam d5 was added to each sample. Injection (1 ml) was based on an on-line solid phase extraction (SPE) system using two valves for column switching; 1.0 ml was injected using a 1 ml loop, onto an online extraction column (*Oasis HLB*, 20 mm × 2.1 mm, 15 µm) and then onto an analytical column (*Hypersil GOLD aQ*; 50 mm × 2.1 mm, 5 µm, *Thermo Fisher Scientific*, San Jose, CA, USA), following a corresponding guard column (20 mm × 2.1 mm, 5 µm). The total time of the on-line extraction and the LC-MS-MS analysis was 15 min.

The UHPLC was a *Surveyor/Acella* system, and the mass analyser a *TSQ Quantum Ultra EMR*, triple stage quadrupole MS/MS, and the software *Xcalibur*, all made by *Thermo Fisher Scientific* (San Jose, CA, USA).

22.2.4 Stability studies

Unfortunately, the water samples could not be extracted (and analysed) immediately after arrival at the JRC. They were extracted on average after 68 days storage time in the fridge (between 27 and 106 days; 30 samples were stored for longer than 70 days). Therefore, in order to get some insight into sample stability (storage at 4°C in aluminum containers), three exemplary parallel water samples (JDS11, JDS12, JDS16) were re-extracted and re-analysed again after 173 days from reception. This "118 day variation" (in%) between the two analyses is shown in Table 62.

Table 62: Percentage concentration decrease (or increase) between the two analyses after 55 and 173 days ("118 day variation")

Unit: (%)

	JDS11	JDS12	JDS16
1H-Benzotriazole	-26	-57	-45
Methylbenzotriazoles	210	38	118
2,4-D	-16	-30	-23
Carbamazepine	11	-21	-11
Carbamazepine-metabolite	-4	-12	-22
Cybutryne	> 75	n.d.	n.d.
DEET	-95	-95	-94
Diclofenac	-50	-87	-76
MCPA	4	-12	31
Metolachlor	-27	-64	-39
Sulfamethoxazole	-11	-46	-21
Terbutryn	-21	-27	-28
PFBS	-58	n.d.	n.d.
PFHxA	-37	-7	-41
PFHpA	> 75	n.d.	n.d.
PFOA	-32	19	-44
PFNA	-71	> 75	n.d.
PFOS	-18	1	0

These results show that most substances studied were relatively stable under the storage conditions; these substances were 1H-benzotriazole, 2,4-D, carbamazepine and its metabolite, MCPA, metolachlor, sulfamethoxazole, terbutryn, PFHxA, PFOA, and PFOS. The concentration increase for the methylbenzotriazoles cannot be explained. Substances less stable were DEET, diclofenac, and cybutryne. Perfluorinated substances are usually "persistent" compounds; therefore, the concentration decrease for PFBS, PFHpA, and PFNA is not clear.

However, when looking at the monitoring data for the individual samples and comparing samples stored for 55 and 100 days, the results are quite uniform and no big difference can be observed, with the exemption of cybutryne which shows clearly lower concentrations in the longer stored samples.

22.3 Results

22.3.1 Polar organic substances in the dissolved water phase

Table 63 gives a statistical summary of the analytical results for the polar (emerging) organic substances in the water samples (dissolved phase). In addition to the 68 official JDS samples, the 3 samples JDS51a, JDS51b (Olt River), and JDS63a were analysed.

Overall, the detection frequency for most compounds was very high (> 90%); only four substances were detected less frequently, which were diclofenac (75%; LOQ: 0.86 ng/l), cybutryne (24%; LOQ: 0.18 ng/l), PFHpA (38%; LOQ: 3.20 ng/l), and PFNA (79%; LOQ: 0.66 ng/l).

The highest concentrations were detected for the corrosion inhibitor 1-H-benzotriazole; its average level was 287 ng/l, median 260 ng/l, 90th percentile (Per 90) 462 ng/l, and its maximum concentration 1550 ng/l found in the Vah tributary (JDS18). The maximum concentration for the methylbenzotriazoles (290 ng/l) was found in the Arges tributary (JDS58). Other substances detected at elevated concentrations were carbamazepine (average 26 ng/l, median 25 ng/l, 90th percentile 36 ng/l, and max 68 ng/l (in the Arges tributary; JDS58), 10,11-dihydro-10,11-dihydroxy-carbamazepine (CBZ-metabolite) (average 53 ng/l, median 43 ng/l, 90th percentile 86 ng/l, and max 161 ng/l (in the Arges tributary; JDS58), DEET (average 13 ng/l, median 10 ng/l, 90th percentile 23 ng/l, and max 81 ng/l (in the Morava tributary; JDS12), diclofenac (average 10 ng/l, median 3.6 ng/l, 90th percentile 15 ng/l, and max 255 ng/l (in the Arges tributary; JDS58), sulfamethoxazole (average 23 ng/l, median 18 ng/l, 90th percentile 40 ng/l, and max 141 ng/l (in the Arges tributary; JDS58), PFOA (average 8.1 ng/l, median 4.9 ng/l, 90th percentile 18 ng/l, and max 36.5 ng/l (in the Danube River downstream Budapest; JDS22), and PFOS (average 7.2 ng/l, median 5.9 ng/l, 90th percentile 13 ng/l, and max 26.2 ng/l (in the Danube River in Szob before Budapest; JDS20).

Table 63: Monitoring results for polar organic emerging substances in the dissolved water phase of the Danube River and tributaries

N, number of samples = 71; unit: ng/l; the results < LOQ were replaced by zero, and in case of cybutryne and PFHpA due to the low detection frequency by LOQ/2.

	D. F. (%)	Min	Average	Max	Median	Per 90	LOQ	EQS
1H-Benzotriazole	100	1.5	287	1550	260	462	0.66	-
5-Methyl-benzotriazole	100	9.5	67	290	57	115	0.53	-
2,4-D	96	< LOQ	2.9	21.6	1.7	4.8	0.22	100
Carbamazepine	100	3.6	26	68	25	36	0.15	500
Carbamazepine-metabolite	100	13	53	161	43	86	0.30	-
Cybutryne	24	< LOQ	0.11	0.83	0.09	0.5	0.18	2.5
DEET	100	< LOQ	13	81	10	23	1.93	-
Diclofenac	75	< LOQ	10	255	3.6	15	0.86	100
MCPA	93	< LOQ	2.2	12.0	1.7	4.0	0.15	100
Metolachlor	99	< LOQ	6.3	38.7	5.4	9.0	1.73	200
Sulfamethoxazole	100	3.7	23	141	18	40	0.10	100
Terbutryn	96	< LOQ	3.1	10.6	2.9	4.4	0.64	65
PFBS	94	< LOQ	1.6	3.7	1.4	2.6	0.55	-
PFHxA	92	< LOQ	4.0	8.5	4.0	6.7	1.10	-
PFHpA	38	< LOQ	2.4	18.8	1.6	6.6	3.20	-
PFOA	100	< LOQ	8.1	36.5	4.9	18	1.07	-
PFNA	79	< LOQ	1.2	3.3	1.1	2.7	0.66	-
PFOS	94	< LOQ	7.2	26.2	5.9	13	1.09	0.65

D. F. = Detection frequency; Per 90 = 90th percentile

These results show that the highest concentrations are usually found in the tributaries of the Danube River. The Inn River, main source of PFOA in 2007, was not analysed.

The water EQS limit values were only exceeded for PFOS which has an AA-EQS of 0.65 ng/l. The blank value dependent LOQ was higher than the EQS level. The 4 samples below the LOQ were JDS15, JDS35 (Tisa River), JDS56 (R. Lom River), and JDS64 (Prut River). The (lowest German national) water AA-EQS limits for 2,4-D (0.1 µg/l), carbamazepine (0.5 µg/l), diclofenac (0.1 µg/l), MCPA (0.1 µg/l), metolachlor (0.2 µg/l), sulfamethoxazole (0.1 µg/l) and the new priority substances cybutryne (2.5 ng/l) and terbutryn (0.065 µg/l) were not exceeded on any sites. The maximum concentrations found for diclofenac (255 ng/l) and sulfamethoxazole (141 ng/l) exceeded the AA-EQS limit of 0.1 µg/l.

22.3.2 PFOS in fish liver

The analytical monitoring results for PFOS in fish liver from JDS3 and JDS2 are shown in Table 64. Four samples were analysed from JDS3 (year 2013), and three (plus 1 filet) from JDS2 (year 2007). The biota EQS limit value for PFOS of 9.1 µg/kg was exceeded in all cases, also for the filet analysed from JDS2 (26 µg/kg). Due to the low number of samples analysed, no clear temporal or local trend of PFOS contamination can be identified.

It should be noted that the EQS protection goal for PFOS is human consumption of fish; therefore, fish muscle or filet should be analysed for EQS compliance checking. However, most biota monitoring studies so far focused either on liver, as a target organ for PFOS accumulation, or on blood or whole body homogenates, respectively (Berger et al., 2009; Houde et al., 2006; 2011). Analyses of ten harbor seal organs showed that perfluorinated compounds (PFCs) tend to accumulate primarily in blood (38% of the total PFC burden) > liver (36%) > muscle (13%) > lung (8%) > kidney (2%) > blubber (2%) > heart (1%) > brain (1%) > thymus (<0.01%) and thyroid (<0.01%) (Ahrens et al., 2009).

Table 64: Monitoring results for PFOS in fish liver from 2013 and 2007

N, number of samples (2013) = 4; (2007) = 4; unit: µg/kg; LOD = 0.2 µg/kg; LOQ = 0.5 µg/kg.

	Location name	Year 2013	Year 2007
JDS2	Kelheim – gauging station (DE)	529	329
JDS20	Szob (HU)	329	
JDS27	Hercegszanto (HU)	284	26 (filet)
JDS63	Siret Tributary (RO)		864
JDS65	Reni (RO/UA)	109	
JDS68	Sf.Gheorghe – Sf.Gheorghe arm (RO)		1007

22.3.3 PFOS in SPM

The analytical monitoring results for PFOS in SPM from JDS3 and JDS2 are shown in Table 65, and their statistical summary in Table 66.

In case of SPM analyses for PFOS, the concentrations in 2013 were only slightly lower compared to 2007; the median in 2013 was 3.75 µg/kg compared to 3.99 µg/kg in 2007.

Table 65: Monitoring results for PFOS in SPM from 2013 and 2007

N, number of samples (2013) = 23; (2007) = 15; unit: µg/kg; LOD = 0.2 µg/kg; LOQ = 0.5 µg/kg.

	Location name	Year 2013	Year 2007
JDS2	Kelheim – gauging station (DE)	9.27	8.60
JDS6	Jochenstein (DE)	3.77	2.78
JDS9	Klosterneuburg (AT)	2.92	1.31
JDS13	Bratislava (SK)	2.28	9.18
JDS19	Iza/Szony (HU)	3.32	3.99
JDS20	Szob (HU)	3.11	12.98
JDS21	Budapest upstream – Megyeri Bridge (HU)	3.42	
JDS22	Budapest downstream – M0 bridge (HU)	3.99	
JDS24	Dunafoldvar (HU)	4.30	22.91
JDS27	Hercegszanto (HU)	3.22	9.60
JDS33	Downstream Novi-Sad (RS)	4.35	9.12
JDS36	Downstream Tisa / Upstream Sava (Belegis) (RS)	4.83	
JDS39	Downstream Pancevo (RS)	3.75	7.06
JDS43	Banatska Palanka / Bazias (RS/RO)	9.57	
JDS49	Pristol / Novo Selo Harbour (RO/BG)	3.26	
JDS53	Downstream Zimnicea / Svishtov (RO/BG)	9.67	
JDS55	Downstream Jantra (RO/BG)	4.77	
JDS57	Downstream Ruse / Giurgiu (RO/BG)	5.24	
JDS59	Downstream Arges, Oltenita (RO/BG)	2.02	3.45
JDS60	Chiciu / Silistra (RO/BG)	2.28	2.61
JDS62	Braila (RO)	4.81	2.47
JDS65	Reni (RO/UA)	2.32	2.07
JDS67	Sulina – Sulina arm (RO)	1.96	< LOQ

Table 66: Statistics for PFOS in SPM from 2013 and 2007

N, number of samples (2013) = 23; (2008) = 15; unit: µg/kg; LOD = 0.2 µg/kg; LOQ = 0.5 µg/kg.

	Year 2013	Year 2007
Min	1.96	0.00
Average	4.28	6.54
Max	9.67	22.9
Median	3.75	3.99
Per 90	8.46	11.6

22.3.4 Benzodiazepine anxiolytics in the water phase

Table 67 gives the summary statistics of the analytical results for the benzodiazepine anxiolytic drugs analysed in the water samples.

In the Danube River and its tributaries eight out of the 13 benzodiazepines were found (alprazolam, clobazam, diazepam, flunitrazepam, midazolam, oxazepam, prazepam, and temazepam). The two most relevant compounds were oxazepam and clobazam with detection frequencies of 85 and 31%, respectively. The maximum concentration detected for oxazepam at the location JDS10 (Wildungsmauer, Austria) was 14.9 ng/l, and for clobazam 10.9 ng/l in sample JDS2 (Kelheim – gauging station, Germany). The average, median and Per 90 concentrations for oxazepam were 5.1, 4.6, and 9.7 ng/l, respectively. Clobazam had average and Per 90 levels of 1.8, and 6.7 ng/l; its median was zero because the detection frequency was < 50% (the below-LOQ-levels were replaced by zero). Temazepam was detected in 19% of the samples, prazepam in 10%, midazolam in 9%, alprazolam in 6%, flunitrazepam in 4%, and diazepam in 3%, with maximum concentrations of 6.7 ng/l for temazepam, 4.4 ng/l for prazepam, 2.3 ng/l for midazolam, 1.6 ng/l for alprazolam, 2.0 ng/l for flunitrazepam, and 2.3 ng/l for diazepam.

Table 67: Monitoring results for benzodiazepines in the dissolved water phase of the Danube River and tributaries

N, number of samples = 68; unit: ng/l; the results < LOQ were replaced by zero.

	D. F. (%)	Min	Average	Max	Median	Per 90	LOQ
Alprazolam	6	< LOQ	0.1	1.6	0.0	0.0	1.0
Clobazam	31	< LOQ	1.8	10.9	0.0	6.7	1.0
Diazepam	3	< LOQ	0.0	2.3	0.0	0.0	0.5
Flunitrazepam	4	< LOQ	0.1	2.0	0.0	0.0	1.0
Midazolam	9	< LOQ	0.2	2.3	0.0	0.0	0.5
Oxazepam	85	< LOQ	5.1	14.9	4.6	9.7	0.5
Prazepam	10	< LOQ	0.3	4.4	0.0	0.4	1.0
Temazepam	19	< LOQ	0.6	6.7	0.0	2.8	1.0

22.3.5 Comparison of individual results for JDS3 and JDS2

The following tables show a comparison of the statistical results for individual substances for JDS3 (year 2013) and JDS2 (year 2007). In 2007, the JRC analysed 64 water samples compared to 71 samples in 2013. Most of the sampling stations were identical.

22.3.5.1 2,4-D

In 2013 the concentrations for 2,4-D were considerably lower compared to 2007 (Table 68).

Table 68: Statistical monitoring results for 2,4-D in water from 2013 and 2007

Unit: ng/l

Location name	Year 2013	Year 2007
N, number of samples	71	64
Detection frequency (%)	96	89
Minimum	< LOQ	< LOQ
Average	2.9	17
Maximum	21.6	188
Median	1.7	9
Per 90	4.8	33
LOQ	0.22	1.0

22.3.5.2 Carbamazepine

Carbamazepine concentrations were lower in 2013 (Table 69).

Table 69: Statistical monitoring results for carbamazepine in water from 2013 and 2007

Unit: ng/l

Location name	Year 2013	Year 2007
N, number of samples	71	64
Detection frequency (%)	100	100
Minimum	3.6	2.9
Average	26	58
Maximum	68	945
Median	25	37
Per 90	36	56
LOQ	0.15	1.0

22.3.5.3 Diclofenac

Diclofenac concentrations were higher in 2013 (Table 70). However, in 2007 we had problems with our diclofenac analytical standard, and therefore the reported concentrations were wrong (too low) (noted by M. Clara (UBA Austria) and see Loos et al. 2013). Note that Table 70 reports the "wrong" values from 2007 as they have been published in Loos et al. (2010).

Table 70: Statistical monitoring results for diclofenac in water from 2013 and 2007

Unit: ng/l

Location name	Year 2013	Year 2007
N, number of samples	71	64
Detection frequency (%)	75	73
Minimum	< LOQ	< LOQ
Average	13	1.7
Maximum	255	36
Median	3.6	0.8
Per 90	15	3.2
LOQ	0.86	1.0

22.3.5.4 Sulfamethoxazole

For sulfamethoxazole, the average, median, and Per 90 concentrations were similar in 2013 and 2007.

Table 71: Statistical monitoring results for sulfamethoxazole in water from 2013 and 2007

Unit: ng/l

Location name	Year 2013	Year 2007
N, number of samples	71	64
Detection frequency (%)	100	100
Minimum	3.7	3
Average	23	24
Maximum	141	204
Median	18	16
Per 90	40	44
LOQ	0.10	1.0

22.3.5.5 PFOA

The concentrations of PFOA have more or less halved since 2007 (Table 72), but it is still an important pollutant in the Danube River basin.

Table 72: Statistical monitoring results for PFOA in water from 2013 and 2007

Unit: ng/l

Location name	Year 2013	Year 2007
N, number of samples	71	64
Detection frequency (%)	100	100
Minimum	<LOQ	0.9
Average	8.1	17.5
Maximum	36.5	60
Median	4.9	14.3
Per 90	18	32.4
LOQ	1.07	1.0

22.3.5.6 PFOS

Average and median PFOS concentrations have decreased only slightly from 2007 to 2013 (Table 73), which shows the different emission pathways of PFOS and PFOA.

Table 73: Statistical monitoring results for PFOS in water from 2013 and 2007

Unit: ng/l

Location name	Year 2013	Year 2007
N, number of samples	71	64
Detection frequency (%)	94	100
Minimum	< LOQ	0.7
Average	7.2	9.7
Maximum	26.2	100
Median	5.9	7.2
Per 90	13	11.8
LOQ	1.09	1.0

22.4 Conclusions

Concentrations of the selected emerging organic micropollutants in the Danube River are low. Higher concentrations were found in some cases in the tributaries, especially in the Arges River. The water AA-EQS limit values were only exceeded for PFOS (by a factor of 10 and in 94% of the samples) in both the Danube River and its tributaries. Also the biota EQS limit value for PFOS was exceeded in fish liver and one filet analysed (by a factor of approximately 3 in the filet). The concentrations for most of the contaminants were lower in 2013 compared to 2007, which indicates a decrease of water contamination.

22.5 References

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23 Spatial and temporal trends of Dioxins, PCBs and BDE-209 in suspended particulate matter and fish – JDS3 versus JDS2

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23.1 Introduction

We report here on the occurrence of the 17 toxic congeners of 2,3,7,8 chlorinated polychlorinated dibenzo –p- dioxins and dibenzofurans (PCDD/Fs), the sum of the six *marker* or *indicator* polychlorinated biphenyl congeners IUPAC# 28, 52, 101, 138, 153 and 180 (EC-6 PCBs) the 12 *dioxin-like* PCB congeners IUPAC# 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189 (DL-PCBs, "WHO-PCB") and decabromodiphenylether (BDE-209) in selected samples of suspended particulate matter and fish (*Abramis brama*) obtained from the second (JDS2, summer 2007) and third Joint Danube surveys (JDS3, summer 2013) from Germany to the Black Sea.

All investigated compounds fall into the category of semivolatile organic compounds (SOCs). SOCs got high octanol/water partition coefficients (Kow) and low vapour pressures. Due to lipophilicity, persistence and low volatility, PCDD/Fs and PCBs accumulate in sediments and biota of aquatic systems. The transport of SOCs with log Kow >6 within the water column is mainly associated with the hydraulic remobilization of sediments and the subsequent transport and re-sedimentation of SPM.

While BDE 209 is usually not found to considerable amounts detected in aquatic biota, PCDD/F and PCBs instead, due to their higher resistance to metabolism, are ubiquitously found in fish. Although production and emissions are strictly regulated in the EU, there is still a notable contamination of PCDD/F and in particular of DL-PCB in fish samples, often above the limits for food given by EU legislation, especially for the big rivers Rhine and Elbe and their tributaries Saar and Saale (Stachel et al. 2007, Neugebauer et al. 2012).

Deca-BDE has long been erroneously characterized as an environmentally stable and inert product that was stable in the environment, not toxic, and therefore of no concern (Alcock and Busby, 2006). Meanwhile it has been demonstrated, that BDE-209 present in sediments and SPM enters the aquatic food web, and, being rapidly metabolized in fish, contributing to the load of lower brominated (toxic) PBDEs (Vigano et al. 2012).

While PCDD/F were never produced (they are unintentional by-products of poor combustion and a variety of chemical processes), PCBs and PBDEs such as BDE-209 are intentionally produced chemicals with a broad spectrum of industrial and domestic applications such as dielectric fluids, paints, hydraulic oils, plasticisers, flame retardants etc. (De Wit 2000, OECD 1994). In contrast to PCDD/F and PCBs, PBDEs still display rising trends in some environmental compartments including human tissue. Legislation to ban decaBDE is in place. The European Union's Restriction of Hazardous Substances Directive (RoHS) has prohibited the use of DecaBDE in electronics and electrical equipment since July 2006. In 2009, the US EPA launched a 'decaBDE Phase-Out Initiative' to eliminate the production, importation and sale of decaBDE by 2013.

The toxic effects of PCDD/Fs and PCBs include dermal toxicity, immune toxicity, carcinogenicity, and adverse effects on reproduction, development, and endocrine functions. Although the toxic properties of PBDEs are not entirely evaluated, their structural similarity to PCDD/Fs and PCBs suggests similar toxicological endpoints.

Due to their similar behaviour and toxicological endpoints, PCDD/Fs and DL-PCBs are often evaluated and reported together. Both compound classes are included in a toxicity evaluation scheme that sums up the toxicity of the individual congeners of both classes (17 PCDD/Fs and 12 DL-PCBs)

expressed as a concentration of toxicity equivalents (TEQs) of the 2,3,7,8-Tetrachloro dibenzo-p-dioxin (TCDD). The toxicity (Toxicity Equivalency Factors, TEFs) of the individual congeners may vary by orders of magnitude. An early classification limited to the 17 PCDD/Fs is the I-TEQ scheme (reported by Van den Berg et al., 1998). It has been updated by the WHO in 1998 and 2005 by two schemes including also the 12 DL-PCBs (Van den Berg et al., 1998, 2006). In existing quality standards both the 1998 and 2005 WHO-TEQ is used, but also the old I-TEQ schemes can be found.

Due to the risk for wildlife and humans arising from PCDD/Fs in sediments a "safe sediment value" of 20 pg I-TEQ/g d.w. was proposed (Evers et al. 1996). For the EC-6 PCBs German quality standards of 20 ng/g exist for sediment/suspended solids for each individual PCB (ARGE Elbe 2010).

What regards human risk through the aquatic foodchain, the relevant EU food limit values are 3.5 pg WHO_{05}-TEQ/g for PCDD/F only and of 6.5 pg WHO_{05}-TEQ/g for combined DL-PCB and PCDD/F toxicity equivalents, both on a fresh weight base. The food limit for combined DL-PCB and PCDD/F toxicity is identical with the EQS recently set for biota in EU surface waters (COM Dir 2013). The limit for the sum of the EC-6 PCBs in freshwater fish is 75 ng/g fresh weight base (COM Reg 2011).

23.2 Methods

23.2.1 Experimental approach

The objective was to investigate the spatial and temporal trends of PCDD/Fs, PCBs and BDE-209 in SPM and fish (*Abramis brama*).

Samples/results were obtained as far as possible from those sites where the JDS3 exercise provided a spatial overlap with the '23 JRC supersites' investigated during JDS2. The data for SPM presented for the comparison with 2007 (JDS2) were generated in 2008 at the JRC and reported in the JDS2 final report. Fish data presented from 2007 (JDS2) were obtained from frozen fish samples stored at the JRC and analysed together with the samples from 2013 (JDS3).

We decided that during JDS3 only SPM is sampled, since it appeared during JDS2, that the SPM associated portion of the investigated compounds sufficiently represents the total amount in water. Since BDE-209 dominated by far the PBDEs detected in SPM during JDS2, the current study is limited to BDE-209.

The bream was selected for this study, since it is a common and wide-spread species at higher trophic level, which allows conclusions on the status of the aquatic environment and links to food and the related legislation (Klein et al. 2010).

SPM was sampled on board of the Argus using a continuous centrifuge approach. Details on sampling locations, conditions, equipment and sample treatment can be obtained from Chapter 2 and the overview map in the JDS3 final report.

23.2.2 Analyses

The freeze-dried solid samples were extracted with a mixture of n-hexane/acetone (220/30 for SPM and 1/1 for fish tissue) by Soxhlet for 48 h after spiking with isotope-labelled surrogate standards. For SPM copper powder was added to the solvent during the extraction to remove Sulphur.

After treatment of the raw extract with conc. H_2SO_4 extract purification was executed with an automated clean-up system (Power-Prep P6, Fluid Management Systems (FMS) Inc., Watertown, MA, USA). This system was previously described (Abad et al. 2000) and uses a multi-layer silica column (acid/neutral), basic alumina and carbon column combination. Two fractions were collected, one containing Mono-ortho PCBs, Indicator-PCBs and PBDEs and one for non-ortho PCBs and PCDD/Fs.

The instrumental analysis of PCDD/Fs, PCBs and PBDEs was based on isotope dilution using HRGC-HRMS (high resolution gas chromatography – high resolution mass spectrometry) for quantification on the basis of EPA1613, EPA 1668 and EPA 1614 methods.

Sediment reference materials were analyzed in parallel with SPM samples for PCDD/Fs, DL-PCBs. The concentrations detected were in accordance with the reference values.

Levels of analytical blanks obtained during the clean-up process were at least 5-10 times lower of the reported concentrations for all compounds studied. The blank level was not subtracted. The reported detection limits were calculated on the bases of a signal to noise ratio of 3/1.

A detailed description of the methodology, including the results for all compounds and each individual site is provided in the full report on CD.

23.3 Results

DL-PCBs and PCDD/F concentrations are reported in toxicity equivalents (TEQ) using the WHO toxicity equivalency factors (TEFs) established in 2005.

23.3.1 PCDD/Fs, PCBs and BDE-209 in SPM compared to 2007(JDS2)

Concentrations/TEQs in SPM are reported on a dry weight base.

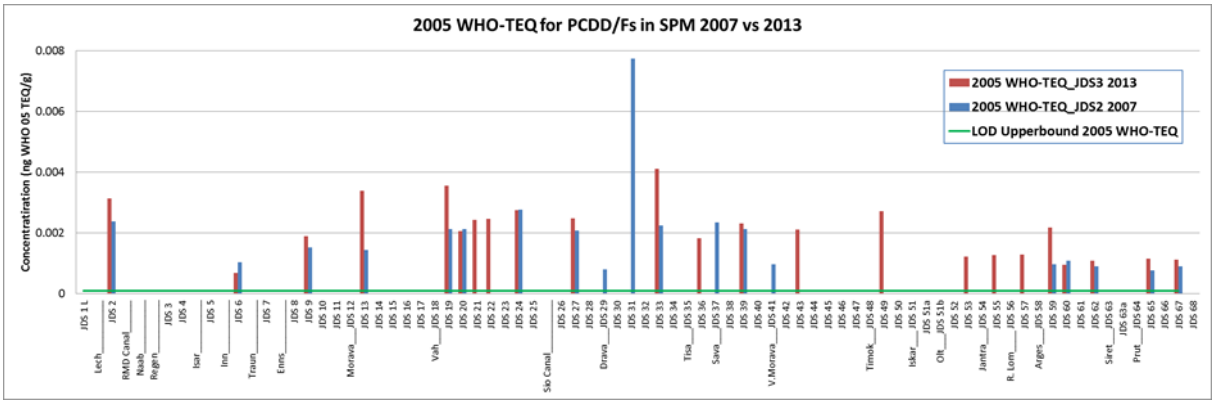

Figure 120: PCDD/Fs in SPM, 2013 versus 2007

Table 74: PCDD/Fs – SPM summary

PCDD/F (ng WHO_{2005} TEQ/g)	JDS2 2007	JDS3 2013	Observation
N	19	23	The 2013 downstream profile in Figure 120 shows that an equilibrated spatial pattern of the PCDD/Fs within a concentration range between 0.00069 and 0.0041 ng WHO_{05}-TEQ/g (JDS33) and an average of 0.0021 ng WHO_{05}-TEQ/g. Almost identical concentrations were observed in the 2007 survey with an average of 0.0019 ng WHO05-TEQ/g and a range between 0.00077 – 0.0077 ng WHO_{05}-TEQ/g. Also the spatial pattern with slightly higher concentrations in the upper/middle stretch results similar from both surveys. The safe sediment value" PCDD/F of 0.020 ng I-TEQ/g is not exceeded.
min	0.00077	0.00069	
mean	0.0019	0.0021	
max	0.0077	0.0041	
C50	0.0015	0.0021	
C90	0.0028	0.0035	

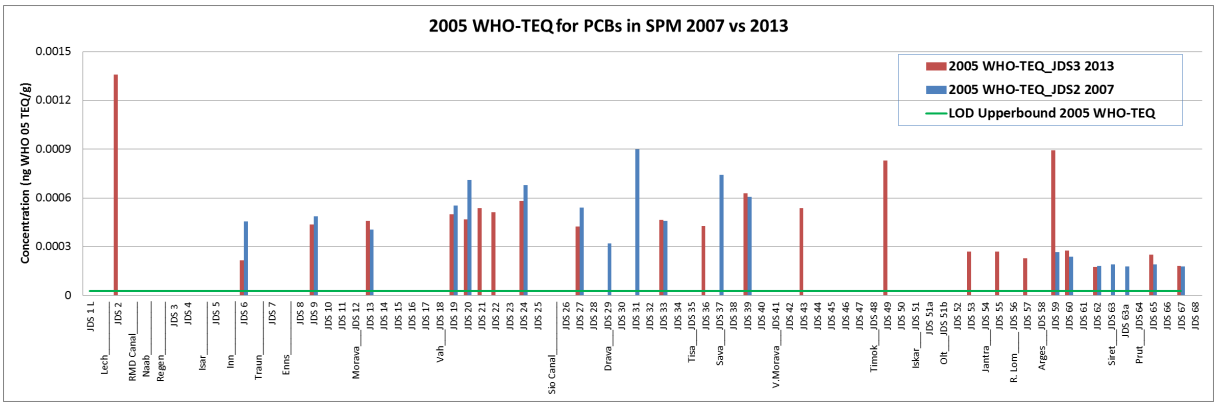

Figure 121: Dioxin-like PCBs in SPM, 2013 versus 2007

Table 75: Dioxin-like PCBs – SPM summary

Dioxin-like PCB (ng WHO_{2005} TEQ/g)	JDS2 2007	JDS3 2013	Observations
N	19	23	Dioxin-like PCBs display a similar spatial pattern as seen for PCDD/Fs and at concentration ranges of around 25% of those of the PCDD/Fs on a TEQ basis, which is a typical observation in soils and sediments.
min	0.00018	0.00018	
mean	0.00044	0.00048	The 2013 downstream profile in Figure 121 shows that an equilibrated spatial pattern of the DL-PCBs within a concentration range between 0.00018 and 0.0012 ng WHO_{05}-TEQ/g (JDS2) and an average of 0.00048 ng WHO_{05}-TEQ/g. Almost identical concentrations were observed in the 2007 survey with an average of 0.00044 ng WHO_{05}-TEQ/g and a range between 0.00018 – 0.00090 ng WHO_{05}-TEQ/g. As for the PCDD/Fs above (Figure 120), the spatial pattern with slightly higher concentrations in the upper/middle stretch results similar from both surveys.
max	0.00090	0.0014	
C50	0.00045	0.00046	
C90	0.00074	0.00087	

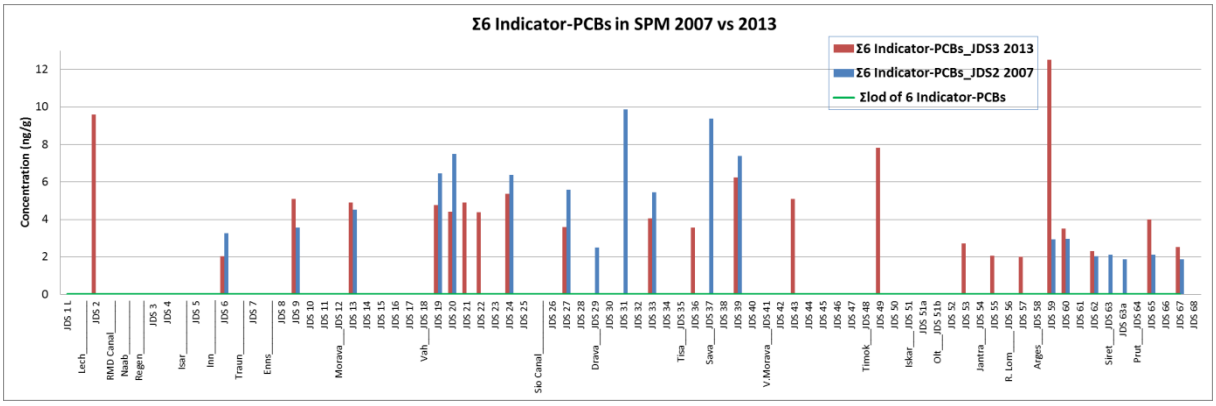

Figure 122: Indicator PCBs in SPM, 2013 versus 2007

During the 2007 survey, the sum of the EC-6 PCBs was equally distributed between the dissolved phase and the SPM. This needs to be considered if attempting to estimate total water concentrations from the SPM associated concentrations provided during JDS3.

Table 76: Indicator PCBs – SPM summary

Σ6 Indicator-PCBs (ng/g)	JDS2 2007	JDS3 2013	Observations
N	19	23	The 2013 downstream profile in Figure 122 displays an equilibrated spatial pattern of the EC-6 PCBs within a concentration range between 2 – 12.5 ng/g (max at JDS59, under the influence of River Arges) and an average of 4.67 ng/g. Overall lower concentrations were observed in the middle stretch compared to 2007, while the upper and lower stretches display minor variations. The 2013 maximum concentration value at JDS59 is 3 times higher though compared to 2007. The mean value and the range are almost identical with that of 2007. The German quality standard for each indivifdual of the EC-6 PCBs of 20 ng/g in sediment/suspended solids is not exceeded.
min	1.88	2.00	
mean	4.62	4.67	
max	9.87	12.50	
C50	3.55	4.39	
C90	9.37	8.88	

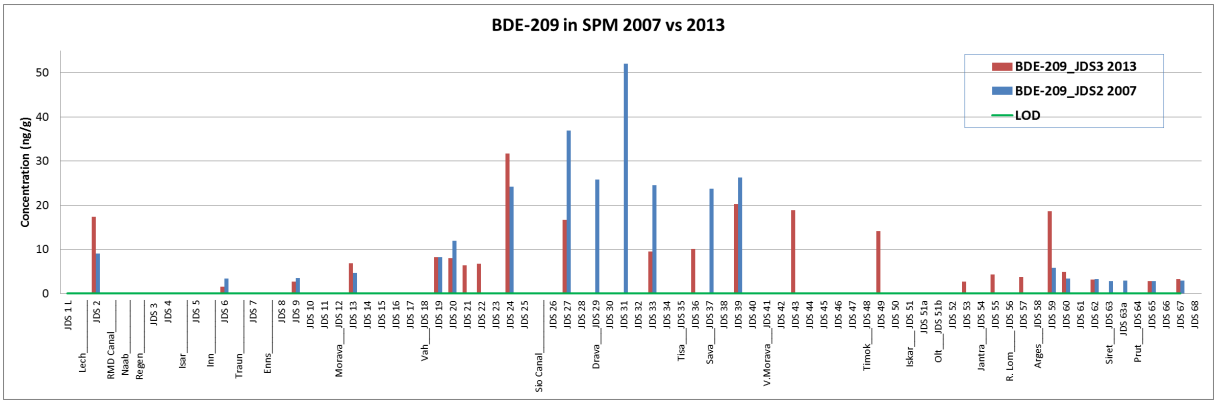

Figure 123: BDE-209 in SPM, 2013 versus 2007

During JDS2 BDE-209 represented typically around 90% of the total content of PBDEs in SPM, all of them analysed in 2007. Moreover, since BDE-209 in the water column was to more than 99% associated with SPM, the total water concentration of BDE-209 in JDS3 exercise can be calculated by using the SPM contents recorded at the individual sampling sites.

Table 77: BDE-209 – SPM summary

BDE-209 (ng/g)	JDS2 2007	JDS3 2013	Observations
N	20	23	The downstream profile in Figure 123 shows that the spatial pattern, with a tendency of higher concentrations in the middle stretch seen during 2007, is observed similarly in 2013.The 2007 maximum seems to have shifted more downstream in 2013. This can occurr when local emissions decrease. In the absence of fresh inputs, the contaminated sediments are bit by bit remobilized by extreme events, and deposited more downstream.The fact that the maximum in the 2013 SPM has shifted downstream since then, suggests a decrease of inputs from sources and tributaries in the middle stretch, and a tendency of PBDEs being cleaned out of the catchment. Also the temporal trend suggests a moderate (approx. 30%) decrease of BDE-209 since 2007. Average concentrations decreased from around 14 – 10 ng/g, together with a decrease of the concentration ranges from 2.84-52.1 ng/g in 2007 to 1.53-31.7 ng/g in 2013. Finally, the decreasing concentration data in data fish point towards the same direction (Table 82, Figure 127).
min	2.84	1.53	
mean	13.93	9.69	
max	52.1	31.7	
C50	7.02	6.88	
C90	35.9	19.7	

23.3.2 SPM – Comparison with other surface waters in Europe

Data on SPM are scarcely available, and our comparison is mainly limited to data from the River Elbe acquired by the authors in an extensive campaign during 2008 (Umlauf et al. 2010, 2011). In Table 78 existing data for SPM are summarized in comparison with the outcomes of JDS2 and JDS3.

PCDD/Fs concentration in settling material from the Danube was approximately one order of magnitude lower than in the River Elbe in 2008, where an average of 0.020 (0.0039-0.068) ng WHO_{05}-TEQ/g, is reported.

DL-PCB concentration in settling material from the Danube was approximately half an order of magnitude lower compared to the River Elbe in 2008, where an average of 0.0029 (0.00098-0.0058) ng WHO_{05}-TEQ/g is reported

6 Marker PCBs in Danube SPM generally range more than one order of magnitude below the concentrations reported from the Elbe River. ARGE Elbe (2010) reports yearly averages for the sum of PCB 138, 156 and 180 of 30-132 ng/g. Umlauf et al. (2010, 2012) report a concentration average of the EC-6 PCBs of 71 ng/g (11.5-180 ng/g) for the entire river from the Czech Republic until Hamburg.

Few data on BDE-209 are available for SPM in Dutch rivers. De Boer et al. (2003) report a median of 71 ng/g at a range between <9 – 4600 ng/g, considerably higher than observed during JDS2 and JDS3.

Table 78: PCDD/Fs, PCBs, and BDE-209 in SPM – JDS3 in comparison with literature

PCDD/Fs, Dioxin –like PCBs, Marker PCBs and BDE-209 in SPM, JDS3 comparison with literature data						
Unit	pg WHO_{05} TEQ/g	pg WHO_{05} TEQ/g	ng/g	ng/g	Reference	Comment
Compound	**PCDD/Fs**	**DL-PCBs**	**EC-6 PCBs**	**BE 209**		
Danube incl Drava & Sava	0.69-4.1; **2.1**	0.18-1.36; **0.48**	2.0-12.5; **4.7**	1.5-32; **9.7**	**This study**	**2013** JDS3; Min-max; **average**
	0.77-7.7; **1.9**	0.18-0.90; **0.44**	1.9- 9.9; **4.6**	2.8-52; **14**	Umlauf et al., 2007, 2008, 2009,	2007 JDS2; Min-max; **average**
Elbe	3.9-67.8; **20**	0.98-5.8; **2.9**	11.5-180; **71.0**		Umlauf et al. 2010, 2011	2008, Min-max; **average**
			30- 132**		ARGE Elbe 2010	2008 **annual average (sum PCB 138, 153, 180)
	7-150				Stachel et al. 2004	2002
Dutch rivers				71 (<9–4600)	De Boer et al. 2003	Median(range)

23.3.3 PCDD/Fs, PCBs and BDE-209 in fish compared to 2007(JDS2)

We report on bream filet on a wet weight basis. This way the EU food limits for PCDD/Fs and dioxin like PCBs can be compared. Dry weight based data can be approximated by assuming 25% dry mass.

Due to the low numbers of samples obtained during both surveys, the data are indicative rather than being interpreted as spatially or temporarily representative. With this respect, it should also be noted, that the 2007 data cover only 2 sites in the upper and one site in the middle stretch.

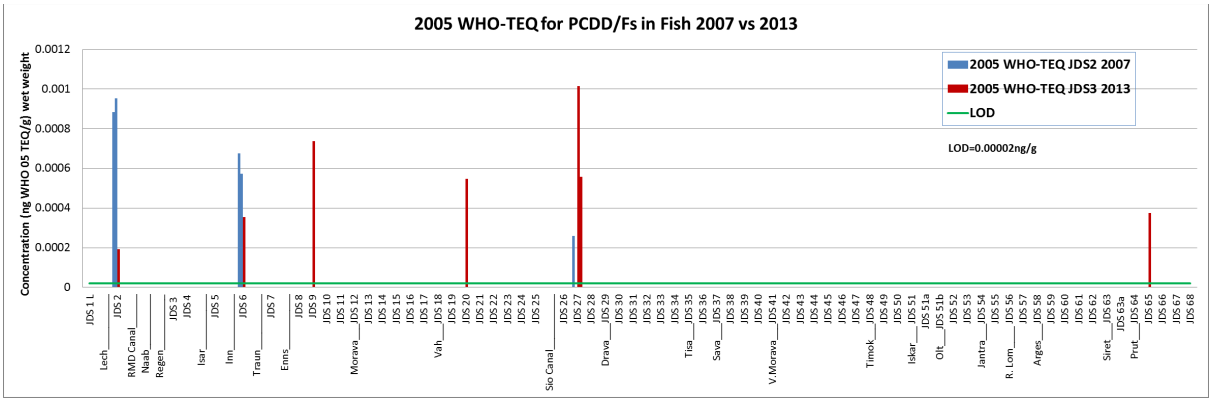

Figure 124: PCDD/Fs in Fish, 2013 versus 2007

Table 79: PCDD/Fs – Fish summary

PCDD/F (ng WHO_{2005} TEQ/g) wet weight	JDS2 2007	JDS3 2013	Observations
n	5	7	The average value during JDS3 of 0.00054 ng WHO05-TEQ/g was slightly (approx. 20%) lower compared to JDS2 with 0.00067 ng WHO05-TEQ/g. Maximum value during JDS3 was at JDS27 with 0.001 ng WHO05-TEQ/g, while the maximum in 2007 was 0,0095 ng WHO05-TEQ/g at site JDS2. The relevant EU food limit value for PCDD/F alone of 0.0035 ng WHO05-TEQ/g in fresh weight (COM Reg 2011) is not exceeded, both in the 2007 and the 2013 samples
min	0.00026	0.00019	
mean	0.00067	0.00054	
max	0.00095	0.0010	

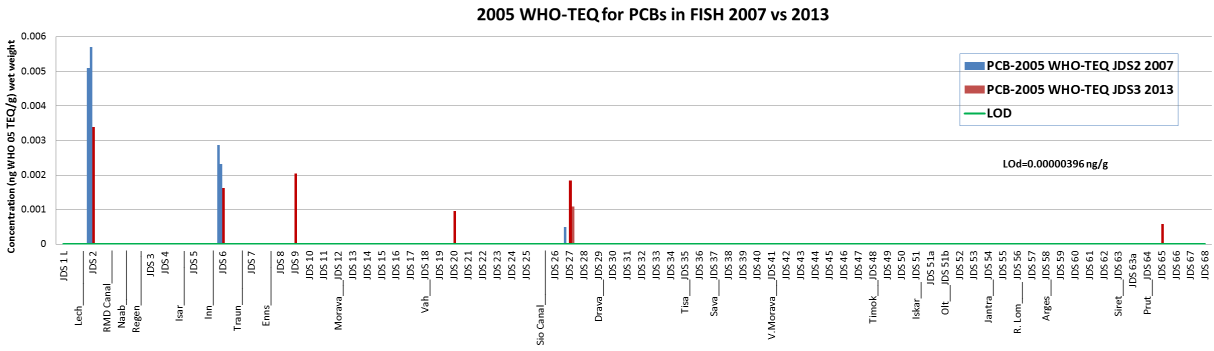

Figure 125: Dioxin-like PCB in Fish, 2013 versus 2007

Table 80: Dioxin-like PCBs – Fish summary

dioxin-like PCB (ng WHO_{2005} TEQ/g) wet weight	JDS2 2007	JDS3 2013	Observations
n	5	7	The average value during JDS3 of 0.016 ng WHO05-TEQ/g was almost 50% lower compared to JDS2 with 0.0033 ng WHO_{05}-TEQ/g. Maximum value during JDS3 was 0.0034 ng WHO_{05}-TEQ/g at site JDS2, which displays also the 2007 maximum of 0.0057 ng WHO_{05}-TEQ/g.
min	0.0006	0.0005	
mean	0.0033	0.0016	
max	0.0057	0.0034	

The toxicity of the dioxins and PCBs in bream is dominated by the PCBs, both in 2007 and 2013.

EU legislation provides a combined limit for dioxin-like PCBs and PCDD/Fs of 0.0065 ng WHO_{05}-TEQ /g of on a fresh weight basis for food, which corresponds to the EQS for surface water biota.

The limit was not exceeded in any sample. One site close to the limit is JDS2 sampled in 2007. However, in 2007 the limit for combined PCDD/F and PCB toxicity in fish was 0.0080 ng/g WHO_{98}-TEQ (COM Reg 2006), which was not exceeded either during that time.

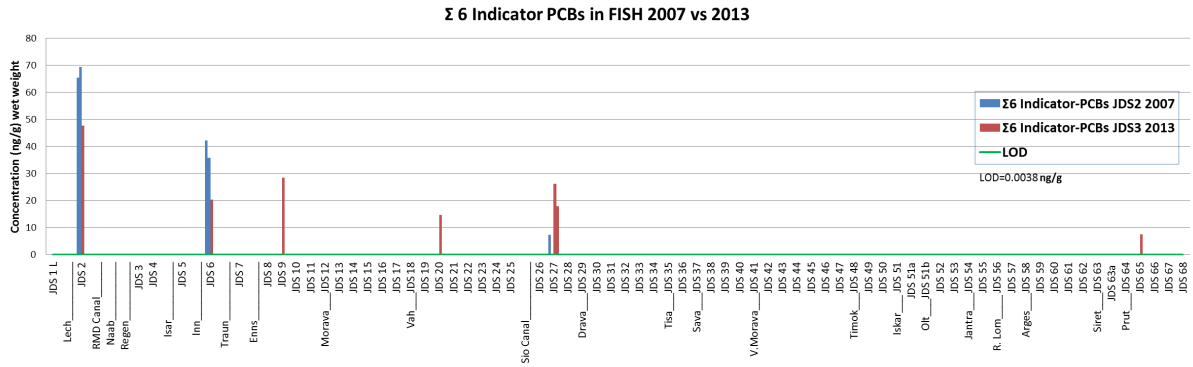

Figure 126: Indicator PCBs in Fish, 2013 versus 2007

Table 81: Indicator PCBs – Fish summary

Σ6 Indicator PCBs (ng/g) wet weight	JDS2 2007	JDS3 2013	Comments
n	5	7	The average value during JDS3 of 23,2 ng/g was almost 50% lower compared to JDS2 with 44 ng/g. The maximum concentration during JDS3 was 47.6 ng/g at site JDS2, which displayed also the 2007 maximum with 69.4 ng/g. The EU food standard of 75 ng/g fresh weight for the Σ6 Indicator PCBs (COM Reg 2011) is not exceeded both in the 2007 and the 2013 samples.
min	7.3	7.5	
mean	44	23.2	
max	69.4	47.6	

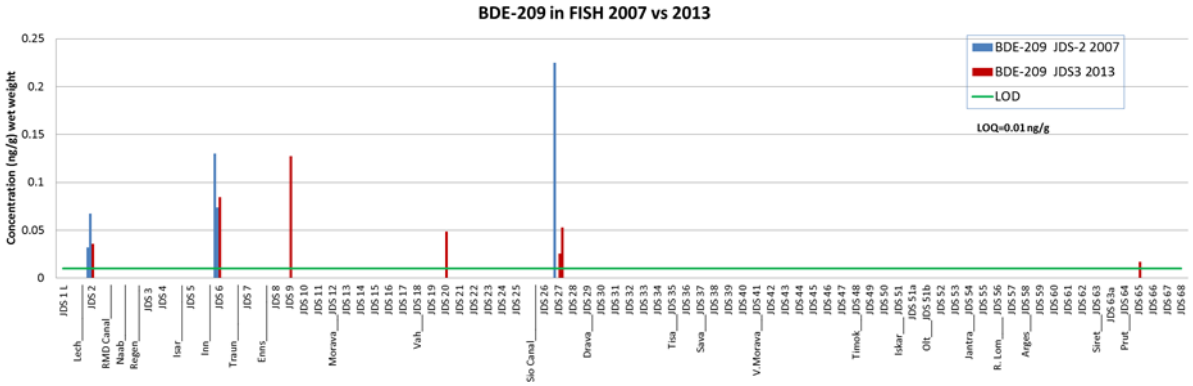

Figure 127: BDE-209 in Fish, 2013 versus 2007

Table 82: BDE-209 – Fish summary

BDE-209 (ng/g) wet weight	JDS2 2007	JDS3 2013	observations
n	5	7	The average value during JDS3 of 0.056 ng/g was about 50% lower compared to JDS2 with 0.106 ng/g. Maximum concentration during JDS3 was 0.127 ng/g at site JDS9, while the 2007 maximum was higher with 0.225 ng/g at site JDS27.
min	0.032	0.017	
mean	0.106	0.056	
max	0.225	0.127	

23.3.4 Fish – Comparison with other surface waters in Europe

In Table 83 existing data for fish are summarized together with the outcomes of JDS2 and JDS3.

The comparison of PCDD/Fs and DL-PCBs during JDS2 and JDS3 with earlier data (2003-2008) from Neugebauer et al. (2012) support the decreasing concentration trends observed in the Danube between JDS2 and JDS3.

The predominance of the PCBs in the total dioxin-like toxicity observed in the Danube is reported similarly for the other rivers, except for the River Mulde (Neugebauer et al., 2012), which is impacted by a particular PCDD/F emission source (Umlauf et al., 2005). The difference to SPM results from the

poor bioavailability of the higher molecular PCDD/F with higher log Kow when compared to PCBs. For humans (Moser and McLachlan, 2002) and chicken (Pirard and De Pauw, 2005) it has been demonstrated that for compounds with a log Kow<7 the absorption percentage decreases drastically.

The concentrations of PCDD/Fs and DL-PCBs found during JDS3 generally fit into the low end of the ranges reported by Neugebauer et al. (2012) for the Rivers Elbe, Rhine, and their tributaries.

For the EC-6 PCBs no data for bream on a wet weight basis were found. However, with the DL-PCBs low, the marker PCBs are supposed to follow this trend.

Data from BDE-209 are very scarce and reported either on a lipid weight or a dry weight base. Assuming a dry weight/fresh weight relation of 1:4, the LODs reported by De Boer et al. (2003), suggests that no BDE-209 was found in bream above 1.25 ng/g on a fresh weight base in Dutch waters. Lepom et al. (2002) report a median BDE-209 concentration of 0.97ng/g lipid weight based from the River Elbe. The calculation of our Danube results for BDE-209 on a lipid weight base reveals comparable concentrations[6]: The BDE-209 average concentration in 2013 is 1.1 (0.11-6.1) ng/g lw and that of 2007 is 1.32 (0.22-4.52) ng/g lw. Both data sets contain each one outlier, caused by the fact that the respective bream contained almost no fat. Eliminating these outliers the Danube, the averages are 0.27 ng/g lw in 2013 and 0.52 in 2007. The similarity of the BDE-209 concentrations in comparison with the River Elbe is interesting, since PCDD/Fs and PCBs were much higher concentrated there. This could point to a comparatively high relevance of the brominated flame retardants in the Danube.

Table 83: PCDD/Fs, PCBs, and BDE-209 in fish – JDS3 in comparison with literature

PCDD/Fs, Dioxin –like PCBs, Marker PCBs and BDE-209 in fish muscle tissue , JDS3 comparison with literature data Fish

Unit	pg WHO_{05} TEQ/g	pg WHO_{05} TEQ/g	ng/g	ng/g	Reference	Comment
Compound	**PCDD/Fs**	**DL-PCBs**	**EC-6 PCBs**	**BDE-209**		
Danube incl Drava & Sava	0.19-1.0; **0.54**	0.50-3.4; **1.6**	7.5-48; **23**	0.017-0.13; **0.056**	**This study**	2013 JDS3;
	0.26-0.95; **0.67**	0.60-5.7; **3.3**	7.3-69**; 44**	0.032-0.23; **0.11**		2007 JDS2; Min-max; **av.**
Danube	1-3.5	2.5 – 10			Neugebauer et al. 2012	Bream, German stretch. Ulm, Kehlheim, Jochenstein 2003–2008. WHO_{98} TEQ
North Atlantic –				0.04 – 2.8	Paepke,Herrmann, 2004	German fish market mix; ; lipid weight
River Vero				86	Eljarrat et al. 2007	2004; lipid weight
				195		2005 Barbel ; lipid weight
Elbe	0.8-8.5	2-5			Neugebauer et al. 2012	Bream 2003 – 2008, Prossen, Barby, Blankenese. WHO_{98} TEQ
Elbe				<LOQ – 37.3 ng/g Med = 0.97ng/g	Lepom et al. 2002	Bream; lipid weight
	0.48–12	1.2–14			Stachel et al. 2007	1989- 2003 – Bream, some Chub and ide: WHO_{98} TEQ
Elbe tributary Mulde	1.8-2.3	0.4-1.8			Neugebauer et al. 2012	Bream 2003 – 2008, Prossen, Barby, Blankenese; WHO_{98} TEQ
Elbe tributary Saale	1.0-2.1	4-6			Neugebauer et al. 2012	Bream 2003 – 2008, Prossen, Barby, Blankenese; WHO_{98} TEQ;
Rhine	1-9	3-16			Neugebauer et al. 2012	Bream , German stretch 2003 – 2008. WHO_{98} TEQ
Saar	1.5-3	7-20			Neugebauer et al. 2012	Bream , German stretch 2003 – 2008.; WHO_{98} TEQ
Dutch rivers				<5 (<0.2–<21)	De Boer et al. 2003	Bream, Median(range), nothing detected. Dry weight.

Note: the concentrations found for the comparison of bream based on fresh weight/wet weight (ww) are highlighted in blue

[6] the lipid content except of the outliers was between 2.5 and 6.4% of dry matter (See full report on CD in Supplement 7)

23.4 Conclusions

For the investigated compounds in SPM the spatial patterns for PCDD/F and PCBs are similar in 2007 and 2013, while for BDE-209 the concentration maximum from 2007 shifted from the middle stretch more downstream. From the downstream concentration profile, there is no indication of relevant point sources. Concentrations in SPM are tendencially stable since 2007 except for BDE-209, displaying a 30% decrease in concentration. The observed concentrations in SPM ranged between half- and more than one order of magnitude lower compared to the River Elbe.

Concentrations in fish show a decreasing trend since 2007, PCDD/Fs decreased about 20%, PCBs, both dioxin-like and the sum of 6 marker PCBs and BDE-209 by approximately 50%. The concentrations of PCDD/Fs and DL-PCBs found during JDS3 generally fit into the low end of the ranges reported for the Rivers Elbe, Rhine, and their tributaries. For the EC-6 PCBs no data for bream on a wet weight basis were found. However, with the DL-PCBs low, the marker PCBs are supposed to follow this trend. The few BDE-209 data available suggest that the concentrations in Danube bream are similar to the River Elbe. Since most other organic pollutants appear up to one order of magnitude lower in the Danube-Elbe comparison, this could be an indication for a higher relative relevance of the brominated flame retardants in the Danube.

For PCDD/F and PCBs none of the existing EQS values for aquatic biota and suspended solids/sediments, and none of the EU food limits concerned were exceeded.

23.5 References

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24 Organophosphorus compounds (OPCs) in surface waters of the Danube and selected tributaries

Gunther Umlauf, Gert Suurkuusk, Giulio Mariani, Simona Tavazzi, Bruno Paracchini

24.1 Introduction

In this study we report on the occurrence of chlorinated and non-chlorinated organophosphorus compounds (OPCs) in selected water samples obtained from third Joint Danube Survey (JDS3, summer 2013) from Germany to the Black Sea.

Flame retardants (FRs) are used in a variety of products, such as electronic equipment, plastics products, rubbers, textiles and building materials (EFRA 2014).

Since the many brominated flame retardant (BFRs) were banned in the recent years, an increase in the usage of chlorinated and non-chlorinated OPCs, as a substitute for PBDEs is observed.

Due to their widespread usage, OPCs have already been detected in several environmental matrices (Van der Veen and de Boer 2012).

The persistence of OCPs together with their toxic properties suggests adverse health effects on man. Some OPCs such as triphenyl phosphate (TPhP), tri-n-butyl phosphate (T*n*BP) and tritolyl phosphate (o-, m-, p-Tris (methylphenyl) phosphate TMPP) are supposed to be neurotoxic, and chlorinated OPCs such as (TCEP) and tris(2-butoxyethyl) phosphate (TBEP) act carcinogenic (Van der Veen and De Boer 2012; WHO1990, 1991a, 1991b, 1998, 2000). Meeker and Stapleton (2010) report associations between levels of TPhP and Tris(1,3-dichloro-2-propyl)phosphate (TDCPP) in house dust and reduced semen quality in men, suggesting endocrine disruption.

Although the focus on OPCs is mainly on human exposure in indoor environments (Marklund et al. 2003, Saito et al. 2007), their presence and fate in aquatic environments and their foodwebs have gained rising scientific attention.

OPCs have been found ubiquitarily distributed in effluents from sewage treatment plants (STPs) in concentrations ranging from ng/l up to several µg/l. Especially the chlorinated OPCs tend to pass through the STPs without being removed, while alkyl-OPCs, are more successfully retained (Marklund et al., 2005). Consequently OPCs are observed in freshwaters (Sundkvist et al. 2010, Yan et al. 2012, Cristale et al. 2013), to some extent in groundwater (Fries and Püttmann, 2003) and in marine environments (Sundkvist et al. 2010). Investigations on the removal of OPCs within a waterworks facility revealed the presence of chlorinated OPCs such as Tris (1-chloro-2-propyl) phosphate (TCPP) and Tris(2-chloroethyl) phosphate (TCEP) also in the state of the art treated drinking water (Stackelberg et al. 2004, Cristale et al. 2012).

Apart from the major input into aquatic systems via municipal and industrial waste water discharge as suggested by Fries and Püttmann (2003), the findings from Bacaloni et al. (2008), who detected OPCs in volcanic lakes without direct urban impacts, suggest also long range atmospheric transport. This is supported by Moeller et al. (2011), who detected OPCs in the in oceanic and arctic air masses, with highest concentrations observed in continental air masses.

Some OPCs are bioaccumulative and can be found in freshwater and marine biota (Sundkvist et al., 2010) as well as in breast milk from remote locations (Sundkvist et al. 2010, Kim et al. 2014)

Aquatic toxicity to fish, daphnia and algae in the mid to low mg/L concentration ranges is reported for OPCs (Verbrueggen et al. 2005). However, compared to the PBDEs they are replacing, acute aquatic

toxicities of OPCs range generally between 2-4 orders of magnitude lower (Cristale et al. 2013). So far TCEP, TCPP, and TDCPP are registered in the European Commission priority lists (Reemtsma et al. 2008, CommissionRegulation (EC) No 2268/95, Commission Regulation (EC) No 2364/2000).

24.2 Methods

24.2.1 Experimental approach

The sampling plan should provide an overview on current spatial distribution and inputs of OPCs through analyses of dissolved phase water samples in the Danube and in selected tributaries. Table 84 gives an overview on the compounds investigated.

Sampling was done on board of the Argus using 1l aluminium bottles. The locations and details of the sampling can be obtained from Chapter 2.

Table 84: Investigated compounds

Analyte Abbreviation	Analyte Common Name	CAS nr	Analyte CA Name
T*n*PP	Tri-n-propyl phosphate	513-08-6	Phosphoric acid, tripropyl ester
T*i*BP	Tris(isobutyl) phosphate	126-71-6	Phosphoric acid, tris(2-methylpropyl) ester
T*n*BP	Tris(butyl) phosphate	126-73-8	Phosphoric acid, tributyl ester
TCPP #	Tris(1-chloro-2-propyl) phosphate	13674-84-5	2-Propanol, 1-chloro-,2,2´,2´´-phosphate
TEHP	Tris(2-ethylhexyl)phosphate	78-42-2	Phosphoric acid, tris(2-ethylhexyl) ester
TCEP	Tris(2-chloroethyl) phosphate	115-96-8	Ethanol, 2-chloro-, phosphate (3:1)
TBEP	Tris(2-butoxyethyl) phosphate	78-51-3	Ethanol, 2-butoxy-, 1,1´,1´´-phosphate
TDCPP$	Tris(1,3-dichloropropyl) phosphate Tris(2,3-dichloropropyl) phosphate	13674-87-8 78-43-3	1-Propanol, 2,3-dichloro-, 1,1´,1´´-phosphate
TPhP	Triphenyl phosphate	115-86-6	Phosphoric acid, triphenyl ester
EHDP	2-ethylhexyl diphenyl phosphate	1241-94-7	phosphoric acid, 2-ethylhexyl diphenyl ester
TMPP&	Tris(methylphenyl) phosphate, Tritolyl phosphate	1330-78-5	Phosphoric acid, tris(methylphenyl) ester
T35DMPP	Tris(3,5-dimethylphenyl) phosphate	25653-16-1	Phosphoric acid, tris(3,5-dimethylphenyl) ester
T2*i*PPP	Tris(2-isopropylphenyl) phosphate	64532-95-2	Phosphoric acid, tris(2-isopropylphenyl) ester

We report on the technical mixture of Tris(1-chloro-2-propyl) phosphate (TCPP, CAS No. 13674-84-5). TCPP is manufactured to a purity of 75 ± 10%. Major impurities are bis (1-chloro-2-propyl)-2-chloropropyl phosphate (20-30%) and bis (2-chloropropyl)-1-chloro-2-propyl phosphate (3-5%). Both Fyrol PCF and Antiblaze 80 (trade names) have a similar composition/purity. http://www.inchem.org/documents/sids/sids/13674845.pdf

$ We report on the commercial product which consists mainly of 1,3-dichloro-2-propyl groups but can contain trace amounts of tris(2,3-dichloropropyl) phosphate (CAS 78–43–3). In literature TDCPP has been mistakenly referred to as TCPP, which is tris(1-chloro-2-propyl) phosphate (CAS 13674–84–5).

&TMPP concentration was measured as a sum of its orto-, meta- and para-isomers

24.2.2 Analyses

An internal standard solution mix of OPCs (2 ng/µl) were added to 1 l water samples, and samples were extracted on SPE (OASIS HLB) cartridges. Samples were eluted with 10 ml ethyl acetate at a flow rate of 5 ml/min. Half of the received extract (i.e. about 5 ml) was evaporated to dryness and reconstituted in 0.2 ml of reconstituting solution for LC-MS/MS analysis (polar compounds reported in chapter 22). The remaining aliquot (i.e. about 5 ml) was used for GC-MS determination of the OPCs.

An Agilent 5869N GC system coupled to an Agilent 5973 Mass Selective Detector was used. Identification and quantification were based on isotope dilution.

It must be noted that for TEHP and EHDP the results are not supported by the quality control measurements and the method needs further improvement. For this reason the findings for TEHP are not reported and results for EHDP can only be taken as an indication, bearing in mind that due to the insufficient recovery an underestimation of the concentration by a factor 3-4 must be considered.

A methodological underestimation of the total water concentration is also suggested for, T35DMPP and T2*i*PPP who got low water solubility (log Kow 7.98 and 9.07). These compounds are associated to a significant extent with suspended matter, which is only partially captured on the SPE cartridges employed for extraction.

A detailed description of the methodology, including the results for all compounds and each individual site is provided in the full report on the attached CD.

24.3 Results

The results presented for the OPCs refer to the dissolved fraction of surface water.

All 68 JDS3 sites were analysed. In addition 3 extra sites were sampled, that had not been foreseen in the survey plan: Upstream River Olt (JDS51a), River Olt (JDS51b) and Upstream Prut (JDS63a). In order to see if degradation of the OPCs might have occurred during storage, 3 exemplary water samples taken in parallel were extracted and analysed again after an extended storage period (118 days) and discussed together with the results.

24.3.1 Spatial distribution of OPCs in the dissolved phase

The observed downstream profile of the OPC's concentration displays some similarities between specific groups of compounds. The profiles of some exemplary OPCs are plotted below.

TDCPP, TCPP (with some isolated maxima, Figure 128), T*n*BP, T*i*BP, and T*n*PP are present in comparable concentrations along the whole Danube.

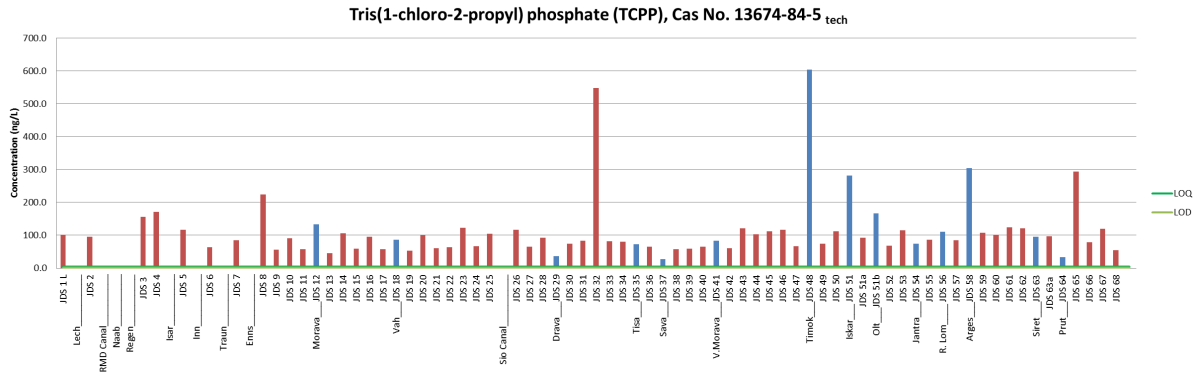

Figure 128: TCPP – Example of an equilibrated downstream profile

TBEP (Figure 129), TPhP, are more abundant in the upper stretch with a decreasing trend downstream but still present in considerable amounts.

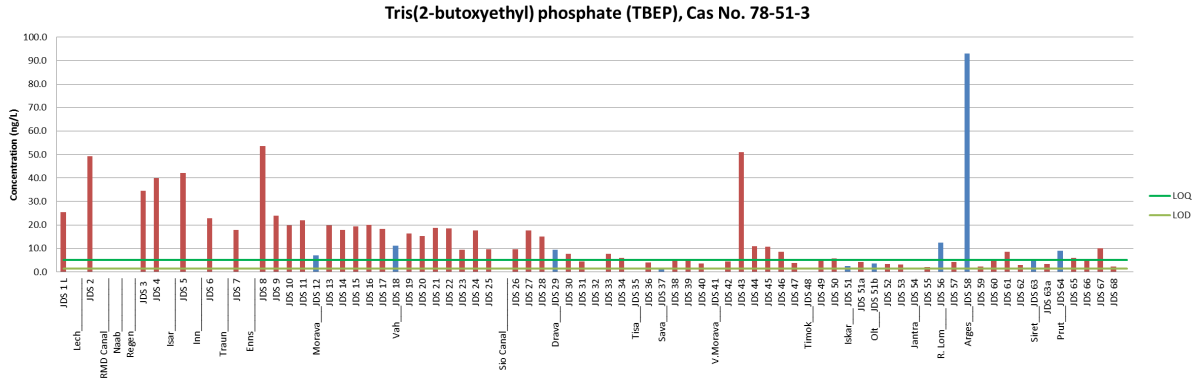

Figure 129: TBEP – Example of a diluted downstream profile

TMPP (Figure 130), T35DMPP, T2*i*PPP, EHDP (to lesser extent) were almost exclusively detected in fairy variable concentrations in the upper/middle stretch, not further downstream than JDS27 (rkm 1434).

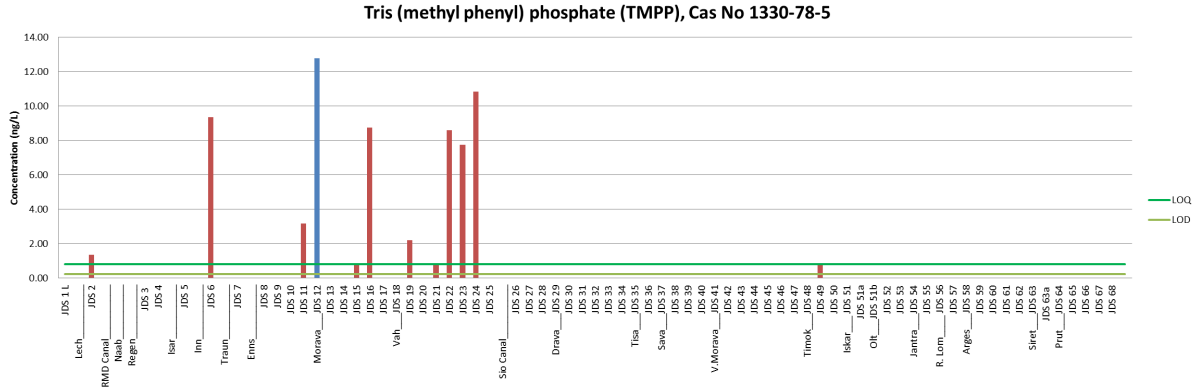

Figure 130: TMPP – Example of an incongruent downstream profile

For most OPCs the concentrations in the tributaries do not differ too much from the Danube itself.

Remarkable concentrations above those in the Danube itself (tributaries in the order of concentration) were seen only for T*i*BP (Arges >Vah), T*n*BP (Vah>>Iskar>Arges, Prut), TBEP (Arges), TPhP (Morava, Arges, Jantra, Tisa), TCPP (Timok> Arges, Iskar), TDCPP (Arges, Morava, Iskar), TCEP (Iskar, Arges). Thereby one order of magnitude of concentration difference was never exceeded.

As a consequence of the low contributions of the tributaries to the overall discharge of the Danube, the partially higher concentrations in the tributaries display no visible impact on the concentration downstream their confluence.

Also along the Danube itself an overall low impact of the sites with higher concentrations on the sites downstream is observed. The few 'hot spot s' display only local impact. It has to be taken into consideration that the higher concentrations observed locally might be due to incomplete mixing, rather than providing a representative value for the whole water column. The overall low variability points to a situation with diffuse emissions along the whole Danube and its tributaries. That would fit to the fact that OPCs are mainly used in open applications and enter the aquatic environment from diffuse urban sources rather than from industrial hotspots.

For those OPCs that were found predominantly in the upper middle stretch (TMPP, T35DMPP, T2*i*PPP, EHDP) one might assume that they entered more recently into production and are therefore released mainly in zones of a higher industrialization, while the diffuse emissions from the open use are still low. In such a scenario they would be simply diluted further downstream. In addition TMPP, T35DMPP, T2*i*PPP, EHDP got the highest log Kows (5.11, 7.98, 9.07, 5.73). As a consequence their

higher association with settling material would privilege a higher sedimentation rates, thus providing an efficient removal mechanism from the water column.

In summary, the concentration differences we observed for the investigated OPCs do not identify any local emission source of concern for the Danube catchment, which would require specific action.

24.3.2 Ranking and potential impact on aquatic biota

The concentration data of T35DMPP, T2*i*PPP and EHDP are underestimated by the methodology applied, and their ranking within the observed concentration ranges for OPCs may be misleading.

In terms of concentrations TCPP clearly dominates, both in the Danube and in the tributaries.

In the 57 samples from the Danube the ranking of the concentrations detected (number of positive samples, average value, range) is as follows:

TCPP (57pos, **av103**, 45-594 ng/L) >> T*i*BP (57pos, **av19**, 6.8-48) > T2iPP (8pos, **av15.1**, 6.5-27) ≥ TBEP (56pos, **av14**, 2-54ng/L) ≥ T35DMPP(15pos, **av14**, 0.76-54) ≥ TDCPP (56pos, **av11.4**, 6-22 ng/L) ≥ TCEP (57pos, **av11**, 4-33) > TMPP(11pos, **av5.0**, 0.81-10.8) ≥ T*n*BP (57pos, **av4.3**, 0.42-9.4) > EDHP (17pos, **av1.6**, 0.079-5.9) ≥ TPhP (55pos, **av1.4**, 0.3-5.6) > T*n*PP (55pos, **av0.35**, 0,075-2.1)

In Figure 131 the ranking is displayed. The compounds that we believe being underestimated are labelled with a dashed box.

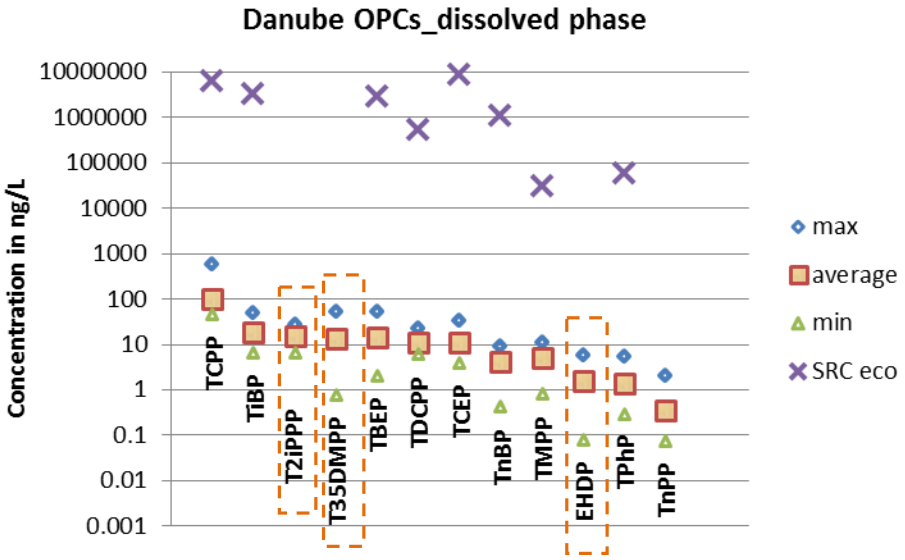

Figure 131: Summary of concentration ranges and average of OPCs in the Danube

In the 14 samples from tributaries the ranking of the concentrations detected (number of positive samples, average value, range) are as follows:

TCPP (14pos, **av151**, 28-603 ng/l) >> T*i*BP (14pos, **av23**, 2.5-97) > TBEP (10pos, **av15.4**, 1.3-93ng/l) ≥ TCEP (14pos, **av13**, 2.4-41) ≥ TMPP(1pos, **12.8)** ≥ TDCPP (14pos, **av10.8**, 8-28 ng/l) ≥ T*n*BP (57pos, **av10.6**, 0,26-70) > T35DMPP(2pos, **av3.2**, 3.1-3.3) > TPhP (14pos, **av2.4**, 0.24-7.6) > EDHP (4 pos, **av0.89**, 0.38-1.8) > T*n*PP (13pos, **av0.42**, 0,085-1.0) >T2iPP (0pos).

In Figure 132 the ranking is displayed. The compounds we believe are underestimated again labelled.

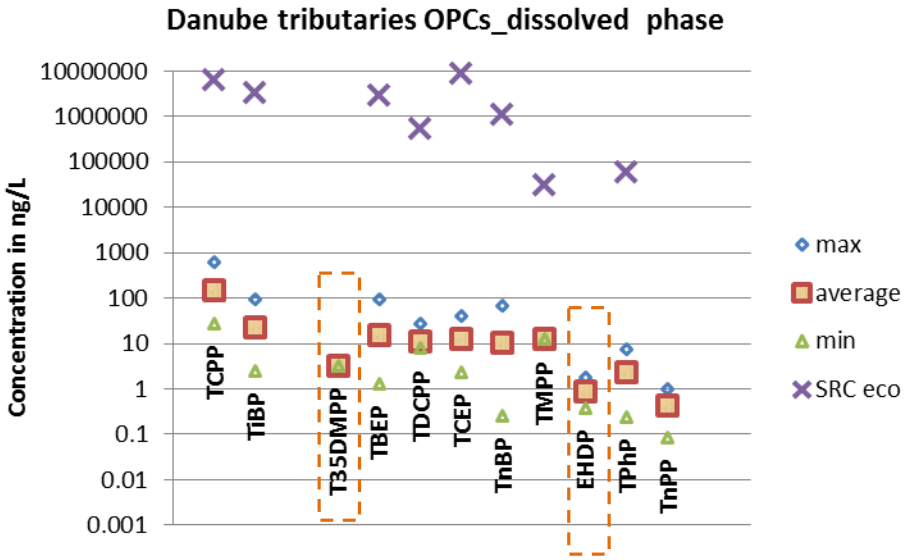

Figure 132: Summary of concentration ranges and average of OPCs in the Danube tributaries

In terms of concentrations TCPP clearly dominates, both in the Danube and in the tributaries, the other OPCs were at ranging 1-2 orders of magnitude lower in concentration.

In order to get an indication about in how far the ranking of the concentrations reflects the actual risk, we compare the concentrations with the so called *'Serious Risk Concentration for surface waters, SRCeco'* which is derived from a risk assessment approach employing acute and chronic toxicity test data for number of aquatic organisms (Verbrueggen et al. 2005). The *SRCeco*, as far as available, is displayed together with the concentrations in Figure 131 and Figure 132.

Including the *SRCeco* values into the attempt of ranking the OPCs, the picture changes considerably (Figure 133). TCPP, although dominating the concentration in the dissolve phase, is now ranking behind TDCPP, TPhP and TMPP, the later one dominating by far the ranking. The situation in the tributaries is analogue. For T35DMPP, T2*i*PPP and EHDP no *SRCeco* values were available.

From this, and since the chlorinated OPCs have been phased out to a large extent, it can be concluded that among the OPCs investigated here only TMPP and to a lesser extent TPhP deserve – if at all – scientific attention what regards their temporal trends in the effluents into the Danube basin.

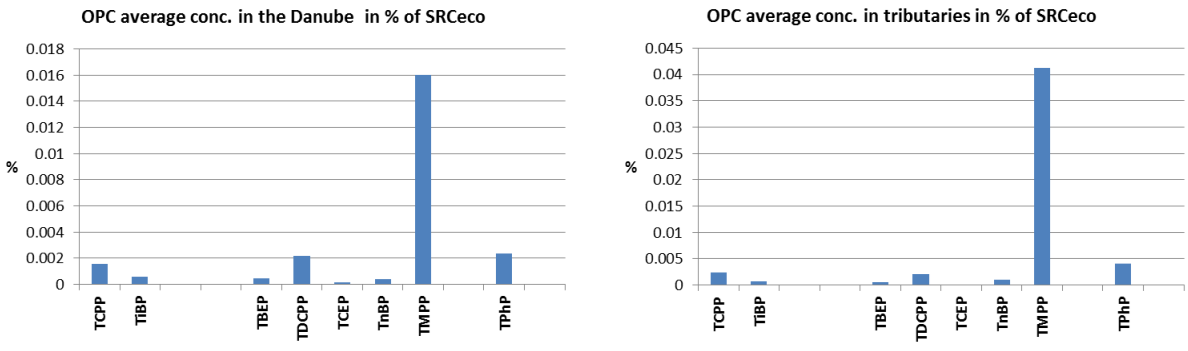

Figure 133: average OPC concentrations in% of the Serious Risk Concentration

24.3.3 Comparison with other surface waters in Europe

In Table 85 the OPC concentrations obtained from this study are resumed, in comparison with data available from literature. It should be kept in mind, however, that the JDS3 data can only be considered as a snapshot, and we do not have reliable information on the temporal variability on OPCs (as observed by Bollmann et al. (2012) in other European Rivers) from the Danube.

The comparison with the data from Martinez Carballo et al. (2007), obtained from the Danube around Vienna suggests lower concentrations in 2013 for T*n*BP and TBEP, while the concentrations of TCPP and TDCPP were higher.

The comparison with the ranges available from other surface waters suggests that the concentrations obtained during JDS3 for OPCs in the Danube are within the (large) ranges typically observed. Also the dominance of TCPP can be seen in most of the other surface waters investigated.

24.4 Conclusions

Among the investigated OPCs, TCPP clearly dominates, both in the Danube and in the tributaries. Looking into their toxicities, the concentrations for OPCs are several orders of magnitude below their effect levels for aquatic biota. Under the toxicity aspect, TMPP and TPhP, although lower in concentration, are ranking at first place. Although being far below any effect level, they may deserve further attention regarding their temporal trends.

For most OPCs the concentrations in the tributaries do not differ much from the Danube itself. Consequently, the partially higher concentrations in the tributaries display no discrete impacts on the concentration downstream their confluence, thus to the overall discharge of the Danube.

Generally the observed concentration differences do not reveal any local OPC emissions of concern for the Danube catchment, which would require action on hot spots or specific zones of emissions.

Table 85: OPCs in surface waters, JDS3 in comparison with literature data

OPCs in surface waters, JDS3 in comparison with literature data, concentrations in ng/L^{-1}

Compound	T*n*PP	T*i*BP	T*n*BP	TCPP	TBEP	TDCPP	TCEP	TPhP	TMPP	Reference	Comment
Danube	0.075- 2.1**; 0.35**	6.8-48; **19**	0.42-9.4; **4.3**	45-549; **103**	2-54; **14**	6-22; **11.4**	4.0-33: **11**	0.3-5.6; **1.4**	0.8-11; **5**	This study	Min-max; **average** of positive samples
Danube trib.	0.085-1.0; **0.42**	2.5-97; **23.4**	0.26-70; **10.6**	28 –603; **151**	1.3-93;**15.4**	3-28; **10.8**	2.4- 41,.: **13**	0.24-7.6; **2.4**	< 0.24-13	This study	
Danube			110, 20	43, 33	52, 24	7, < 3	23, 13	6 < 4.4 –	< 7.9	Martinez Carballo et al. 2007	Up and down Vienna (Nussdorf, Haslau)
Elbe	< 0.86	4.3 – 19.2 10 – 50	< 0.25-7.5	44.0 – 134 40 – 250 20-520; 72-217 <25-88	< 2 -94.3 < 2 – 80	6.4-31	9.3 – 35.5 5 – 20 <10-150; 52-66 <25-100	< 1.2 -10.3	< 2.0	Bollmann et al. 2012 ARGE Elbe 2000	August 2010 March – October 2010 1996 1998
Mulde				160-450; 284 71-79			<10-150; 79 45-57			ARGE Elbe 2000	1996 Dessau 1998 Dessau
Saale				130-780; 205 <25-140			50-220; 112 <25-98			ARGE Elbe 2000	1996 Rosenburg 1998 Rosenburg
Schwarze Elster				33-720			30-52			ARGE Elbe 2000	1998 Gorsdorf
Weser	< 0.86	< 1.3 – 13.2		24.3 – 167	< 2-48.4	5.3-27	3.3 – 34	< 1.2	< 2.0	Bollmann et al. 2012	August 2010
Ems	< 0.86	4.81-11.1		89.9 – 175	38.9 – 42.7	8-35	11.5 – 34.2	< 1.2	< 2.0	Bollmann et al. 2012	August 2010
Rhine	< 0.86	16.8-84.0 30-50	6 – 28 30-120	74.8 – 159 30-150 80-100	28.5- 53.9 80-140	13-31 13-36	12.4 – 25.8 5-500 80-100	< 1.2	< 2.0	Bollmann et al. 2012 Knepper et al. 1999 Andresen et al. 2004	August 2010, Colone September 2002, instant extraction
Meuse	< 0.86	20.7		196	103	37	38.4	3.6	< 2.0	Bollmann et al. 2012	August 2010
Scheldt	< 0.86	5.04-5.23		164 – 570	< 2 – 72	19-67	19-69.9	< 1.2	< 2.0	Bollmann et al. 2012	August 2010
Ruhr		< LOD – 150	30 – 110	20-200	10 – 200	50	50	<10 – 40		Andresen et al. 2004	September 2002, instant extraction
Aire				113 – 26050 Average 6040		62-149	119 -316	6.3 – 22		Cristale et al. 2013	
Tiber	15-62	98 – 137	82-114	54-117	87-323	< 0.7	< 1.5-7	11-165	< 0.1	Bacaloni et al. 2007	June and November 2006, Rome
Spanish rivers	< 0.2	11 – 89	<10-50	28-430	< 10 – 2700	< 2-70	0.8-85	< 2 – 35		Garcia-Lopez et al. 2010	2009, no location
Lake Taihu	1,2-9.4		2.2-12	7.7-19		7.4-42	260-2406	<0.8-1.8		Yan et al. 2012	
Urban lakes		8-10	17 -32	85-126	<30-53		23-61			Regnery and Puettmann 2010	2007 – 2009, range of mean

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***Note**: a list of references including the titles can be found in the full report.*

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25 Emerging substances in surface and groundwater

Florian Rüdiger Storck, Doreen Richter and Heinz-Jürgen Brauch

25.1 Introduction

25.1.1 Emerging substances

A huge number of anthropogenic chemicals have been found in water resources in low concentrations (low µg/l – ng/l range). Among those, emerging substances comprise potentially hazardous contaminants for which information on possible toxic effects for aquatic organisms and humans is often not available. They are usually not included in routine monitoring programs in major river basins and health-based or ecology-based standard or guideline values have not been set so far. Some examples of emerging substances are pharmaceuticals, hormones, perfluorinated compounds (PFCs), flame retardants, benzotriazoles, artificial sweeteners, siloxanes, musks, algal toxins, perchlorate or pesticide transformation products. A general compilation of current research, regulation and analytical methods on emerging substances is given by Richardson and Ternes (2011).

25.1.2 Occurrence in the Danube catchment area

Information on emerging substances in the Danube catchment is rare and Joint Danube Survey campaigns which provide a more comprehensive view on the state of pollution, give only a short-term impression. The field of emerging substances develops faster than the six year cycle of JDS. However, up-to-date information on emerging substances from single sampling campaigns is available for parts of the catchment (e.g. Storck et al., accepted). Moreover, the International Association of Water Supply Companies in the Danube River Catchment Area (IAWD) publishes up-to-date results on pollution in its bi-annual report (www.iawd.at).

25.1.3 Groundwater

Drinking water production in many cities along the Danube and its tributaries is based on natural treatment of surface water by means of bank filtration or artificial recharge. Many classes of emerging pollutants like benzotriazoles or artificial sweeteners are easily water soluble and have been recognized to be very mobile in soil and especially in subsurface systems for drinking water treatment like bank filtration sites (Scheurer et al. 2010, 2011). Still, the behaviour of many compounds and their removal or retention during natural water treatment depends – besides compound specific properties – on different site specific factors like the redox setting and the retention time (e.g. Storck et al. 2012a,b). Information on the linkage of emerging substances in surface water and groundwater helps to make a rough evaluation of the quality and cleaning capacity of these natural water treatment systems.

25.2 Methods

25.2.1 Sampling, transport and storage

Surface water samples were taken by the JDS3 team as described in Chapter 2. In addition, ground water and bank filtrate samples from 10 sites near the Danube were taken by local water suppliers. Polypropylene bottles containing 2 l of water sample were shipped and stored cool until analysis.

Sample temperature was controlled as described in Chapter 2. However, a few bottles were broken during transport and the volume of water available for analysis was smaller than expected. The latter caused partly higher levels of the limit of quantification due to necessary dilution with ultrapure water. In addition, for 3 sites a subset of parameters could not be determined at all due to the small volume of water.

25.2.2 Analysis

A set of 49 compounds was analysed according to standard routines which comprised benzotriazoles, artificial sweeteners, betablockers, lipid-lowering drugs, nonsteroidal anti-inflammatory drugs, cytostatic drugs and other pharmaceuticals, iodinated X-ray contrast media (X-RCM), the stimulant caffeine and the preservative salicyclic acid. Moreover, drug metabolites clofibric acid, 4-acetylaminoantipyrine and 4-formylaminoantipyrine (AAA and FAA) were included. Appropriate internal standard cocktails were added to the sample before the analytes were pre-concentrated using different methods of solid phase extraction (SPE). SPE cartridges were eluted and the extracts were further processed (e.g. change of solvents, volume reduction) to match conditions for analysis by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). In addition to the samples, field blanks were checked. The laboratory is accredited according to ISO 17025 for all parameters investigated.

25.3 Results

25.3.1 Benzotriazoles

1-*H*-benzotriazole concentrations ranged from 200 to 400 ng/l in the Danube and were mostly similar or lower in most tributaries (Figure 134). The only exception was the Vah, where 840 ng/l were observed. Concentrations of 4-Methyl-1-*H*- and 5-Methyl-1-*H*-benzotriazole were generally lower (approximately 50% and 25% of 1-*H*-benzotriazole concentrations). In Arges and Morava and near Bratislava (rkm 1869) elevated concentrations of the Methyl-benzotriazoles were detected. Concentrations of benzotriazoles generally decreased from the source to the Danube Delta. The confluence of the rather unpolluted Inn water remarkably lowered concentration levels. Benzotriazole and tolyltriazoles concentrations of 130-380 ng/l and 62-130 ng/l had been reported during JDS2 by Loos et al. (2008, 2010). 4-Methyl-1-*H*- and 1-*H*-benzotriazole were detected at several sites in bank filtrate, but concentrations were mostly below 100 ng/l.

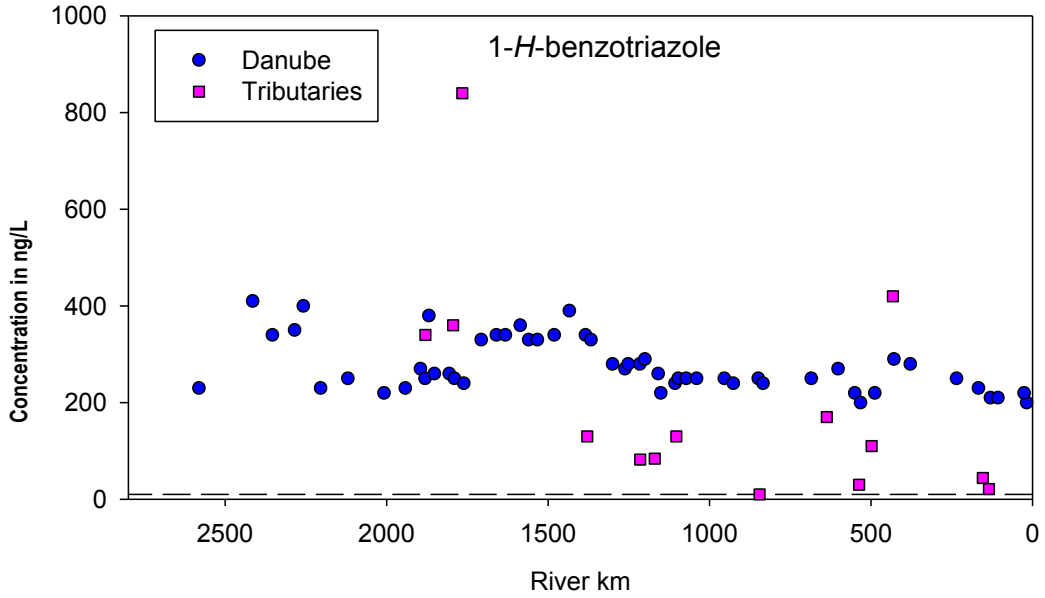

Figure 134: Concentration of 1-*H*-benzotriazole in the Danube and its tributaries. Dashed line indicates limit of quantification (LOQ)

25.3.2 Iodinated X-ray contrast media

Concentrations of diatrizoic acid in the Danube and its tributaries did not exceed 100 ng/l. Exceptions were the German stretch of the Danube (up to 160 ng/l) and the Moson Danube (510 ng/l). Similarly, iopromide and iohexol were rarely found in concentrations >100 ng/l, but at Velika Morava and Arges concentrations ranged up to 700 ng/l (Figure 135). Iomeprol concentration reached up to 350 ng/l at the first five sampling points and declined continuously in the further course of the Danube to approximately 70 ng/l in the Delta region. Polluted water from Iskar and Arges (1300 and 1100 ng/l) did not significantly increase iomeprol concentration levels in the Danube. Iopamidol concentrations were below 190 ng/l, except for Vah, Moson Danube and Arges, which were stronger polluted. In Arges, highest iopamidol concentrations of 1600 ng/l were observed. Generally, dilution with cleaner Inn water caused an abrupt concentration decrease for all iodinated X-ray contrast media. Interestingly, Drava, Tisa and especially Sava had significantly lower concentrations for most X-ray contrast media than the Danube. However, concentration lowering in the Danube after confluence was not observed. Therefore, it can be assumed that the waste water input from the Belgrade metropol region in this river stretch compensates the dilution effect. In bank filtrate, iopamidol and diatrizoic acid were detected at most sites, with concentrations between 10 and 90 ng/l.

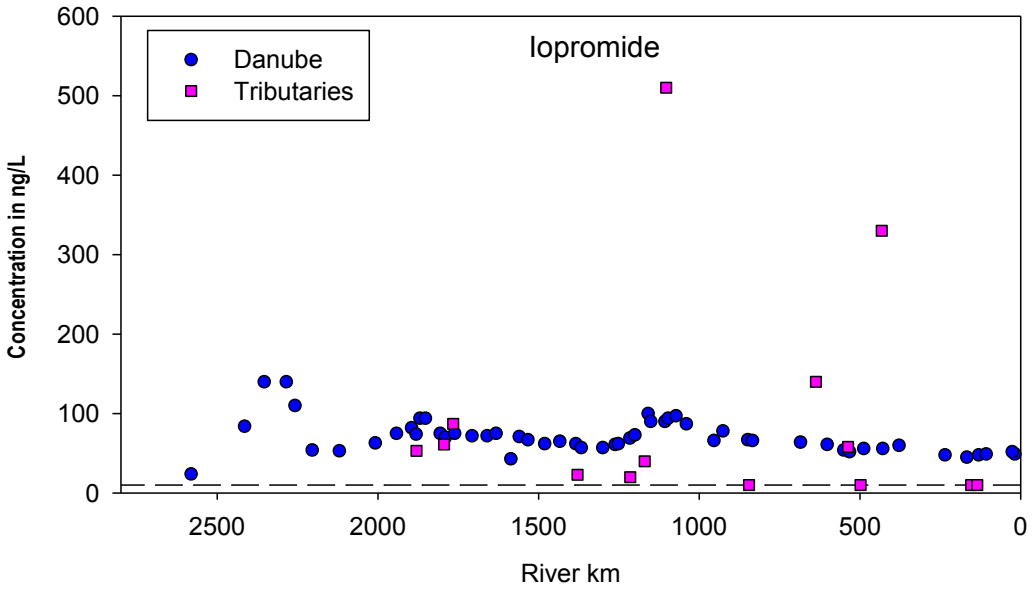

Figure 135: Concentration of iopromide in the Danube and its tributaries. Dashed line indicates limit of quantification (LOQ)

25.3.3 Artificial sweeteners

Acesulfame was the compound with highest concentrations observed both in the Danube (1.1 µg/l) and its tributaries (2.9 µg/l) and in groundwater (0.45 µg/l). In the Danube, acesulfame concentrations were highest in the upper catchment. After confluence with the Inn, acesulfame concentrations were lowered by 50% to 0.55 µg/l but then stayed very constantly on this level on the further way down to the Black Sea. However, maximum concentrations of the other sweeteners cyclamate, saccharine, and sucralose ranged from 0.5 to 2.1 µg/l in the Danube tributaries while maximum concentrations were mostly by far lower in the Danube itself (0.11 to 0.46 µg/l). Cyclamate and saccharine, which have been reported to be easily biodegradable (Scheurer et al. 2010), exceeded concentrations of 0.3 µg/l only in Russenski Lom and Arges. The comparatively high concentrations observed in the latter rivers could be due to the release of a bigger proportion of untreated municipal waste water in their catchments. However, the reason should be closer evaluated to exclude industrial waste water discharge and to avoid wrong conclusions. Besides acesulfame, which was detected in almost every bank filtration well, cyclamate and sucralose were observed in two of ten sampled wells in concentrations up to 80 ng/l. Especially the presence of cyclamate in the abstraction wells points to short retention times and/or rather low capacity to retain organic pollutants at this site.

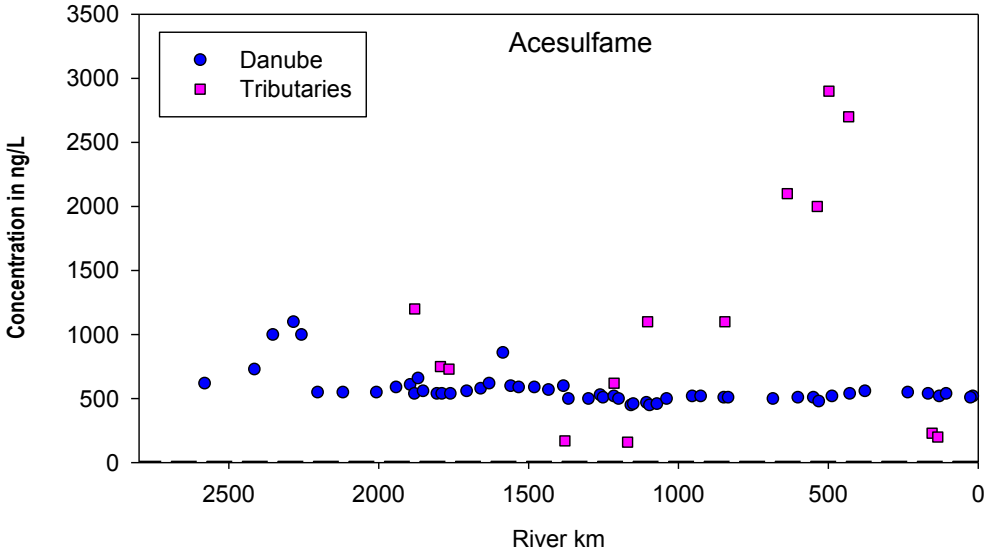

Figure 136: Concentration of acesulfame in the Danube and its tributaries. Dashed line indicates limit of quantification (LOQ)

25.3.4 Caffeine

Caffeine concentrations in the Danube ranged from 60 to 340 ng/l. Areas with comparatively higher concentrations comprised Geisling in Germany, Szob in Hungary, the sections from rkm 1533-1379 and 1159-834 and at rkm 378. In the metropol region of Belgrade, caffeine concentrations were more than double compared to the upstream section. Elevated caffeine concentrations may indicate the release of untreated municipal wastewater or a malfunction of biological wastewater treatment steps, as caffeine is easily biodegradable. Highest concentrations were observed in the tributaries Velika Morava, Russenski Lom, and Arges (800-1700 ng/l), while concentrations in all other tributaries were similar or lower than in the Danube. Caffeine was detected in one bank filtrate abstraction well. The latter may reflects the rather short retention time at this site. However, other parameters (biodegradable X-RCM) did not indicate low cleaning capacity of this bank filtration site. During JDS3 caffeine concentration levels were often similar to JDS2, but especially in Arges concentrations had been higher during JDS2.

25.3.5 Pharmaceuticals

Carbamazepine concentrations ranged from < 20 to 49 ng/l in the Danube and from < 20 to 140 ng/l in the tributaries with Arges showing highest pollution. In 2007 during JDS2, concentrations were in a similar range, while Arges, Sio, and Ipoly had been even stronger polluted (up to 945 ng/l, Loos et al. 2008, 2010). Traces of carbamazepine up to 23 ng/l were found in wells at three bank filtration sites.

Diclofenac concentrations were generally below 40 ng/l, mostly even below 20 ng/l. The only exceptions were Russenski Lom (46 ng/l) and Arges (280 ng/l). For comparison: Loos et al. (2010) reported < 1 to 7 ng/l in the Danube and < 1 to 52 ng/l in the tributaries.

The drug metabolites 4-acetylaminoantipyrine and 4-formylaminoantipyrine (AAA and FAA) were detected in concentration ranges from 20 to 160 and 9 to 100 ng/l in the Danube (Figure 137). Downstream of the mouth of Velika Morava concentrations were somewhat lower than in the upper part of the Danube catchment. For the tributaries, the situation was different: Especially in Iskar, Jantra, Russenski Lom, and Arges concentrations were higher than in the Danube and reached up to 960 and 480 ng/l for AAA and FAA, respectively. During JDS1 only a few samples had been analysed for both metabolites, but most of these tributaries had been reported to be stronger polluted in the year 2001 (AAA: 1700 to 2700 ng/l and FAA: 750-1600 ng/l). However, in the Danube concentration levels had increased since JDS1. While AAA was not detected in groundwater, FAA occurred in two wells in concentrations up to 27 ng/l.

For betablockers, lipid-lowering and nonsteroidal anti-inflammatory drugs and other pharmaceuticals concentrations were below 40 ng/l in most samples (Tables 86 and 87), similar to results from JDS1 and JDS2. Concentrations above LOQ occurred mostly in the tributaries (Table 87) and at single hotspots like Arges highest concentrations were observed, e.g. 550 ng/l of ibuprofen, 170 ng/l of pentoxifyllin, 470 ng/l of paracetamol, and 660 ng/l of metoprolol. Except for the few examples described above, pharmaceuticals were not detected in bank filtrate.

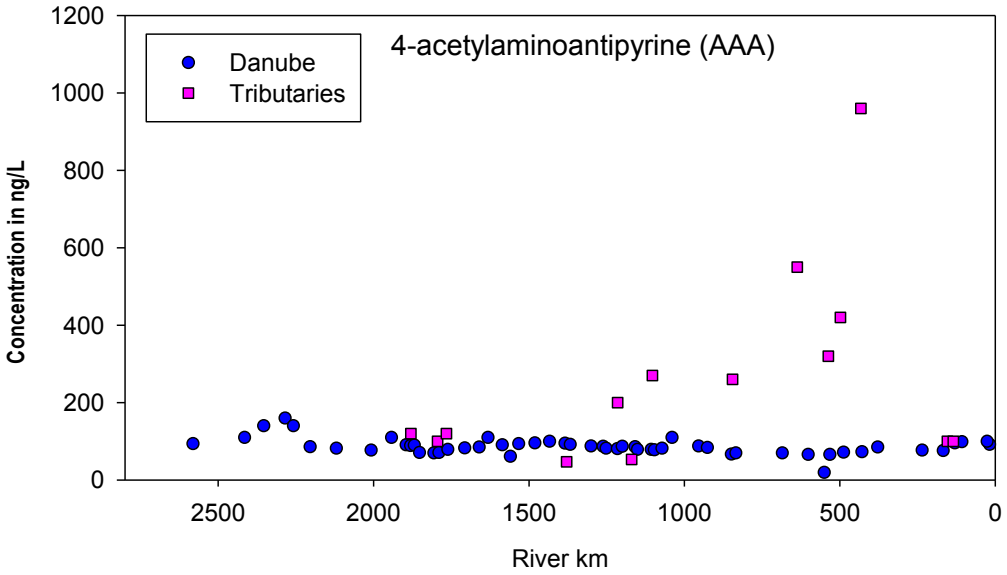

Figure 137: Concentration of 4-acetylaminoantipyrine (AAA) in the Danube and its tributaries

Table 86: Pharmaceuticals and metabolites with concentrations below limit of quantification in the Danube and its tributaries in all samples

Determinand name	Use
Dimethylaminophenazone	analgesic
Phenacetin	analgesic
Propyphenazone	analgesic
Diazepam	anticonvulsant
Fenoprofen	anti-inflammatory
Ketoprofen	anti-inflammatory
Betaxolol	betablocker
Pindolol	betablocker
Clofibric acid	clofibrate metabolite
Cyclophosphamide	cytostatic
Ifosfamide	cytostatic
Bezafibrate	lipid-lowering drug
Etofibrate	lipid-lowering drug
Fenobric acid	lipid-lowering drug
Gemfibrozil	lipid-lowering drug
Simvastatin	lipid-lowering drug
Fenofibrate	lipid-lowering pro-drug
Salicylic acid	preservative, metabolite
Clenbuterol	sympathomimetic
Salbutamol	sympathomimetic
Terbutaline	sympathomimetic

Table 87: Pharmaceuticals occasionally detected in the Danube (D) and its tributaries (T)

Determinand name	Group / Use	Number of samples > limit of quantification
Diclofenac	analgesic	2T
Paracetamol	analgesic	3D, 3T
Phenazone	analgesic	4T
Ibuprofen	anti-inflammatory	3T
Indomethacin	anti-inflammatory	1T
Naproxen	anti-inflammatory	1T
Atenolol	betablocker	1D, 2T
Bisoprolol	betablocker	1T
Metoprolol	betablocker	6D,1T
Propanolol	betablocker	1T
Sotalol	betablocker	3T
Pentoxifylline	blood-thinner	1T

25.4 Conclusions

25.4.1 General concentration trends

Many pharmaceuticals occurred mostly in concentrations below 40 ng/l. Pollutants with generally higher concentration levels were the metamizol metabolites FAA and AAA, the artificial sweeteners acesulfame and sucralose, benzotriazoles, iodinated X-ray contrast media and the stimulant caffeine. Concentrations in the Danube itself were quite uniform for many parameters for long distances. For several parameters, increasing concentrations in the Upper Danube were observed until "cleaner" water from Inn caused dilution. Overall, concentration levels slightly decreased downstream the Danube to the Black Sea for many parameters. Country-specific use patterns may explain general trends of decreasing concentrations from source to mouth of the Danube for acesulfame and vice versa for sucralose.

25.4.2 Comparison with former campaigns

A comparison of JDS3 data for emerging substances with former campaigns is mostly not possible due to the rapid development of the field and lacking data. However, concentration levels observed in the Danube during JDS3 were in the same order of magnitude as reported by Storck et al. (accepted) for a sampling campaign hold in the year 2011 during low discharge conditions. Comparing JDS3 results with JDS1 and JDS2, a slight improvement with lower concentrations in the tributaries could be suspected for carbamazepine in the lower catchment. Caffeine concentrations in the upper catchment were higher during JDS3 compared to JDS2, while concentrations in tributaries were lower during JDS3. However, concentration levels of AAA and FAA in the Danube seemed to have increased.

25.4.3 Impact of wastewater

There was a clear impact of municipal wastewater released from metropolis areas like Belgrade. Far lower concentrations of several parameters in Sava, which has high discharge, were not reflected by decreasing concentrations in the Danube after confluence, underlining the latter observation. Similarly, highest concentrations of many compounds were often detected in Arges, most probably due to the waste water burden from Bucharest. Due to the comparatively small discharge of most tributaries, concentrations were higher there than in the Danube for most substances. The Danube itself hardly showed higher concentrations after afflux of polluted tributaries. Thus, the load of the tributaries seemed to be small compared to the load of the Danube and dilution prevented elevated concentrations in the Danube after confluence. Occurrence of elevated concentrations of rather easily biodegradable compounds like caffeine, cyclamate and saccharine in surface water could be due to a release of bigger portions of untreated wastewater or malfunctions of biological wastewater treatment steps.

25.4.4 Groundwater and bank filtrate

A number of emerging substances was detected in abstraction wells at bank filtration sites. The latter can be expected for substances like amidotrizoic acid, iopamidol, acesulfame, benzotriazole or carbamazepine which are known to be quite persistent in the aquatic environment and which are mostly not completely retained by bank filtration. Due to the comparatively low concentration levels in the Danube, concentrations in the abstraction wells were mostly below 0.1 µg/l for most substances. An exception was the artificial sweetener acesulfame which occurred in concentrations up to 1.1 µg/l in the Danube and was detected in most abstraction wells with a maximum concentration of 0.45 µg/l. Acesulfame is used as a food additive and the observed concentrations are not considered to be harmful for humans. However, acesulfame can act as an example for a more or less persistent and very mobile substance which is consumed in large quantities. Therefore, future potentially increasing pollution of the Danube and its tributaries with compounds obtaining similar properties like acesulfame, especially when they are harmful, must be prevented.

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26 Chemical and immunochemical analysis of anthropogenic markers and organic contaminants

Arnold Bahlmann, Tina Lochen, Tobias Schulze, Alexander Kirschner, Werner Brack, Rudolf J. Schneider, Martin Krauss

26.1 Introduction

Municipal wastewater is a main point source for the input of xenobiotics into the river Danube. Raw wastewater contains many different potentially hazardous organic and inorganic contaminants. Many of these compounds require at least a secondary wastewater treatment for efficient removal. This high level of wastewater treatment is not available in all parts of the Danube basin, thus raw wastewater is continuously released into the Danube (Figure 138).

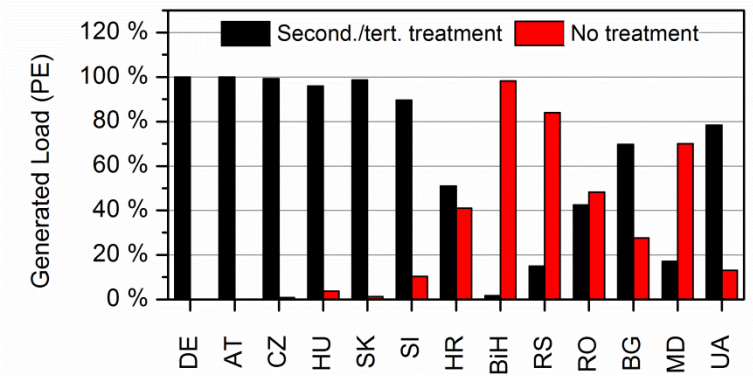

Figure 138: Population equivalents (PE) of treated urban wastewater in the Danube river basin in 2009/2010 (data source: ICPDR)

Different anthropogenic markers – indicative of human presence or activity – have been discussed to track the origin and type of contamination sources. Caffeine (CAF), the antiepileptic drug carbamazepine (CBZ) and the artificial sweetener acesulfame (ACE) have been proposed as possible markers for wastewater (Buerge, et al. 2003, Buerge, et al. 2009, Clara, et al. 2004). CAF is efficiently removed (> 99%) in wastewater treatment plants (WWTP), while CBZ and ACE are not significantly degraded by activated sludge, thus passing almost unchanged to the receiving water bodies. Therefore, CAF is suitable to indicate the presence of untreated wastewater, while the latter two indicate wastewater in general (treated or untreated). Untreated wastewater may contain very high levels of CAF up to 500 µg/l, while the concentrations of ACE and CBZ are usually below 50 and 5 µg/l, respectively.

All three compounds are likely to occur in all countries in the Danube basin. Beverages containing CAF and ACE are widely used in Europe. As far as CBZ is concerned, sales data indicate high amounts above 100 mg consumed per capita in Austria, Bulgaria, Germany, Hungary, Romania and Slovakia (Zhang and Geißen 2010).

Apart from effluents of WWTP, there are other inputs of domestic wastewater into the environment. During intense rainfall, combined sewers are likely to overflow releasing the runoff alongside raw sewage, sewer deposits and sewer slime into the receiving water body. Combined sewers are still in

use for many cities in Europe. For well degradable contaminants such as CAF, combined sewer overflows are the main input pathway into surface water (Weyrauch, et al. 2010).

In addition to rainfall episodes, sewer leakage and damaged pipelines may also contribute to the CAF input into surface waters (Buerge, et al. 2003). At least 5% of the sewage is believed to exfiltrate from the sewers through broken pipes, joint failures and faulty connections (Reynolds and Barrett 2003).

26.2 Methods

CBZ and CAF were measured with two enzyme-linked immunosorbent assays (ELISA) at the BAM Federal Institute for Research and Testing in Berlin, Germany, according to previously published methods (Bahlmann, et al. 2009, Bahlmann, et al. 2012, Carvalho, et al. 2010). No sample pretreatment was applied; the limits of quantitation (LOQ) were 20 ng/l for CBZ and 30 ng/l for CAF.

The concentrations of ACE were measured with a newly developed method using liquid chromatography tandem mass spectrometry (LC-MS/MS) at the UFZ in Leipzig. No sample enrichment or other pretreatments were applied, except for the addition of isotopically labeled internal standards. The LOQs for CBZ, CAF and ACE were 1 ng/l, 80 ng/l and 20 ng/l, respectively. The same method was applied to determine the concentrations of 35 additional organic compounds, among them four of the priority compounds according to the water framework directive (atrazine, cybutryne, diuron, n-nonylphenol) as well as other pharmaceuticals, pesticides, personal care products and industrial chemicals. A detailed list of all analytes is included in the extended version of this chapter.

A total of 180 samples were measured with both the ELISA and the LC-MS/MS method, comprising left, middle and right profile samples of the 68 locations (see chapter 12).

For CBZ and CAF, both methods showed a high level of correlation. For the discussion in this report, the mean value of the CBZ concentrations obtained by both methods was calculated. For CAF, only the ELISA results were used because the LC-MS/MS results suffered from a significantly higher LOQ and a lower repeatability.

Quality assurance included the analysis of blank samples that were taken at six locations along the Danube. Using distilled water produced in situ from drinking water stored in the ship tank, these blank samples were processed and stored in the same way as all other samples. Since previous studies had indicated frequent CAF contaminations in field blanks (Focazio, et al. 2008), special care was taken to minimize the risk of contamination. Still, a single blank sample contained detectable concentrations of CAF (240 ng/l) as well as the nicotine metabolite cotinine (49 ng/l). Presumably, this sample was contaminated by human contact.

Furthermore, the insect repellent diethyltoluamide (DEET) was found in all six blank samples at concentrations between 9 and 91 ng/l. Obviously, these samples were accidentally contaminated either during sampling or sample preparation. Since the concentrations of DEET found in the Danube samples were similarly elevated (6-110 ng/l), we omitted these findings.

A brief interlaboratory comparison was conducted using the analytical results obtained from the laboratories of Croatian Waters (CW, lab no. 4, see chapter 2, CBZ and CAF), the Joint Research Centre of the European Commission (lab no. 9, CBZ only) and Zweckverband Landeswasserversorgung Langenau (ZLBF, lab no. 33, CBZ and CAF). Results from all 68 JDS sampling locations were provided, all of which were taken from the middle of the river except JDS1 (left side). Although taken approximately at the same time and location as the samples analyzed by us, these samples and the samples analyzed by us were no aliquots. Thus, small variations between the analytical results were to be expected.

In general, a sufficient correlation between our results and the results obtained in the three aforementioned labs was observed for most samples. For CAF, seven out of 68 samples differed by more than 50% compared to each of the labs. The biases found in these seven outliers were visible with both methodologies applied in our lab (ELISA and LC-MS/MS), hinting at an alteration of the sample (see above) rather than a methodological bias. The CAF results obtained from the middle of

the river at stations 17, 19, 50, 51, 58, 62, 63 were therefore neglected in the discussion. For CBZ, no outlier matching the aforementioned criteria was found. No results for ACE were available to us.

26.3 Results

26.3.1 Marker for untreated wastewater – caffeine

CAF was abundantly present in the river Danube and its tributaries (Figure 139). The median concentration found in the Danube was 93 ng/l, while a slightly higher median concentration of 132 ng/l was observed in the tributaries. These results are in good agreement with the median concentration of CAF found during the previous Danube expedition JDS2 (80 ng/l) (Loos et al. 2008).

In Germany and most parts of Austria, CAF concentrations were below the median of the whole river at 60 and 39 ng/l, respectively. An enormous increase of the CAF concentration was observed at JDS11 (Hainburg, AT) and JDS13 (Bratislava, SK), which were both taken on the same day during intense rainfall. These high concentrations of CAF hint at the presence of large amounts of untreated wastewater, which was most likely discharged by an overflow of combined sewers in the upstream regions.

Interestingly, the CAF concentrations found at the right side of JDS13 (870 ng/l) and at the left side of JDS11 (470 ng/l) were among the highest of the entire campaign. Unfortunately, these results could not be confirmed by other participating laboratories because only samples from the middle of the river were measured. Still, the lab of Croatian Waters found a higher than average concentration of CAF of 198 ng/l at JDS11 (middle), slightly less than the result obtained by us (270 ng/l). The inhomogeneous distribution of CAF on the different banks of the river at these locations can be explained by a short-termed release of CAF from various potential point sources in the upstream regions. JDS13 was sampled six hours later than JDS11, thus a short and intense rainfall event may explain the observed differences between the two locations. Together with the input of CAF, increased concentrations of the pharmaceuticals CBZ (70 ng/l), diclofenac (120 ng/l), tramadol (80 ng/l) and others were detected in JDS11 (left), which confirms the increased presence of wastewater at this location.

The magnitude of the aforementioned rainfall event was also documented by the hydromorphological team. At these two locations, the river discharge was temporarily increased by approximately 60% compared to the discharge at JDS10 taken on the day before.

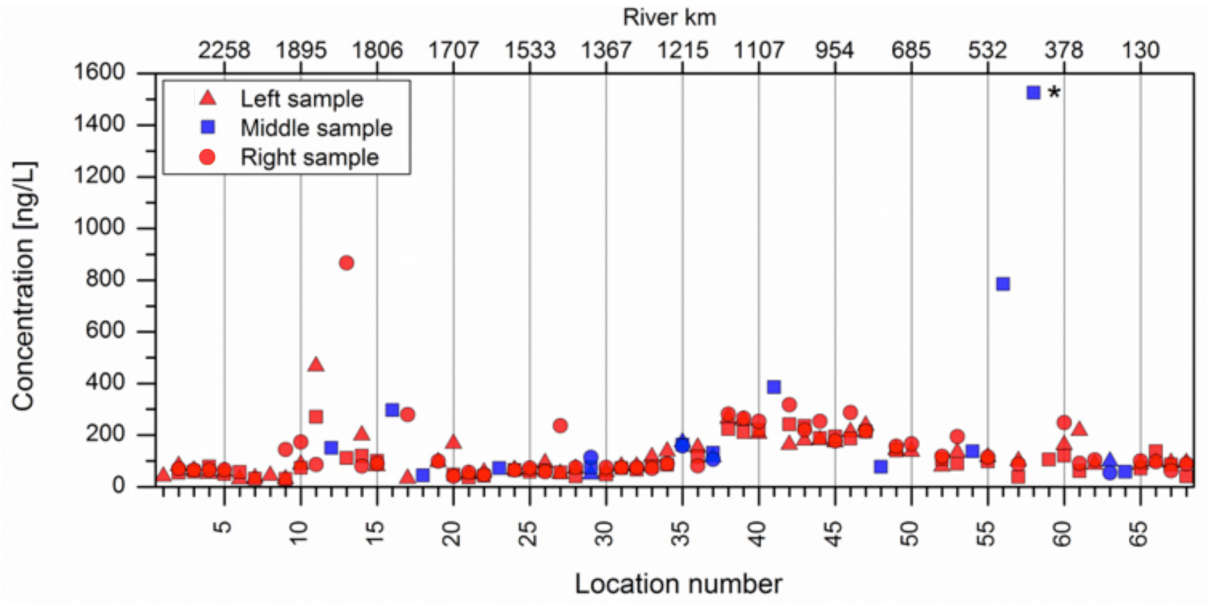

Figure 139: Caffeine concentrations in the Danube (red) and its tributaries (blue).
***For JDS58, the mean result obtained by the laboratories of CW and ZLBF is shown**

Moreover, elevated concentrations of CAF were found at JDS17 (Klizska Nema, SK/HU) in the middle and on the right side of the river, while a low concentration was found on the left side. It is plausible that this increase is related to the Moson arm (JDS16, HU) re-entering the Danube from the right side 4 km earlier. The concentrations of CAF found in the Moson arm and in these Danube samples were similarly high at approximately 300 ng/l.

Beginning with JDS38 (Belgrade, RS), the CAF level raised again above 200 ng/l for the next several hundred river kilometres. This observation can be explained by the input of untreated wastewater from Belgrade, the largest Serbian city. The input of the river Sava (JDS37, RS), one of the main Danube tributaries, seems to play a minor role, as the CAF concentration was relatively low.

Furthermore, the concentration of CAF was elevated in the tributary Velika Morava (JDS41, RS). For the pharmaceutical metabolite N-acetyl-sulfamethoxazole, the highest concentration of the whole campaign (43 ng/l) was measured at this location. Like CAF, this compound is known to be well degradable during wastewater treatment confirming the presence of untreated wastewater. Entering the Danube from the right side, the input of Velika Morava resulted in increased concentrations of CAF on the right side of the Danube in the following sample JDS42 (RS).

A very high concentration of CAF (790 ng/l) was found in the tributary Russenski Lom (JDS56, BG). This river carried also high concentrations of various other compounds such as ACE, CBZ and N-acetyl-sulfamethoxazole. However, the relatively small Russenski Lom did not noticeably impact the following Danube samples.

The highest CAF concentration of the entire campaign was found in the river Arges (JDS58, RO). Concentrations of 1.25 µg/l and 1.8 µg/l were reported by the laboratories of CW and ZLBF (see section Methods), indicating a high level of untreated wastewater. In agreement with this observation, the highest load of microbiological faecal pollution in the whole river basin was found in this tributary (see chapter 12). Furthermore, for numerous compounds the highest concentrations of the entire campaign were found in this river, such as N-acetlyaminoantipyrine (1500 ng/l), metoprolol (820 ng/l), diclofenac (320 ng/l), sulfamethoxazole (210 ng/l), CBZ (130 ng/l) and atrazine (70 ng/l). Therefore, the Arges was identified as the river with the highest relative portion of untreated wastewater during this survey.

In conclusion, Figure 140 shows the median concentrations of CAF found in each country, in relation to the percentage of secondary and tertiary wastewater treatment. Due to the relatively low level of wastewater treatment, the highest median concentration was determined in the Serbian part of the river. The lowest median concentrations were found in Austria and Germany, owed to their high level of wastewater treatment.

Despite the high level of wastewater treatment, the concentrations of CAF in the Slovakian part of the Danube (SK) were relatively high. In this region, the input of wastewater was presumably temporally elevated due to a massive rainfall event during sampling resulting in combined sewer overflows.

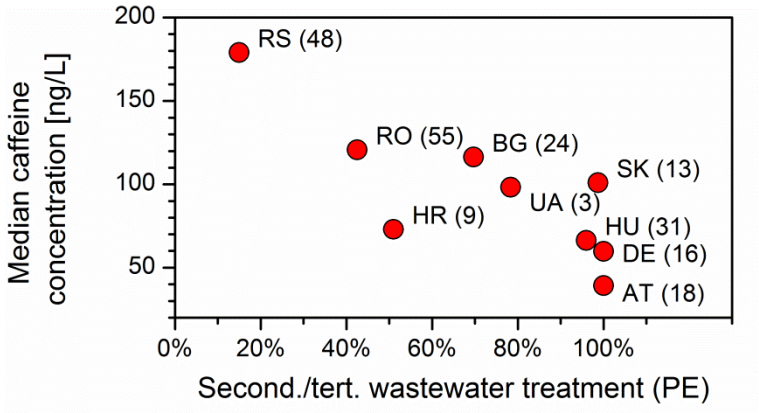

Figure 140: Median caffeine concentrations in the countries in the Danube basin (number of samples in brackets).

26.3.2 Markers for treated and untreated wastewater – carbamazepine and acesulfame

The two anthropogenic markers for treated wastewater, the pharmaceutical CBZ and the artificial sweetener ACE, were found in all analysed samples from the Danube and its tributaries. The median concentration of CBZ was 30 ng/l in the Danube and 40 ng/l in the tributaries (Figure 141). This is in good agreement with the results of the last expedition JDS2, where a median of 37 ng/l was reported (Loos et al. 2008). The artificial sweetener, which was not analysed during the last expedition, was found at median concentrations of 460 ng/l in the Danube and 470 ng/l in the tributaries (Figure 142).

Along the whole river span, the level of CBZ stayed rather constant at concentrations between 20 and 50 ng/l (70 ng/l at JDS11, Hainburg, AT), showing much less variation than the level of CAF, as described in the previous section.

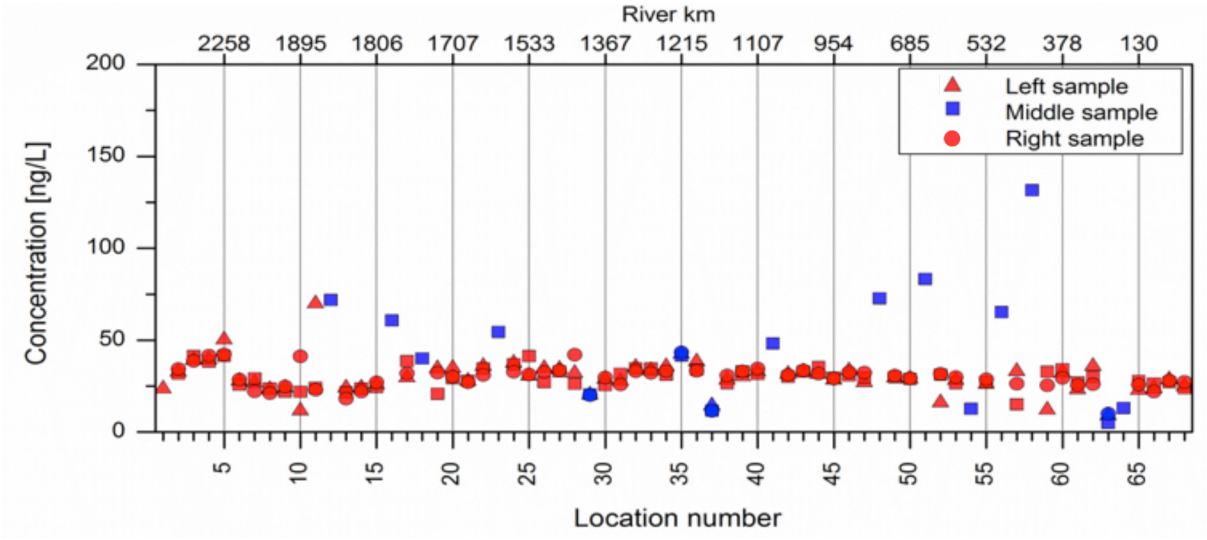

Figure 141: Carbamazepine concentrations in the Danube (red) and its tributaries (blue).

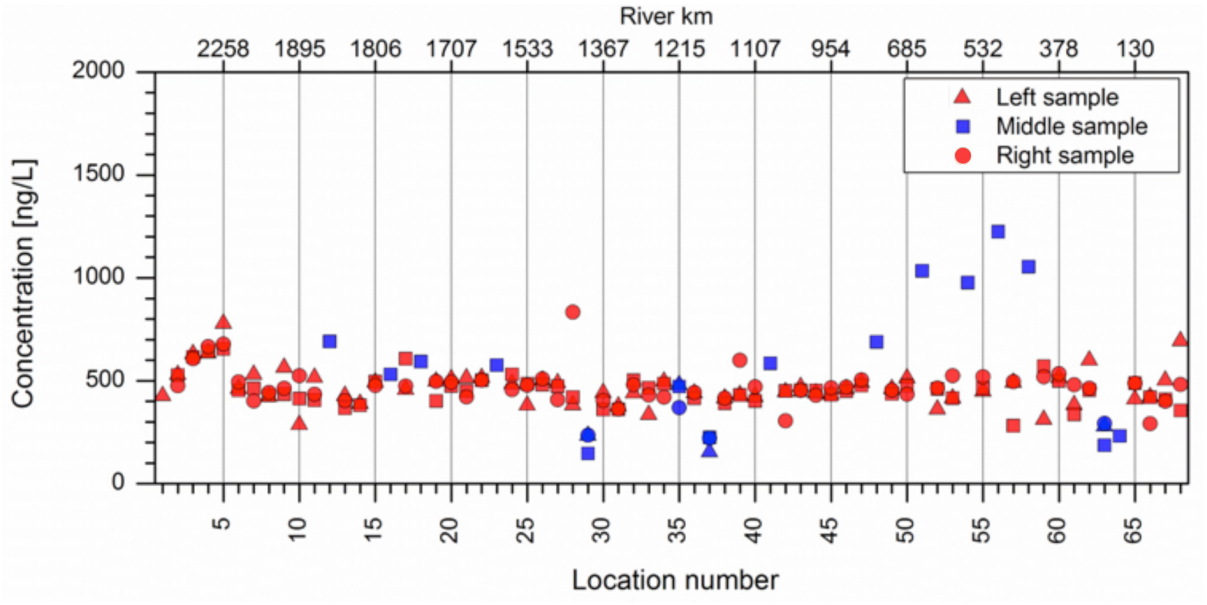

Figure 142: Acesulfame concentrations in the Danube (red) and its tributaries (blue).

The highest concentration of ACE in the Danube was found at JDS28 (HR) on the right side with a concentration of 830 ng/l, while the concentration of CBZ was only slightly increased to 42 ng/l at this location.

In the tributaries, the highest concentration of CBZ was found in the river Arges (JDS58, RO) with 130 ng/l. Elevated concentrations were also found in the rivers Morava (JDS12, SK), Timok (JDS48, RS/BG), Iskar (JDS51, BG) and Russenski Lom (JDS56, BG), while the concentrations in the rivers Sava (JDS37, RS), Jantra (JDS54, BG), Siret (JDS63, RO) and Prut (JDS64, RO/MD) were lower compared to the Danube.

The highest overall concentration of ACE was found in the river Russenski Lom (JDS56, BG) with 1200 ng/l, while the lowest concentrations were present in the rivers Drava (JDS29, HR), Sava (JDS37, RS), Siret (JDS63, RO) and Prut (JDS64, RO/MD).

In general, similar results were obtained for the two markers CBZ and ACE. For both compounds, high concentrations were found in several tributaries. This indicates a high percentage of wastewater in these rivers. In a few cases, the results obtained for ACE and CBZ seem to be contradictory, e.g. in JDS54 a low concentration of CBZ was found, while the concentration of ACE was high. However, variations in the ratio between CBZ and ACE can be explained by local differences, e.g. the presence (or absence) of hospitals in specific river segments, different usages in each country, etc. In conclusion, a low concentration of one of the markers does not necessarily mean the absence of wastewater.

26.4 Conclusions

The analytical results obtained for CAF and CBZ were in good agreement with the previous Joint Danube Survey. The concentrations obtained for CBZ and ACE were similar to other European streams like Elbe or Rhine. The concentrations of CAF, the marker for untreated wastewater, were considerably higher in the middle and lower sections of the Danube, compared to Elbe and Rhine. This indicates a higher amount of untreated wastewater present in the Danube which can be attributed to the lower level of wastewater treatment in this river basin. As there are currently no EQS defined for the three marker substances, the findings shown in this report imply no need for direct action according to EU legislation. Nevertheless, the discharge of untreated wastewater poses a considerable risk for the environment as numerous substances with known (and unknown) toxicity along with microbial contamination enter the river.

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27 Large volume sampling and effect-based screening

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27.1 Introduction

Many organic compounds and their transformation products occur in waters and may pose a risk to human and environmental health. Their chemical structures are often unidentified and they are mainly present in low concentrations with an unknown contribution to mixture toxicity effects (Escher et al. 2014, Escher et al. 2013, Umbuzeiro et al. 2011). Therefore, traditional water monitoring using priority lists or river basin specific compounds is increasingly supplemented by multi-target, non-target and bioanalytical techniques (Hecker and Hollert 2009, Krauss et al. 2010, Richardson and Ternes 2011, 2014). These approaches aim to unravel adverse effects potentials and link them to known, unknown or so far neglected compounds (e.g., transformation products) in a non-deterministic manner (Brack 2003). Effect-based screening is therefore an important prerequisite for a holistic and risk-based river basin management to support the WFD (Brack et al. 2014, Brack et al. 2009, Malaj et al. 2014).

The effect-based screening in highly diluted large rivers such as the River Danube requires significant pre-concentration and the extraction of large water volumes for subsequently applying a large number of different bioassays and multi-target analysis. At the same time the transport to the laboratory and the preparation of extracts of large water volumes are a big challenge. Therefore, a newly developed mobile large-volume extraction device (LVSPE) was used to extract water samples of up to 1000 litres on-site during the JDS3 (Scholz 2013, Schulze et al. 2014).

The extracts were analysed for 264 water phase relevant organic compounds using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) in support of the effect-based screening with a set of different *in vitro* and *in vivo* bioassays. The bioassays cover a broad range of endpoints including algal growth inhibition (biological quality element), algal photosynthesis inhibition (biological quality element), (anti-)estrogen-like activity (female sex hormone system), (anti-)androgen-like activity (male sex hormone system), glucocorticoid-like activity (development, metabolism, immune response), thyroid hormone-like activity (metabolism, development), mutagenic activity (damages of genes and cells), adaptive stress responses (protective response to chemicals), dioxin-like activity (xenobiotic metabolism, chronic), pregnane X receptor mediated activity (xenobiotic metabolism) and acetylcholinesterase inhibition (neurotoxicity).

The algae bioassays refer directly to a biological quality element and thus represent aquatic ecosystem relevant endpoints. The selected cell-based bioassays present important steps in toxicity pathways; for example induction of xenobiotic metabolism, specific and reactive modes of toxic action, and activation of adaptive stress response pathways (Escher et al. 2014).

The main objective of the LVSPE sampling was to enable combined biological and chemical analysis. However, multi-target enrichment methods have typically recoveries below 100% for some compounds. Thus, the parallel usage for biological and chemical analyses does not allow the compensation of compound losses during *on-site* extraction and sample handling for example by using isotope labelled internal standards. Thus, some uncertainness needs to be accepted for the benefit of

parallel chemical and biological analyses. We estimate this uncertainty by comparison with direct analysis of water samples. The JDS3 was an excellent platform to demonstrate the feasibility of an effect-based screening at a river basin scale.

27.2 Methods

27.2.1 Large volume solid phase extraction (LVSPE)

27.2.1.1 Principles of LVSPE

The LVSPE consists of a vacuum sampling system (borosilicate glass vessel connected to a membrane pump), a filtration cartridge to remove residual suspended particulate matter (glass fibre deep filter with a size cut-off of <0.63 µm), and extraction cartridges filled with different solid phases to adsorb dissolved semi-polar to polar organic compounds (Schulze et al. 2014).

A water volume of 500 ml per sampling step was taken using the vacuum system. The filtration unit was mounted in the inflow tubing. The water was released to a stainless steel chamber from the sampling vessel. In the steel chamber, the water was pressurised and pumped through the extraction cartridges, which were mounted in sequence.

The first, neutral sorbent was a polystyrene-divinylbenzene co-polymer (PS-DVB; 160 g Chromabond® HR-X, Macherey Nagel, Düren, Germany) for the extraction of neutral polar to semi-polar organic compounds. The second sorbent was a weak anionic exchanger (100 g Chromabond® HR-XAW) based on the PS-DVB sorbent for the extraction of acidic compounds, which are anionic at the typical pH of surface water. The third sorbent was a weak cationic exchanger (100 g Chromabond® HR-XCW) also based on the PS-DVB sorbent for the extraction of basic compounds that are cationic at a water pH of 6-8.

27.2.1.2 Sampling

The collection of LVSPE samples was performed at 22 sampling sites during the JDS3 transect including 5 tributaries (Table 88). Total volumes of 650 litres of water were collected within 1 hour after reaching the sampling site, centrifuged for suspended particulate matter (SPM) removal and then stored in a stainless steel chamber. A total of 500 litres was extracted. At two sites (JDS33 and JDS57), samples of 1000 litres were extracted for additional experiments. The samples were stored in isolation boxes for transport at approximately 10 °C. At UFZ, the samples were maintained at 4 °C until further preparation.

27.2.1.3 Sample processing

Freeze-dried solid phases were extracted with ethyl acetate and methanol in series (neutral sorbent), methanol containing 2% of 7N ammonia in methanol (weak anion exchanger) and methanol with 1% formic acid (weak cation exchanger). The extracts were combined, neutralised, filtered (GF/F, Whatman) to remove remaining precipitates and reduced in volume to a final concentration factor of 1000 for aliquotation. For further analysis, aliquots were reduced until dryness using rotary (40 °C water bath temperature) and nitrogen evaporation. All freeze-dried samples and extracts were stored at -20 °C.

27.2.2 Chemical analysis with liquid chromatography coupled to high resolution mass spectrometry

Aliquots of the extracts for chemical analysis were reconstituted in methanol to a concentration factor of 1000 of water sample corresponding to 1 ml of final extract. Before analysis, a mixture of 38 isotope-labelled internal standards was added.

The chemical screening was conducted by liquid chromatography-high resolution mass spectrometry (LC-HRMS) using an Agilent 1200 LC coupled to a Thermo LTQ Orbitrap XL. Samples were analysed by positive and negative mode electrospray ionization at a nominal resolving power of

100,000 (Hug et al. 2014). For calibration, 1 l water sample aliquots from a pristine streamlet (Harz mountains, Germany) were spiked with a mixture of the target compounds at seven concentration levels between 1 and 1000 ng/l and extracted using a multilayer SPE cartridge containing 200 mg of Chromabond HR-X, 100 mg of Chromabond HR-XAW, and 100 mg of Chromabond HR-XCW, eluted as described above and extracts adjusted to a final concentration factor of 1000.

With the LC-HRMS target screening, altogether 264 compounds could be analysed, covering a wide range of compounds from different sources and chemical classes including pesticides, biocides, pharmaceuticals, industrial chemicals, artificial sweeteners, UV filters, and surfactants.

27.2.3 Bioanalysis with in vitro and in vivo bioassays

Aliquots of the extracts were tested in the bioassays detailed below. Most samples were tested with a highest concentration level of relative enrichment factor (REF) of 500 and in dilution series to obtain full dose-response curves. Fabrication and solvent blank samples were tested in parallel using the same relative enrichment factors.

27.2.3.1 Growth inhibition and Photosystem II inhibition of Chlamydomonas reinhardtii

The growth inhibition of green algae represents a standardised acute toxicity endpoint used in chemical risk assessment (OECD 2011) and has been expanded to include Photosystem II (PSII) inhibition (Nestler et al. 2012). Green algae are often among the most sensitive whole organism assays and thus used as a biological quality elements in the WFD. In this study the algae *Chlamydomonas reinhardtii* was used.

27.2.3.2 Mutagenicity

The Ames test is the most widespread test for the detection of the mutagenic potential of chemicals and environmental mixtures (Reifferscheid et al. 2012). The method bases on the chemically-induced reversion of auxotrophic *Salmonella typhimurium* mutants to prototrophic metabolism (Ames et al. 1975). To detect possible bioactivation of substances, rat liver homogenate and cofactors (S9-mix) can be added to the test system to simulate exogenous biotransformation potential (Maron and Ames 1983). We used the Ames fluctuation assay with the tester strain *Salmonella thyphimurium* TA98 with and without S9 accordingly to Reifferscheid et al. (2012).

27.2.3.3 Adaptive Stress Responses

The adaptive stress response pathways are key players in controlling the cell homeostatis and / or for repairing damages by transcriptional activation of cytoprotective genes (Simmons et al. 2010). In order to investigate the p53 mediated apoptosis in response to deoxyribonucleic acid damage, the p53-bla HCT-116 gene-reporter assay was used (Yeh et al. 2014). The NF-κB-bla THP-1 gene-reporter assay was used to analyse the samples for induction of inflammation (Invitrogen 2009). The ARE-bla Hep G2 gene-reporter assay was performed to investigate the samples for the inductions of the Nrf-2 mediated oxidative stress pathway (Invitrogen 2006).

27.2.3.4 Estrogen Receptor

The MELN assay was employed to assess the presence of substances able to interact with and activate the human estrogen receptor (ER), and thus presenting estrogenic activity in the samples (Balaguer et al. 1999).

27.2.3.5 Aryl hydrocarbon Receptor

CAFLUX assay was employed to assess dioxin-like (aryl hydrocarbon receptor – mediated) toxicity. Chronic adverse effects of xenobiotics such as interference with liver functions, immunity, endocrine and nervous system as well as embryo toxicity and carcinogenicity were experimentally related to Aryl hydrocarbon receptor (AhR)-dependent events (Janošek et al. 2006).

27.2.3.6 Pregnane X Receptor

In this study, we explored the potential use of the HG5LN-hPXR assay (Lemaire et al 2006) as a detector of PXR-active substances in JDS samples. The human pregnane X receptor (PXR) is a

nuclear receptor that plays a crucial role in detoxification processes by mediating the transcription of genes that code for xenobiotic biotransformation enzymes. As such, PXR is a molecular target for a wide range of xenobiotics including pharmaceuticals, pesticides, steroids, phthalates or alkylphenols, though at relatively high concentrations, i.e. in the µg/l range and upper (Creusot et al. 2010, Lemaire et al. 2006, Mnif et al. 2007).

27.2.3.7 Glucocorticoid Receptor

The GR CALUX assay was performed to assess the glucocorticoid-like (glucocorticoid receptor – mediated) activity of the JDS3 samples (van der Linden et al. 2008). Glucocorticoids are important steroid hormones controlling metabolism, immune responses and inhibition of inflammation as well as cellular proliferation (Sonneveld et al. 2007, van der Linden et al. 2008).

27.2.3.8 Thyroid Receptor

The potencies of the JDS3 samples to activate the thyroid hormone receptor – which plays a key role in growth, development and energy homeostatis – was investigated by employing the GH3-TRE-luc assay (Freitas et al. 2011).

27.2.3.9 Acetylcholinesterase (AChE) inhibition

The in vitro inhibition of acetylcholinesterase (AChE) is a rapid assay to determine the chemical interference with the enzymatic conversion of AchE to acetyl and choline in the neuronal synaptic cleft. The measurement of AChE inhibition has predominantly been associated with the neurotoxicity of organophosphate and carbamate insecticides, but may also be affected by other organic compounds of unknown structure (Holth and Tollefsen, 2007).

27.3 Results

27.3.1 Results of chemical screening

Of the 264 compounds which were analysed, 91 could be detected in at least one sample. Method detection limits determined based on the replicate analysis of calibration standards were below 2 ng/l for 156 compounds, between 2 and 5 ng/l for 67 compounds, between 5 and 20 ng/l for 30 compounds, and between 20 and 150 ng/l for 11 compounds.

Compared to the direct analysis of water samples (see chapter 26) the concentrations determined were in general up to a factor of 2 to 3 lower, which is exemplified for a set of compounds in Figure 143. The reasons for these differences are losses of compounds occurring during sampling (breakthrough through LVSPE cartridge) as well as sample and extract handling (sample transfer, evaporation). These compound losses could not be compensated for by internal standard addition, as an on-site extraction was conducted and the extracts were used for biological testing.

Furthermore, enriched LVSPE extracts showed about 2 times higher matrix effects as compared to water samples, which could only partly be compensated for by internal standard addition. Thus, it has to be stressed here that the concentrations determined by chemical screening of LVSPE extracts have to be considered as an underestimation of the real water concentrations. However, in contrast to direct water analysis of water samples, LVSPE provides the opportunity to directly compare chemical and ecotoxicological analyses. This needs to be considered when interpreting the chemical analytical and bioassay data.

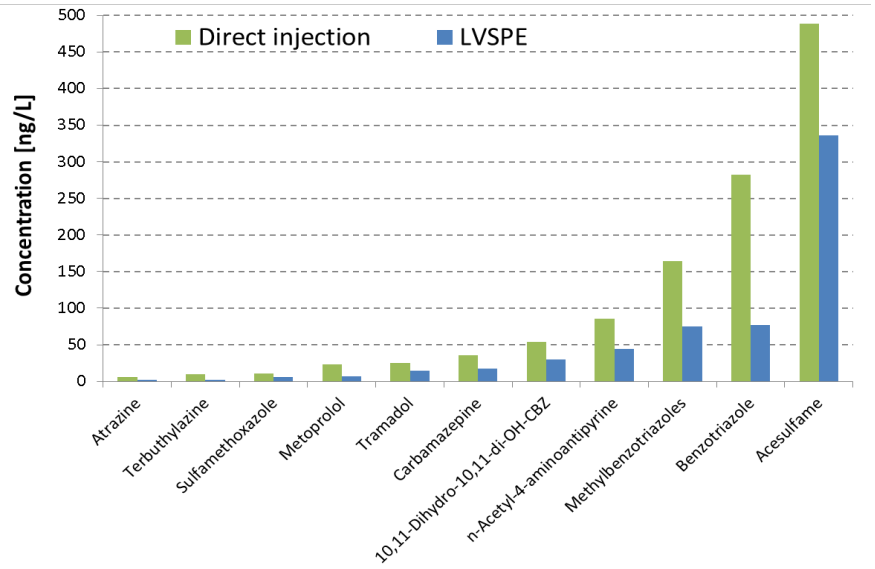
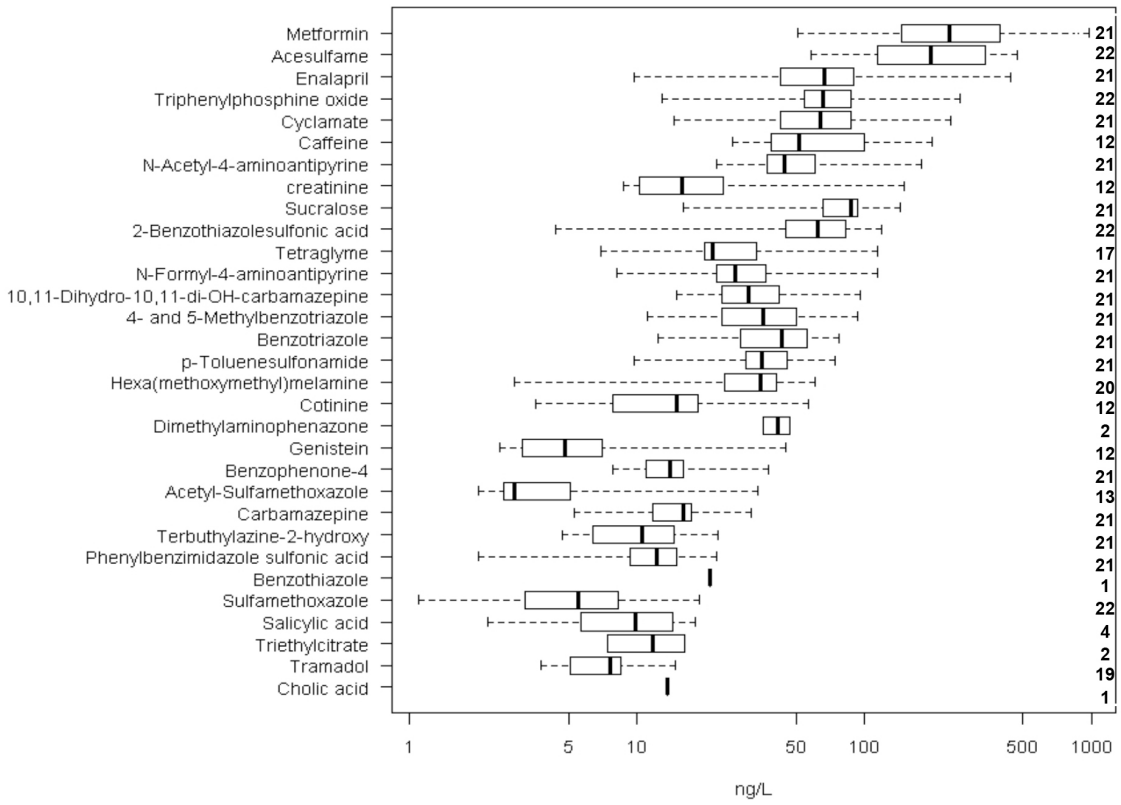
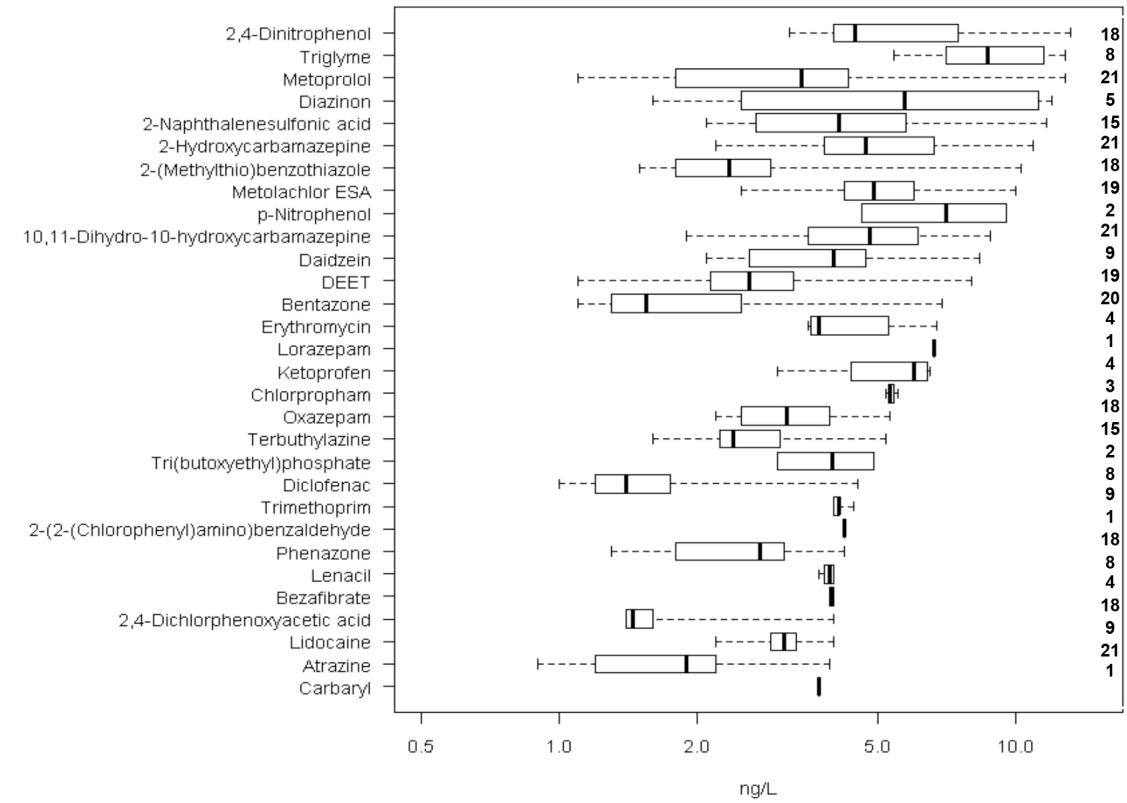


Figure 143: Concentration of selected compounds in sample JDS27 as determined by direct water injection (see chapter 26) and after LVSPE

An overview of the compounds detected and the concentration ranges is given in Figure 144. Among the compounds most frequently detected at relatively high concentrations were pharmaceuticals (metformin, enalalpril, carbamazepine), their transformation products (TPs; N-acetyl and N-formyl-4-aminoantipyrine, both derived from metamizole, TPs of carbamazepine), artificial sweeteners (acesulfame, cyclamate, sucralose), benzotriazoles and methylbenzotriazole corrosion inhibitors, and industrial chemicals such as benzothiazole sulfonic acid, triphenylphosphine oxide, p-toluenesulfonamide and hexa(methoxymethyl)melamine.

Widely used and legacy herbicides and their TPs (bentazone, atrazine, terbuthylazine, metolachlor, metolachlor ESA, isoproturon, mecoprop) were frequently detected at concentrations below 10 ng/l. Only in a small number of samples the insecticides diazinon (n=5), acetamiprid (n=2) and the fungicides carbendazim (n=9) and tebuconazole (n=1) concentrations were detected below 10 ng/l.


Metformin 21
Acesulfame 22
Enalapril 21
Triphenylphosphine oxide 22
Cyclamate 21
Caffeine 12
N-Acetyl-4-aminoantipyrine 21
creatinine 12
Sucralose 21
2-Benzothiazolesulfonic acid 22
Tetraglyme 17
N-Formyl-4-aminoantipyrine 21
10,11-Dihydro-10,11-di-OH-carbamazepine 21
4- and 5-Methylbenzotriazole 21
Benzotriazole 21
p-Toluenesulfonamide 21
Hexa(methoxymethyl)melamine 20
Cotinine 12
Dimethylaminophenazone 2
Genistein 12
Benzophenone-4 21
Acetyl-Sulfamethoxazole 13
Carbamazepine 21
Terbuthylazine-2-hydroxy 21
Phenylbenzimidazole sulfonic acid 21
Benzothiazole 1
Sulfamethoxazole 22
Salicylic acid 4
Triethylcitrate 2
Tramadol 19
Cholic acid 1
1 5 10 50 100 500 1000
ng/L


2,4-Dinitrophenol 18
Triglyme 8
Metoprolol 21
Diazinon 5
2-Naphthalenesulfonic acid 15
2-Hydroxycarbamazepine 21
2-(Methylthio)benzothiazole 18
Metolachlor ESA 19
p-Nitrophenol 2
10,11-Dihydro-10-hydroxycarbamazepine 21
Daidzein 9
DEET 19
Bentazone 20
Erythromycin 4
Lorazepam 1
Ketoprofen 4
Chlorpropham 3
Oxazepam 18
Terbuthylazine 15
Tri(butoxyethyl)phosphate 2
Diclofenac 8
Trimethoprim 9
2-(2-(Chlorophenyl)amino)benzaldehyde 1
Phenazone 18
Lenacil 8
Bezafibrate 4
2,4-Dichlorphenoxyacetic acid 18
Lidocaine 9
Atrazine 21
Carbaryl 1
0.5 1.0 2.0 5.0 10.0
ng/L

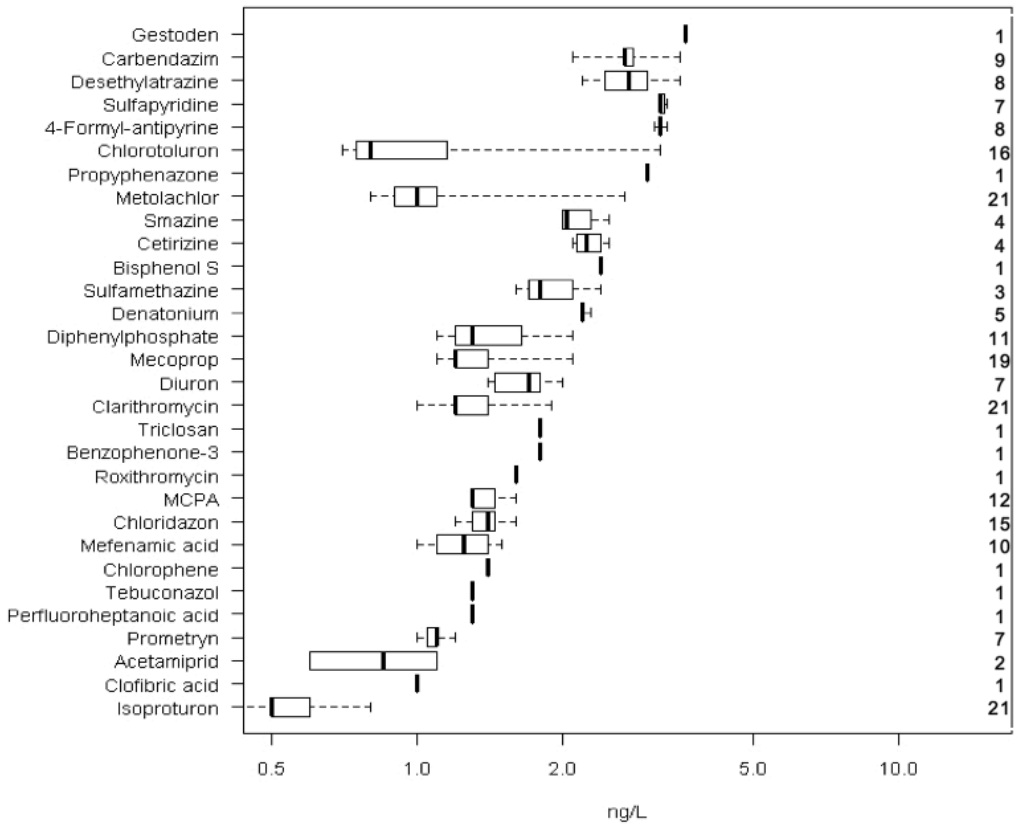


Figure 144: Overview of concentration of all 91 compounds detected in the 22 LVSPE samples; data are shown as median values, 25/75-percentiles (boxes) and maximum/minimum (whiskers); the values on the right denote the number of detections; note the logarithmic scales

27.3.2 Results of biological screening

The assessment of the LVSPE samples on a number of bioassays is currently in progress. Available preliminary results of the bioassays are summarized in Table 88. The conclusions are to be seen as preliminary and can only reflect the state of knowledge based on the currently available data.

Table 88: Summary of preliminary bioassay results as (A) qualitative and (B) semi- quantitative classification; tributaries are highlighted in red[a]

Station code	Name	Algae GI	Algae PSII	Ames -S9	Ames +S9	p53	ARE	NF-κB	ER	PXR	AhR	GR Calux®	GH3-TRE	AChE inhibition
	Classification	**(A)**	**(A)**	**(A)**	**(A)**	**(B)**	**(B)**	**(B)**	**(A)**	**(A)**	**(A)**	**(A)**	(A)	**(A)**
JDS8	Oberloiben	E-	E-	E	E	E-	E-	E	E-	NA	E	N	C	N
JDS22	Budapest downstream M0 bridge	N	E	E-	E	E-	E-	E-	E	E-	E	N	C	N
JDS27	Hercegszanto	E-	N	N	E	E-	E-	E	E	E	E	N	C	N
JDS29	/Drava (rkm 1.4)	P-	E	N	E	E-	E-	E-	E	E	E-	N	C	E
JDS30	Downstream Drava (Erdut/Bogojevo)	N	E	N	E	E-	E-	E	E	E-	E-	E-	C	N
JDS32	Upstream Novi-Sad	NA	NA	N	E	E-	E	E-	E	NA	E	N	C	N
JDS33	Downstream Novi-Sad	N	N	E-	E	E-	E-	E-	E	E	E	E-	C	N
JDS35	/Tisa (rkm 1.0)	E-	E	N	E	E-	E	E	C	E	E	E	C	N
JDS36	Downstream Tisa/Upstream Sava (Belegis)	E	E-	E-	E	E-	E-	C	N	NA	E	E	C	N
JDS37	/Sava (rkm 7.0)	E	E	N	N	E-	E-	E	E-	E	E-	E-	C	E
JDS39	Downstream Pancevo	E-	E	NA	NA	E-	E-	E-	E-	E	E	E-	C	N
JDS41	/Velika Morava	E-	E-	N	E-	C	E	C	E	E	E	N	C	N
JDS44	Irongate reservoir (Golubac/Koronin)	E-	E	N	N	E-	E	E	E	E	E	E-	C	E
JDS53	Downstream Zimnicea/Svishtov	E	E-	N	E	N	E-	E-	N	NA	E-	N	C	N
JDS55	Downstream Jantra	E-	E-	N	E	C	C	E-	N	NA	E-	N	C	N
JDS57	Downstream Ruse	E	E-	N	E-	E-	E-	E-	N	E	E	N	C	N
JDS59	Downstream Arges	E	E-	N	E	E-	E	E-	E-	E	E	N	C	N
JDS60	Chiciu/Silistra	E	E-	N	E	E-	E	E-	N	NA	NA	N	C	N
JDS63	/Siret (rkm 1.0)	E-	E-	E	E	E-	E	E-	C	E	E	N	C	N
JDS64	/Prut (rkm 1.0)	P	N	N	E-	N	N	N	N	NA	NA	N	N	E-
JDS65	Reni	E	E-	E-	E	E-	E	E-	C	NA	NA	N	C	N
JDS67	Sulina – Sulina arm	E-	E-	N	N	E-	C	E-	C	E-	E	N	C	N

[a] E: effect, E-: weak effect; E+: strong effect, N: no effect; C: cytotoxic effect, C-: weak cytotoxic effect, NA: not yet analysed

27.3.2.1 Growth inhibition of Chlamydomonas reinhardtii

Screening the extracts at REF100 in the assay identified that all extracts caused some level of growth inhibition, with 3 extracts being non-toxic, 9 were weakly toxic and 10 were toxic to the algae. Similar screening using PSII inhibition as an endpoint revealed that 2 were non-toxic, 11 were weakly toxic and 9 were toxic to the algae. The corresponding negative controls also showed some degree of toxicity in the bioassays tested at a REF100, thus indicating that introduction of toxic compounds in the extraction process may have occurred and thus potentially overestimating the toxicity of certain extracts.

27.3.2.2 Mutagenicity

All samples were tested in 3 replicates at a REF of 1000, since no cytotoxicity occurred. Such effects would cause a decrease in revertant numbers and thus lead to false positive results. The blanks were not mutagenic. In the combination of TA98 without S9, mutagenicity was found for the sites JDS8 and JDS63. Weak effects were observed for samples of the sites JDS22, JDS33, JDS36 and JDS65. For the combination of TA98 with S9 an increased number of active samples were proven.

All samples – except the sites JDS37, JDS44, JDS67 and JDS39 (not analysed) – showed a mutagenic potential by the use of S9. However, the samples JDS41, JDS57 and JDS64 showed only weak mutagenicity. This indicates that at a large number of sites substances entering the River Danube and not being mutagenic as parent compound might get bioactivate and enhance mutagenic potential upon metabolism by organisms in the water column.

27.3.2.3 Adaptive Stress Responses

The adaptive stress response assays targeted oxidative stress (ARE-BLA), inflammation (NF-κB-BLA) and p53 mediated apoptosis in response to DNA damage (p53-BLA). In the ARE-BLA assay, which responds to chemicals that produce reactive oxygen species and those that are direct electrophiles, 8 samples were positive (JDS32, 35, 41, 44, 53, 59, 60, 65, 67), 11 were weakly positive (JDS8, 22, 27, 29, 30, 33, 36, 37, 39) and one had no effect up to a REF of 500 (JDS64). JSD64 also had no effect in the NF-κB-BLA and p53-BLA assays. Two samples were cytotoxic (JDS55, 67) and thus oxidative stress response could not be ruled out but was masked by cytotoxicity.

The samples tended to have less effect in the p53-BLA assay, which responds to genotoxic chemicals, with no samples having a positive response in the p53-BLA assay. Instead, the majority of the samples were only weakly positive, 4 were cytotoxic (JDS41, 55, 57, 63) and 2 had no effect (JDS 53, 64). In the NF-κB-BLA assay 6 samples were positive (JDS8, 27, 30, 35, 37, 44), 13 were weakly positive (JDS22, 29, 32, 33, 39, 53, 55, 57, 59, 60, 63, 65, 67) and one had a similar effect as the control samples (JDS64). Cytotoxicity masked induction for JDS36 and 41.

27.3.2.4 Estrogen Receptor

Several samples were found to be cytotoxic in the MELN assay at a REF of 300 and above. Also, a slight effect was seen in one of the two blanks samples at a REF of 1000. Therefore only effects observed at a REF of 100 or below were considered as positive in the assessment of estrogenic activity. Estrogenic activity was found in several of the JDS samples, at non cytotoxic concentrations. The most active samples were JDS41 (positive response at a REF<3) and JDS22, 27, 29, 30, 32, 44 (positive response at a REF comprised between 3 and 30). In these samples, concentrations of estradiol-equivalents (E2-EQ) are in the 0.01-0.1 ng E2-EQ / L range. However, these values have to be refined by establishing complementary assays and thus are preliminary.

27.3.2.5 Aryl hydrocarbon Receptor

Most of the samples were cytotoxic in the CAFLUX assay at high concentrations and the cytotoxicity prevented the assessment the dioxin-like potential at relative enrichment factor (REF) of 500 and in case of some samples even at REF of 167. Dioxin-like activity was detectable in most of the JDS samples at non cytotoxic concentrations. Available preliminary data indicate that it may be in some samples below limit of quantification of toxic equivalent (TEQ) relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In case where sufficient induction was reached, the EC_{20} values of dioxin-like response (concentrations causing 20% response relative to maximum induction caused by TCDD in the bioassay) were for most samples in the range of REF from 10 to 100. When quantifiable, the dioxin-like equivalent occurred in the LVSPE samples in the pg TEQ/L range and it did not differ greatly among sites. According to preliminary results, samples JDS22 and JDS59 belong among those with relatively greater dioxin-like activity. Similar levels of TEQ (6-10 pg/l) were also determined from passive sampling with silicone rubber samplers (see chapter 29). Presence of AhR-active substances at comparable concentrations have been previously detected by bioassays in water from other European rivers (Jálová et al. 2013).

27.3.2.6 Pregnane X Receptor

All the 12 samples tested so far were able to activate the PXR in this assay. These effects were observed at relatively high concentrations, i.e. at REF ranging from 30 to 300. No cytotoxic effect was observed at these concentrations and the blanks were negative, thus suggesting that PXR active substances are indeed present in the JDS samples. Similarly to these findings, previous studies have shown the widespread occurrence of PXR activity in different environmental matrices such as wastewater effluents, river surface water or sediments (Creusot et al. 2013, Creusot et al. 2010).

Although identification of environmental PXR ligands is still a matter of research, phthalates and alkylphenols were identified as contributors to PXR activity in French river sediments (Creusot et al. 2013).

27.3.2.7 Glucocorticoid Receptor

All of the 22 samples were tested in 3 replicates at a REF 100, since acute cytotoxicity in the neutral red assay with U2-OS (GR-CALUX) cells were observed for all of the 22 samples at REF >100-250. In the GR-CALUX, cytotoxicity would reduce luminescent cells and thus signal strength, which would deliver false negative findings. Receptor-mediated endocrine activity in the GR-CALUX could be demonstrated for the samples JDS30, JDS33, JDS35, JDS37, JDS39 and JDS44. Blanks showed no activity in the GR-CALUX.

27.3.2.8 Thyroid Receptor

None of JDS samples caused induction in the GH3-TRE assay, but were cytotoxic at high REFs. The lack of induction of the thyroid receptor was not unexpected because most environmental chemicals that interfere with the thyroid function are not binding to the thyroid receptor but are rather goitrogens, which suppress the function of the thyroid gland by interfering with iodine uptake, such as inorganic oxyanions, such as perchlorate and nitrate (Pickford 2010), which would not be extracted by LVSPE samples and which would not be active in the GH3-TRE assay. Relatively few organic chemicals that could be present in are active in the T-Screen assay (Freitas et al. 2011, Schriks et al. 2006). The results of absence of thyroid receptor agonist were consistent with previous studies on water samples (Escher et al. 2014, Inoue et al. 2009, Jugan et al. 2009).

27.3.2.9 Acetylcholinesterase (AChE) inhibition

All extracts from the JDS3 were screened for AChE inhibition using a maximum REF of 100. The results showed that 18 were non-toxic, 1 was weakly toxic and 3 were toxic to the algae. Interestingly, 2 out of 3 solvent blanks caused AChE inhibition at the REF used in the assay. Potential contamination by toxic compounds introduced by the extraction and use of solvents should therefore be determined to assess to which degree this affects the toxicity of the extracts.

27.4 Conclusions

Large volume solid phase extraction was successfully applied at 22 sampling sites of the JDS3 to realise effect-based screening in a river basin scale for the first time. The samples were analysed with liquid chromatography – high resolution mass spectrometry for semi-polar to polar organic compounds as well as a set of 9 *in vitro* and 2 *in vivo* bioassays to assess the mode of action of organic compounds present in the samples.

The chemical screening resulted in the detection of 91 compounds in at least one sample. Among mostly identified in relatively high concentrations were pharmaceuticals, their transformation products, artificial sweeteners, corrosion inhibitors, and industrial chemicals. Widely used and legacy herbicides and their TPs were frequently detected. It must be stressed that the concentrations determined were in general up to a factor of 2 to 3 lower than corresponding analyses by direct water injection and thus an underestimation of real water concentrations is obvious.

Despite the overall low concentrations of organic compounds compared to other rivers in Europe (Loos et al. 2010, ter Laak et al. 2010), all extracts were effective in one or more bioassays with the endpoints mutagenicity, dioxin-like and PXR mediated activity, oxidative stress responses, and estrogenicity as well as growth inhibition and Photosystem II inhibition of green algae. The sample with lowest toxicity was JDS64 (Prut) that only showed weak mutagenicity (after S9 activation) and neurotoxicity. Samples JDS33 (downstream Novi Sad) and JDS63 (tributary Siret) were among the most toxic samples, which were effective in almost all bioassays.

The bioassays are not fully evaluated and thus the toxicological potentials of the samples might be over- or underestimated. Presented conclusions are therefore to be seen preliminary as well and can only reflect the state of knowledge based on the available data.

Finally, this study demonstrated the feasibility of an effect-based screening in a river basin wide scale using on-site LVSPE even under conditions of high dilution such as in Danube River.

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28 Biomarkers: In-situ detection of genotoxicity of the Danube River in mussels and fish

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28.1 Introduction

For the assessment of the ecotoxicological status of European water bodies, biomarker response analyses provide a wide range of valuable information. Biomarkers can be defined as substance or mixture induced variations on different levels of biological organization, which can be measured in tissues, body fluid samples or organisms (Depledge 1993). Under consideration of the ecological relevance, biomarkers have the potential to fill the gap between chemical/*in-vitro* data and effects on organism and population level and enhance the integrative "weight-of-evidence" approach. The utilization of biomarker response analysis in water quality assessment provide a tool for the early detection of effects which are potentially affecting wild living populations and can supply a profile characterization of biological impacts of river sites, even if the classification of the chemical and/or ecological status illustrate no/less anthropogenic interferences (Sanchez and Porcher 2009). Furthermore, *in-situ* analysis in wild fish base on a realistic exposure scenario under consideration of the fate and bioavailability of chemicals (Chapman and Hollert 2006).

The integrity of cellular DNA is continuously attacked by various agents in the environment resulting in DNA lesions such as strand breaks, modified bases, DNA–DNA crosslinks and DNA–protein crosslinks. Unrepaired DNA lesions may block replication and transcription, potentially leading to cell death, or may give miscoding information, generating mutations. As a result, a number of biological consequences can be initiated at the cellular and organ levels, whole animal and finally community and population (Jha 2008). The benefit of using genotoxic endpoints in organisms is the potential correlation of DNA-damages with adverse reproductive effects which can be directly correlated with effects on population level, e.g. mutational meltdown (Boettcher et al. 2010).

Previous studies demonstrated the applicability of freshwater mussels and fish in the ecogenotoxicology (Sunjog et al. 2012; Kolarević et al. 2013; Sunjog et al. 2013; Vuković-Gačić et al. 2013; Gačić et al. 2014; Sunjog et al. 2014). Mussels have several characteristics, such as wide distribution, filter feeding, a sessile life form and an ability to accumulate pollutants, which makes them favourable organisms for estimating the environmental pollution level and the bioavailability of various types of pollutants (Roméo et al. 2003; Andral et al. 2004; Amiard et al. 2006). Fish are also used as sentinel organisms due to their role in food webs, human nutrition, their potential for bioaccumulation of toxic substances, and their sensitivity to even low concentrations of mutagens (Szefer et al. 1990; Višnjić-Jeftić et al. 2010). Due to the importance for human health aspects, changes in the health status of fish and accumulation of hazardous substances in fish tissues are of particular interest for authorities and the public. As a result of the higher length of aquatic food webs – in comparison to its terrestrial pendants – higher predators tend to accumulate higher amounts of pollutant substances (Di Giulio and Hinton 2008) which may result in increased effect levels at the sites of toxic action and leads to potentially higher biomarker responses

The comet assay, also known as single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for the detection of DNA damage in individual cells based on the migration of denatured DNA during electrophoresis, in which damaged nuclei form comet-like shapes. Comet assay has been accepted as one of the major tools for assessing pollution related genotoxicity in aquatic organisms

(Dixon et al. 2002). Losses of chromosomes or chromosome fragments in fish cells which occur during mitosis and were not reincorporated in the nucleus after cell division can be examined by the evaluation of the micronucleus frequency of peripheral erythrocytes.

28.1.1 Aims and goals

The aims of the research were the following:

- Detection of the genotoxic pollution in investigated sections of the Danube River by comet assay in haemolymph of mussels *Unio sp.* (*Unio pictorum* and *Unio tumidus*) as well as *Sinanodonta woodiana,* and peripheral erythrocytes of fish *A. alburnus* as well as *Neogobius sp.;*
- Detection of the genotoxic pollution in investigated sections of the Danube River by micronucleus assay in peripheral erythrocytes of *A. alburnus;*
- Identification of the hotspots of genotoxic pollution;
- Comparison of the response to genotoxic pollution in autochthonous and alochthonous species of mussels and fish;
- Comparison of response to genotoxic pollutions in fish with different habitat preferences;
- Comparison of two different methods for the evaluation of genotoxic biomarker response in fish.

28.2 Methods

28.2.1 Comet assay

Specimens of *Unio sp.* were collected from 31 sites while specimens of *S. woodiana* were collected from 15 sites. Specimens of *A. alburnus* were caught at 12 and *Neogobius sp.* at 13 sites. The research was performed on total of 217 specimens of mussels and 98 specimens of fish. When possible, for each sampling site, samples of haemolymph/blood were collected from 4 specimens of each investigated species. Appropriate dilutions of sampled tissues were made in physiological solutions and subjected to comet assay.

The alkaline comet assay procedure used is basically as described by Singh et al. (1988). Cells were embedded in 1% low melting point agarose on slides precoated with 1% normal melting point agarose and subjected to lysis into freshly made cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1.5% Triton X-100, pH = 10) for 3h. To allow DNA unwinding, slides were placed into electrophoresis chamber containing cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH = 13) for 20 min. Electrophoresis is performed by setting the power supply at 0.75 V/cm and adjusting the current to 300 mA for 20 min. After electrophoresis the slides were placed into freshly made neutralizing buffer (0.4M M Tris, pH = 7.5) for 15 min followed by fixation of the slides in ice cold methanol. Slides were stored in dark boxes and transferred at the laboratory of the Center for Genotoxicology and Ecogenotoxicology-Chair of Microbiology, Faculty of Biology, University of Belgrade, Serbia. Slides stained with acridine orange (2 µg/ml) were examined with a fluorescence microscope (Leica, DMLS, Austria) at 400x magnification with an excitation filter of 510-560 nm, barrier filter of 590nm. Images of 50 cells were analyzed from each slide using Comet IV Computer Software (Perceptive Instruments, UK) and among the parameters available for analyses Tail intensity% (TI%) was chosen as relevant measure of DNA damage (Figure 145).

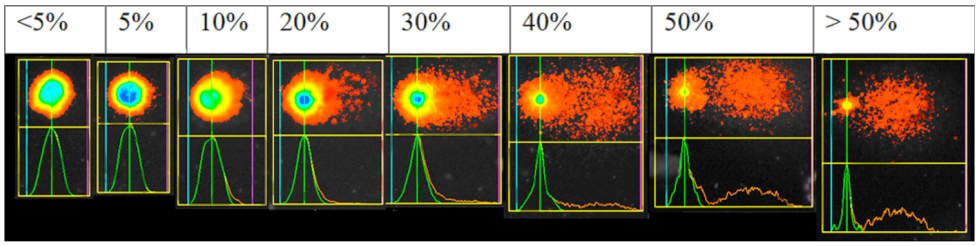

Figure 145: Representative micrographs of scored comets showing different levels of DNA damage (Tail intensity%)

28.2.2 Micronucleus assay

On top of 99% ethanol-cleaned microscope slides two smears per individual were made and subsequently fixed in methanol. The microscopical examination was performed at the Institute for Environmental Research, RWTH Aachen University, Germany. Previous to the visual evaluation of the blood samples, the smears were treated by adding one drop of a 0.2 µm MCE membrane filtered 0.004% acridine orange solution (m/v) in phosphate buffered saline (PBS) on the microscope slide. The samples were evaluated by using a Nikon Eclipse E400 epi-fluorescence microscope at a 1000 fold magnification. The total number of analyzed erythrocytes per individual amounted to 4000 (2000 erythrocytes per smear). Scoring criteria according to Huber et al. (1983) and Titenko-Holland et al. (1998).

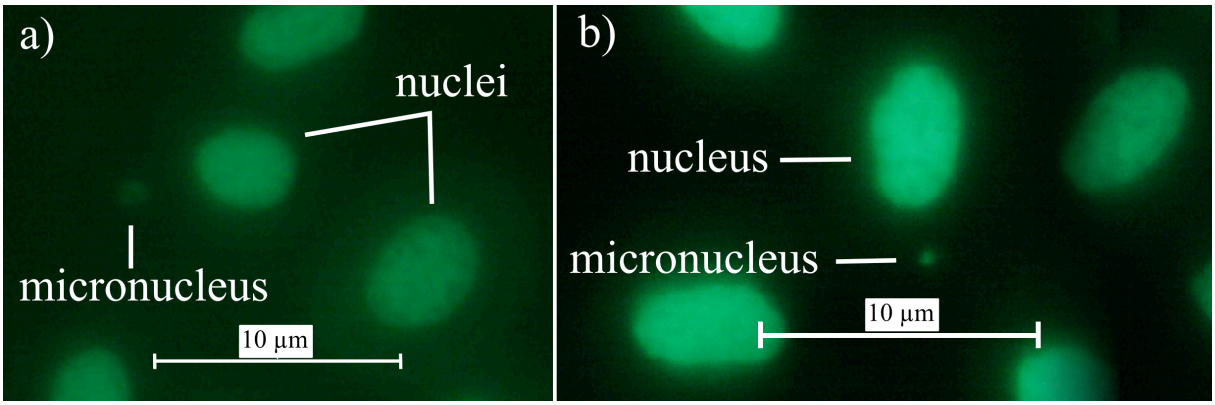

Figure 146: Fluorescence microscope pictures of micro nucleated and non-micro nucleated peripheral erythrocytes of *Alburnus alburnus*

28.2.3 Index of condition (fish)

The index of condition basically represents the nutritional condition of fish and can be used to compare the health/dietary conditions of different fish groups of the same species in a water body. In this study the index of condition was used to exclude a relationship between the health status and genotoxic response in different fish groups collected along the Danube River.

The index of condition was calculated in accordance with Fulton (1902).

28.3 Results

28.3.1 Comet assay

The results of the comet assay are shown in Fig. 147. Shown values are individual average TI% for each specimen. The results are analysed in light of the Danube River section types with borders suggested by Robert et al. (2003).

The lowest level of DNA damage for *A. alburnus* was detected at the site JDS27 (5.7 ± 2.3) while the highest level was recorded in specimens collected at the site JDS47 (28.3 ± 2.8). For *Neogobius sp*., the lowest level of damage was detected at the site JDS67 (3.8 ± 2.5) and the highest level in specimens collected at the site JDS6 (28.3 ± 2.8). For both *Unio sp*. and *S. woodiana*, the lowest level of damage was detected at the site JDS52 (5.8 ± 3.0 and 5.0 ± 1.9 respectively) and the highest level in specimens collected at the site JDS31 (28.8 ± 12.2 and 21.9 ± 9.9 respectively). Significant correlation in the DNA damage level at different sites was detected between autochthous species of mussels *Unio sp.* and allochthonous species *S. woodiana* ($n = 13$, $r = 0.73$, $p = 0.005$). Considering fish, correlation in response between autochthous species *A. alburnus* and allochthonous species *Neogobius sp*. was not significant ($n = 11$, $r = 0.37$, $p = 0.26$).

In section type II, increased values of TI% were noticed in specimens of *Neogobius sp.* and *Unio sp*. which can be the influence of Kelheim and Deggendorf.

In section type III, significant decrease of DNA damage was observed at the site JDS8 in specimens of *A. alburnus* and *Neogobius sp*.

In section type IV, the level of DNA damage in specimens of *A. alburnus* and *Neogobius sp*. did not differ significantly from the values detected in section III. Slight increase of Ti% was noticed in specimens of *S. woodiana* at the site JDS15. The effects of Vienna and Bratislava were not detected.

In section type V, values of DNA damage in specimens of *Unio sp*. and *S. woodana* were similar to ones measured in section type IV. The impact of Budapest was not evident.

In section type VI, the lowest level of DNA damage was recorded in specimens collected at the site JDS27. The influence of the Drava River was evident at the sites downstream the confluence. The highest values of TI% were measured in mussels collected at the site JDS31. The influence of the Sava River was evident at the sites JDS38 and JDS39 where gradual decrease of DNA damage was observed. The effects of industry in towns Smederevo and Kostolac were evident in high levels of DNA damage recorded in specimens at the sites JDS40 and JDS42.

In section type VII, gradual decrease of DNA damage was noticed. Specimens of *Unio sp*. collected at the site JDS44 had significantly lower values when comparing with the site JDS43.

In section type VIII, the highest level of DNA damage was recorded in specimens of *A. alburnus* at the site JDS47. Increased level of DNA damage was also recorded in specimens of *S. woodiana* and *Neogobius sp.* at the sites JDS53 and JDS54 respectively.

In section types IX and X, there were no significant variations in the level of DNA damage in specimens of *A. alburnus* and *Neogobius sp.* In section type X, significantly higher level of DNA damage was detected in specimens of *Unio sp.* at the sites JDS66 and JDS67 comparing to specimens at the site JDS68 and from section type IX.

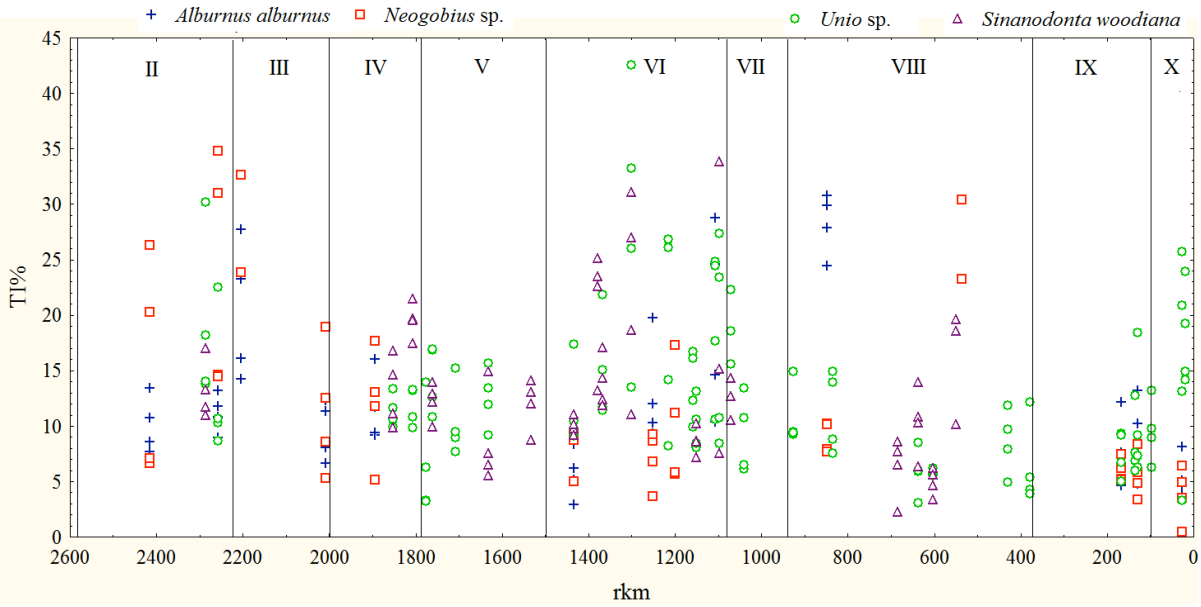

Figure 147: The level of DNA damage expressed as Tail intensity% (TI%) measured in erythrocytes of fish (*Alburnus alburnus* and *Neogobius sp.*) and haemocytes of mussels (*Unio sp.* and *Sinanodonta woodiana*). Values represent average of 50 nuclei scored for each specimen

28.3.2 Micronucleus assay

For the micronucleus assay 18 sampling sites were evaluated. The longitudinal profile of the micronucleus formation in erythrocytes of *A. alburnus* demonstrated differences along the Danube River (Fig. 148). The Danube River section types according to Robert et al. (2003).

Significant differences could be shown for sampling sites JDS10, 31, 36, 60, 65 and 67 in comparison to the reference site JDS48 (site with the lowest values for micronucleus formation). The mean values for the micronucleus formation in erythrocytes ranged between 0.39±0.25 ‰ (JDS48) and 5.76±5.54 ‰ (JDS60).

In section type IV, significantly increased values for micronucleus formation for JDS10 (downstream Vienna) observable. Micronucleus formation may be influenced by the capitol catchment area.

In section type VI, generally elevated values for the micronucleus formation of the Serbian/Croatian stretch of the Danube River (JDS31-40).

In section type VIII, lowest values of all 18 evaluated sampling sites (JDS48 and 54) downstream of the Iron Gate region and the tributaries Timok and Iskar. Increase of the micronucleus frequency on the levels of the Serbian/Croatian stretch downstream the tributaries Jantra and Lom.

In section type IX, significantly elevated values of DNA damages demonstrated for JDS60 downstream the tributary Arges in the catchment area of the metropolitan region of Bucharest/Ruse. Decrease of the micronucleus formation within the following 205 km. In comparison to the reference site (JDS48) significantly evaluated values for JDS65 downstream the tributaries Siret and Prut.

In section type X, significantly elevated micronucleus formation.

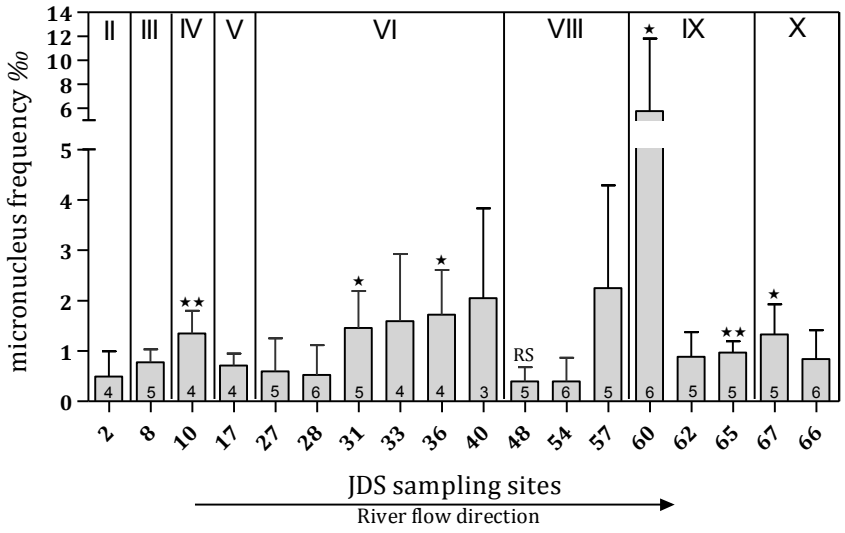

Figure 148: Longitudinal profile of the micronucleus frequency (MN [‰]) in erythrocytes of *Alburnus alburnus* in the Danube River. Each bar represents mean data of *Alburnus alburnus* with at least 4000 erythrocytes counted per fish. Total numbers of fish utilized for determination of the micronucleus frequency are listed in each bar. Error bars represent the standard deviation. Asterisks depict significant differences between sampling sites and reference site (JDS48; Reference site of low micronucleus frequency). Student`s t-test was performed for data which passed the test for normality and variance homogeneity. If data set failed those criteria the Wilcoxon rank-sum test was performed. (*): p ≤ 0.05; (**): p ≤ 0.005.

28.3.3 Correlation of comet and micronucleus data

For 9 sampling sites of the Danube the same blood samples were subjected to corresponding biomarker response analysis in the comet and micronucleus assay. The data demonstrated a significant correlation in response of both biomarker analysis (n = 9, r = 0.72, p = 0.028).

28.3.4 Index of condition (fish)

Under consideration of the index of condition (Fig. 149) as a marker for the dietary and general health status of the fish, no significant correlation of the genotoxicity in *A. alburnus* (r = -0.43, p = 0.16) and *Neogobius sp.* (r = 0.26, p = 0.39) could be observed for the comet assay. The same applies for the micronucleus assay with *A. alburnus* (r = 0.32, p = 0.20). Therefore, it can be assumed that the genotoxic effects in fish of the Danube are directly correlated to the amounts of stressors in the water body, sediments or food web.

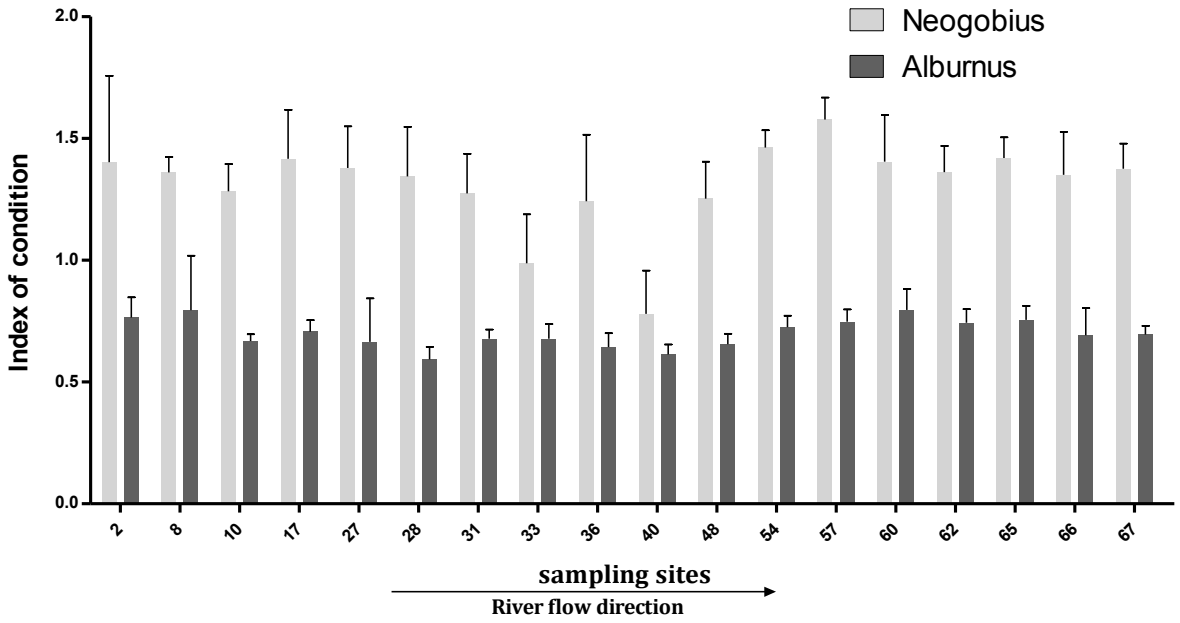

Figure 149: Longitudinal profile of the index of condition of *Neogobius sp.* and *Alburnus alburnus* in the Danube River. Each bar represents mean data of all collected *A. alburnus* and *Neogobius sp.* at the investigated sites. Error bars represent the standard deviation

28.4 Conclusions

- Significant variations in DNA damage levels as determined using the comet assay and the micronucleus assay were observed for different sampling sites for all selected species of mussels and fish. Significantly elevated values of the micronucleus frequency in erythrocytes of *A. alburnus* were demonstrated in the micronucleus assay for the sites JDS10, 31, 36, 60, 65 and 67. In the comet assay, cumulated highest levels of genotoxic response in *Unio sp.* and *Sinanodonta woodiana* were observed for sampling sites in section type XI.
- The highest levels of DNA damages were observed in specimens collected in section VI (comet assay) and sections VI and IX (micronucleus assay).
- In section VI and IX, the effects of urban and industry centres were evident as well as the impact of the tributaries.
- The metropolitan region of Bucharest/Ruse showed significant highest values of micronucleus formation in erythrocytes of *A.alburnus*. Lower values in the comet assay at these sites may indicate different genotoxic modes of action.
- No correlation between the dietary/health status of fish and genotoxicity in erythrocytes was found. Genotoxicity due to chemical stressors in water matrix and sediments can be assumed. Differences in food sources and resulting uptake of pollutants via the gastrointestinal system may also be conceivable.
- Habitat preferences of fish did not affect differences in genotoxicity.

- Significant correlation in the level of DNA damage was detected between autochtonous and alochtonous species of mussels (*Unio sp.* and *S. woodiana*) while between autochtonous and alochtonous species of fish (*A. alburnus* and *Neogobius sp.*) correlation was insignificant.
- Significant correlation between comet and micronucleus data of *A.alburnus* was found for 9 corresponding sampling sites, which indicates the strength of the data sets.

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29 Passive sampling: chemical analysis and toxicological profiling

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29.1 Introduction

Organic pollutants are often present in the water column at trace concentrations that are difficult to detect when conventional low volume spot sampling of water is applied. The scope of the sampling campaign performed using passive samplers was the screening of trace organic pollutants and their toxic potentials in the water column of the Danube, as well as the assessment of their spatial distribution along the river.

Freely dissolved concentrations of priority substances in the water phase (c_{free}) can be derived from the uptake of these substances by passive samplers, and because accumulated contaminants represent a large water volume, low limits of quantification can be obtained. C_{free} is a more stable parameter than a concentration measured in whole water as the level is not influenced by variable amounts of the substance bound to dissolved and suspended particulate organic matter. Thus, it is very suitable for assessment of trends. C_{free} is further considered to play a key role in chemical uptake by aquatic organisms. It is proportional to the chemical activity (Mayer et al., 2003) and if in equilibrium with surrounding environmental compartments it also represents chemical activity of those environmental compartments, including the biota at the base of the food chain (Reichenberg and Mayer, 2006).

We used an "active" passive sampling system (APS) for temporally and spatially integrative sampling of trace organic pollutants. APS is used in a concept similar to that of a Ferry-Box ("Website of the European Ferrybox Community," 2014) to obtain a representative picture of pollution situation along defined stretches or transects of large water bodies including rivers, lakes or seas. The uptake principle in the APS remains the same as in classical static passive sampling and the monitoring results can be evaluated using usual passive sampler calibration parameters. The APS enhances the uptake rate of contaminants into passive samplers, thereby allowing to drastically reduce the exposure time needed for accumulation of sufficient chemicals for analysis.

The application of temporal- and spatial- integrative passive sampling approach resulted in samples that provide a representative picture of pollution situation in eight defined stretches of the Danube River.

29.2 Methods

29.2.1 Passive samplers

Three types of passive samplers were applied: two partitioning samplers for hydrophobic compounds (silicone rubber (SR) and low density polyethylene (LDPE) sheets), and an adsorption sampler for polar compounds based on styrene-divinylbenzene solid phase extraction disks, SDB-RPS Empore disks (ED), respectively.

The SR sampler consisted of a single Altesil® SR sheet with dimensions 14×28 cm and 0.5 mm thickness. The mass of a sampler was cca 23 g and the surface area exposed to water was 392 cm^2 (one side of the sheet). SR samplers (except those intended for the ecotoxicological analysis) were spiked

prior to exposure with a number of Performance Reference Compounds (PRCs) that are partially released during exposure. The residual concentration of PRC is compared with the initial amount of PRCs analysed in samplers that have not been exposed.

The LDPE sampler consisted of two strips 4×28 cm and 80 µm thickness (cut from 2.5 cm wide lay-flat LDPE tubing from Brentwood Plastics Inc, St. Louis, USA). LDPE samplers were also spiked with PRCs and were used for chemical analysis only.

The ED sampler consisted of 10 solid phase extraction disks Empore® SDB-RPS with 47 mm diameter. The mass of a sampler was cca 3.2 g and the surface area exposed to water was 173 cm^2. Before exposure samplers were pre-conditioned and kept immersed in MilliQ water until exposure. These samplers were not spiked with PRCs.

29.2.2 Sampling operation

The "active" passive sampling system was installed on board of the expedition ship Argus to obtain enhanced passive sampler uptake rates in order to achieve sufficient sensitivity despite the short time available for sampling.

The APS device consists of a rectangular stainless steel plate box. During operation the box remained open from two sides and it was fully immersed in water. One end of the box was connected to a submersible pump (cca 9 m^3 h^{-1}) that forced water at high flow velocity (1-2 m s^{-1}) through the exposure chamber. A submersible temperature and light intensity logger was attached to the box during the entire cruise. Two parallel APS devices were in operation during each sampling period. The samplers exposed in one device were used for chemical analysis, and those from the other one for ecotoxicological analysis, respectively.

The APS device was deployed on the frontal deck of the Argus. For sampling, the device was immersed in a flow-through system that consisted of a 600 l stainless steel tank. The river water in the tank was exchanged at a rate cca 3 m^3 h^{-1} by a high performance pump. The water intake to the chamber was by a vertical steel pipe positioned in front of the ship. The water sampling depth was cca 0.5 m below the water level.

The device was operated only during the cruising of the ship or when the ship anchored outside harbours (e.g. for sampling) in areas not visibly impacted by point sources of pollution, e.g. discharge pipes, industrial areas next to the river, oil film visible on the water surface. The device was switched off before the ship entered harbours and switched on again when the cruise resumed. Samplers were mounted to the APS device just before exposure and removed immediately after recovery. The recovered samplers were placed back into their storage containers. They were stored in a refrigerator at 4°C on board of the ship and transported to the processing laboratory once per week, where they were stored in a freezer at -20°C.

Each individual water sampling period took approximately 5 days. During this period ship moved downstream along a defined stretch. The obtained sample contained water pollutants integrated in time and space along that stretch. Samplers were exchanged every 5 days, which resulted in total of eight samples of each type (SR, LDPE and ED) representing eight stretches of the Danube (Table 89). Sampling periods were planned so that exposure was avoided during days when ships stopped in harbours for one day or longer.

Table 89: River stretches sampled with passive samplers deployed from the Argus ship

Stretch number	Stretch start and end	River km	Dates of cruise	Mean water temperature [°C]	Exposure time [d]	Volume extracted by SR [l][1]
1[2]	Regensburg-Passau	2375-2225	13.8.-16.8.	-	-	-
2	Passau-Bratislava	2203-1852	17.8.-22.8.	21.3	2.0	169
3	Bratislava-Budapest	1852-1632	22.8.-26.8.	22.0	1.2	84
4	Budapest-Vukovar	1648-1297	26.8.-2.9.	21.9	1.7	139
5	Vukovar-Belgrade	1297-1154	2.9.-6.9.	22.8	1.6	133
6	Belgrade-Turnu-Severin	1154-930	6.9.-10.9.	22.1	2.0	139
7	Turnu-Severin-Ruse	930-495	11.9.-17.9.	21.9	2.0	129
8	Ruse-Braila	495-170	17.9.-21.9.	19.2	1.4	79
9	Braila-Tulcea	170-71	21.9.-26.9.	18.7	1.3	72

[1] Volume of water extracted by the SR sampler during exposure; it is calculated for a model compound with molecular mass of 300.
[2]The stretch from Regensburg to Passau was not sampled due to initial technical difficulties with sampler installation.

29.2.3 Sample processing

SR samplers (except those intended for the ecotoxicological analysis) were spiked with recovery internal standards. Compounds sorbed in the SR sheet were extracted for 8 hours in methanol using Soxhlet extraction. The volume of the extract was reduced using Kuderna-Danish (K-D) apparatus and under nitrogen flow to a volume of 2 ml. For ecotoxicological analyses, the sample in methanol was divided to aliquots for different types of bioassays. For chemical analyses, a 20% aliquot of the sample was used for instrumental analysis by LC/MS methods. The remaining 80% aliquot of samples for chemical analysis was azeotropically transferred to hexane using K-D apparatus. Aliquots of the extract were divided into vials for different types of GC/MS analysis. The extract aliquots for analysis of PAHs were further cleaned-up by a silica gel column clean up step using diethylether/acetone elution. The extract aliquots for analysis of organochlorine compounds (OCs), PCBs, BDE and PRCs were purified by a cleanup using activated silica gel modified with sulphuric acid. Following cleanup, addition of internal standards and volume reduction using a K-D apparatus, samples were analysed using a GC-MS/MS method for indicator PCBs, BDEs, OCPs and PRCs.

LDPE samplers, including trip controls, were extracted twice by soaking overnight with *n*-pentane (100 ml). Recovery standards (deuterated PAHs and PCBs that do not occur in the environment) were added to the extraction jar during the first extraction. The volume of pentane was reduced to 2 ml by a gentle stream of nitrogen at room temperature.

Extracts were split into two, with one fraction kept for non-target screening. For target analyses, extracts were first split into two equal fractions by volume. One fraction received a general clean-up using gel permeation chromatography (GPC). This post GPC sample was again split into two equal fractions by volume; the first of these was reduced in volume using nitrogen and analysed for PAH; the second received treatment with 2 × 1 ml concentrated sulphuric acid, was reduced in volume, and analysed for PCBs and OCs (Allan et al., 2013).

For non-target analyses, the extracts from samplers without PRCs were reduced by a gentle stream of nitrogen to 50-100 µl, with no clean up in order to preserve the integrity of the samples as much as possible. The extracts were stored at -20 °C until analysis by gas chromatography coupled to high resolution time of flight mass spectrometry (GC-HR ToFMS).

ED samplers for chemical analysis (but not those for ecotoxicological analysis) were spiked with RIS (C_{13} caffeine, C_{13} triclosan, M8PFOA, M8PFOS, D_{13}-alachlor, D_6-diuron, D_{10}-simazine, deuterated EE_2, n-nonylphenol). All samplers where then freeze dried for 24 hours in the original containers that were used for sample storage and transport. The disks were extracted three times by overnight (12 h) slow shaking at room temperature with 70 ml acetone. Combined extracts were reduced by vacuum rotary evaporation. After removal of particles by filtration through a layer of anhydrous Na_2SO_4 the extract was further reduced in volume to cca 1 ml. The acetone extract was transferred to methanol by

addition of methanol (20 ml) and subsequent evaporation and a nitrogen flow to further reduce in volume to 2 ml. Aliquots of the extract were divided into vials for different types of analysis.

29.2.4 Sample analysis

29.2.4.1 Analysis of hydrophobic compounds

SR and LDPE sampler extracts were analysed using a GC-MS/MS (GC 7890 / MS-MS Triple Quadrupole 7000B (Agilent), equipped with an HT8 SGE Analytical Science column for PCBs and OCs. PAHs were analysed using GC 7890 / MS5975 (Agilent) equipped with a J&W Scientific fused silica column DB-5MS column. PBDEs were analysed by a GC equipped with a 15m × 0.25 mm × 0.10 µm RTX-1614 column (Restek, USA) HRMS (AutoSpec Premier) was operated in EI+ mode at the resolution of >10 000.

29.2.4.2 Analysis of polar compounds

Polar pesticides and pharmaceuticals were analysed by liquid chromatography (Waters Acquity) with MS detection (Waters Xevo TQ-S). Analytes were separated on reverse phase column (Waters Acquity UPLC BEH-C18) using gradient elution with methanol and water, both with 0.1% formic acid. Eluting analytes were ionized using electrospray in positive mode and detected in MRM mode.

29.2.4.3 Toxicological profiling

For toxicological profiling, a battery of bioassays has been established. The same tests are employed for assessment of toxic potential of samples from high volume active sampling (Chapter 27). The set consists of eight assays provided by four laboratories (INERIS, RECETOX, RWTH, and University of Queensland (UQ)). The selected bioassays cover several important steps in the toxicity pathway including induction of xenobiotic metabolism, specific and reactive modes of toxic action, activation of adaptive stress response pathways. The diverse modes of action provide broad range of information on toxic potential.

Specifically, there are assays for assessment of endocrine disruptive potential (anti-)estrogenicity (MELN) and (anti-)androgenicity (MDA-kb2), activation of receptors for xenobiotics (CAFLUX and HG5LN-hPXR), immune response (NF-κB-bla THP-1), mutagenicity and DNA damage –related apoptosis (Ames fluctuation assay and p53-bla HCT-116, resp.) and detection of response to oxidative stress (ARE-bla Hep G2). The model cell lines are exposed to dilution series of the ED and SR extracts to describe dose-response relationship of the effects. The potentials are quantified in comparison with negative control and positive control describing the effect of a model chemical with known toxic potency specific for each of the bioassay endpoints.

Table 90: List of bioassays employed in the toxicological profiling of passive sampler extracts

Laboratory	Bioassay	Endpoint
INERIS	MELN	Binding to and activation of human estrogen receptor (ER)[1]
	HG5LN-hPXR	Binding to and activation of the human pregnane X receptor (PXR)[2]
RECETOX	CAFLUX	Binding to and activation of aryl hydrocarbon receptor (AhR)[3]
	MDA-kb2	Binding to and activation or inhibition of activity of human androgen receptor (AR)[4]
RWTH	Ames fluctuation assay	Assessment of mutagenic activity in Salmonella typhimurium after metabolic activation of compounds with S9 liver fraction[5]
UQ	p53-bla HCT-116	Assessment of p53-mediated apoptosis rate in response to DNA damage[6]
	ARE-bla Hep G2	Induction of the Nrf-2-mediated oxidative stress pathway[7]
	NF-κB-bla THP-1	Induction of inflammatory response[8]

[1](Balaguer et al., 1999), [2](Lemaire et al., 2006), [3](Aarts et al., 1998), [4](Wilson et al., 2002), [5](Reifferscheid et al., 2012), [6](Yeh et al., 2014), [7](http://tools.lifetechnologies.com/content/sfs/manuals/cellsensor_AREblaHepG2_man.pdf, n.d.), [8]("http://tools.lifetechnologies.com/content/sfs/manuals/CellSensor_NFkBbla_THP1_man.pdf," n.d.)

29.2.5 QA/QC

The applied quality control measures included the analysis of procedural solvent blanks, fabrication controls, field controls and matrix spikes.

29.2.6 Data analysis

Dissolved water concentrations of were calculated from analyte amounts accumulated in SR and LDPE samplers, the in situ sampling rate (*R*s) of the compounds and their sampler-water partition coefficients (Smedes et al., 2009) as described in Smedes and Booij (2012). Sampling rates were estimated from dissipation of PRCs from samplers during exposure using methods described by Booij and Smedes (2010).

For ED samplers calibration data are not available so far. For compounds under investigation we assumed an integrative uptake with a constant sampling rate. Identification of pollutant gradients along the Danube was performed based on the amount of a compound sampled by the ED in individual stretches, normalised to an average sampler exposure time (1.6 days).

29.3 Results

29.3.1 Analysis of hydrophobic compounds- use of silicone rubber samplers

SR samplers were deployed at 8 successive Danube stretches to characterise the spatial variability of hydrophobic compounds in the water column of the river.

29.3.1.1 Polychlorinated biphenyls and brominated diphenyl ethers

Calculated dissolved PCB concentrations were in sub ng l^{-1} range (Figure 150). Sums of 6 indicator PCB congeners ranged from 158 to 369 pg l^{-1}. Over the set of PCBs investigated there is a decrease in free dissolved concentration as hydrophobicity increases. The highest spatial variability is observed for the more water soluble congeners PCB28, 52 and 101. There was no clear spatial trend of PCB contamination along the river.

Concentrations of freely dissolved PBDEs (referring to the sum of the concentrations of congener numbers 28, 47, 99, 100, 153 and 154) were below the limit of quantification of 3 pg l^{-1} with the exception of the stretch Passau to Bratislava, where the summed concentration of the 6 congeners was 12 pg l^{-1}. Measurement of such low concentrations would require longer exposure times for integrative sampling, which was not available during the JDS3 cruise. A parallel 43 day sampling using a caged SR sampler statically deployed at a sampling site downstream Bratislava in the period August-October 2013 provided a concentration estimate of 2 pg l^{-1} for the sum of 6 PBDE congeners (Vrana, unpublished data).

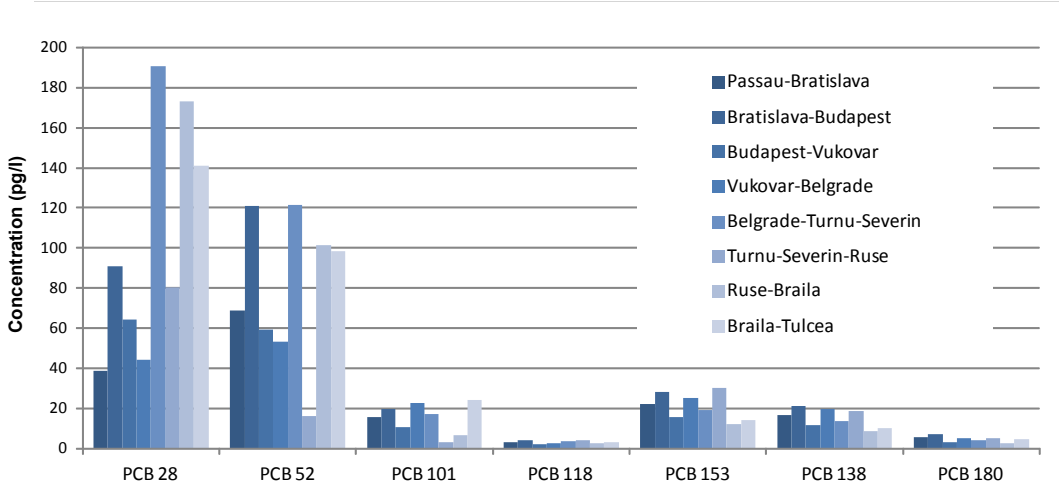

Figure 150: Free dissolved concentration of PCBs measured by SR samplers in 8 Danube stretches

29.3.1.2 Organochlorine compounds

The free dissolved concentrations of OCs were in sub ng l^{-1} range (Figure 151).The highest concentration of pentachlorobenzene (PeCB) up to 96 pg l^{-1} was observed in the stretch between Budapest and Belgrade whereas the highest level of hexachlorobenzene (HCB) of 97 pg l^{-1} was measured in the lowest Danube stretch between Ruse and Tulcea. The spatial variability of PeCB concentration was higher than that of HCB. Among the hexachlorocyclohexane (HCH) congeners, only β-HCH is reported because of low extraction recovery of the remaining isomers. There is an increasing trend of β-HCH concentration along the river, ranging between 9 pg l^{-1} in the upper stretches and 259 pg l^{-1} in the river delta area, respectively. The same spatial trend can be observed also for the sum of total DDT (given as sum of 4 isomers according to the Directive 2008/105/EC) as well as for p,p`-DDT. Concentrations of p,p`-DDT (1-21 pg l^{-1}) comprised only 2-7% of the total DDT, which indicates no current use of DDT in the Danube catchment. In the delta area concentration of DDT metabolites reach levels up to 864 pg l^{-1}.

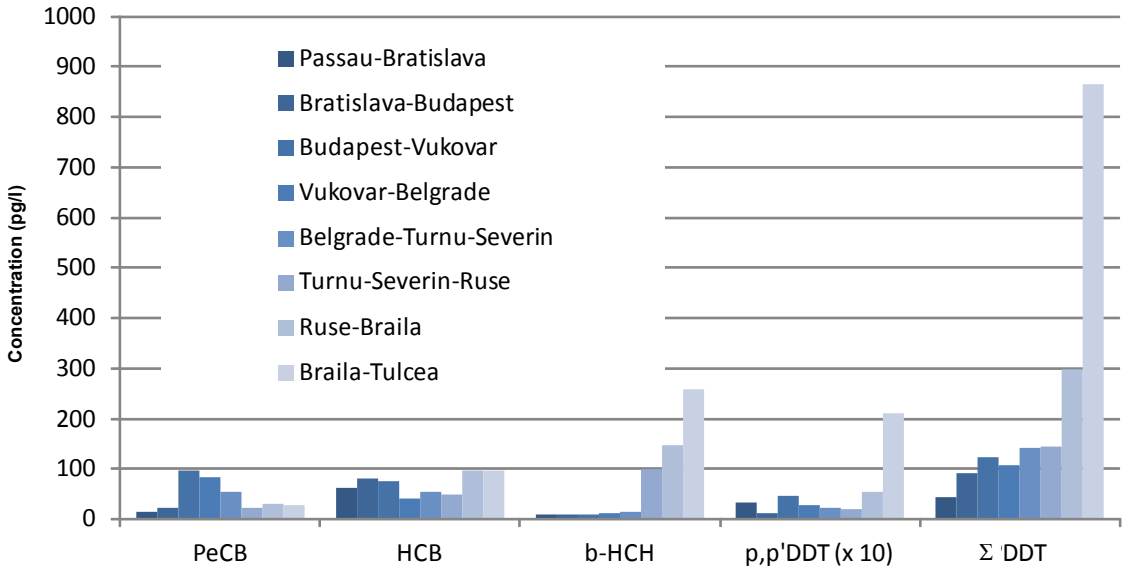

Figure 151: Free dissolved concentration of OCPs measured by SR samplers in 8 Danube stretches

29.3.1.3 Polycyclic aromatic hydrocarbons

Summed concentrations (Σ16 US EPA PAHs) of free dissolved PAHs in the water column ranged between 10.6 ng l^{-1} in stretch 7 and 45.1 ng l^{-1} in stretch 4, respectively. Summed concentrations were largely composed of PAHs with up to 4 aromatic rings. As for PCBs there is a strong decrease of free dissolved concentration with increasing compound hydrophobicity (Figure 152). Concentrations of compounds with 6 aromatic rings were mostly below the limit of quantification (tens of pg l^{-1}). Elevated PAH concentrations were observed in the stretches 4 and 5 (Budapest to Vukovar) and stretch 5 (Vukovar to Belgrade) with distinct pollutant patterns, which indicates different sources of PAHs along those river stretches. Concentrations of individual PAHs measured in stretch 2 (Passau to Bratislava) are within the concentration range that was measured in that stretch in spring till autumn 2011 using SPMD passive samplers (Vrana et al., 2014). This indicates that free dissolved PAH concentrations and their patterns in that Danube stretch in the summer period remained stable over a period of several years. A comparison with free dissolved concentrations measured using passive sampling in other European rivers (Vrana et al., 2014) shows that the concentrations of PAHs in the Danube is comparable to about 10 times lower.

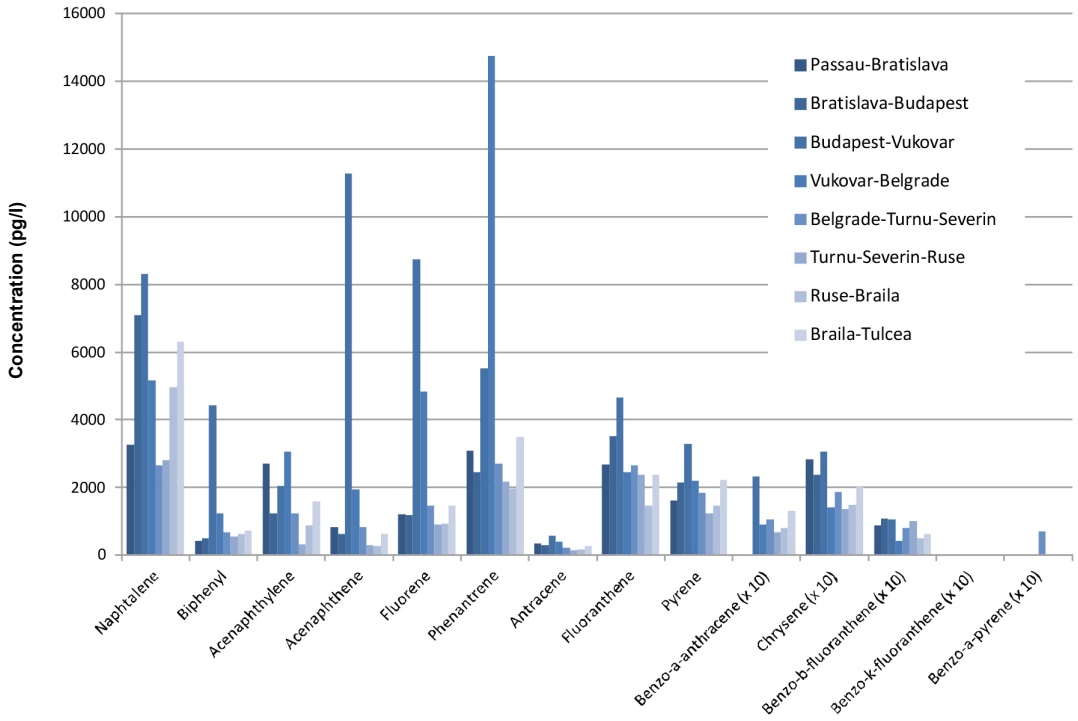

Figure 152: Free dissolved concentration of PAHs measured by SR samplers in 8 Danube stretches

29.3.1.4 Alkylphenols

The highest concentrations of free dissolved 4-nonylphenol (4-NP; 9.2 ng l^{-1}) and that of 4-tert-octylphenol (4-t-OP; 0.36 ng l^{-1}) was observed in the stretch between Vukovar and Belgrade (Figure 153). Concentration of 4-t-OP was on average 50 times lower than that of 4-NP.

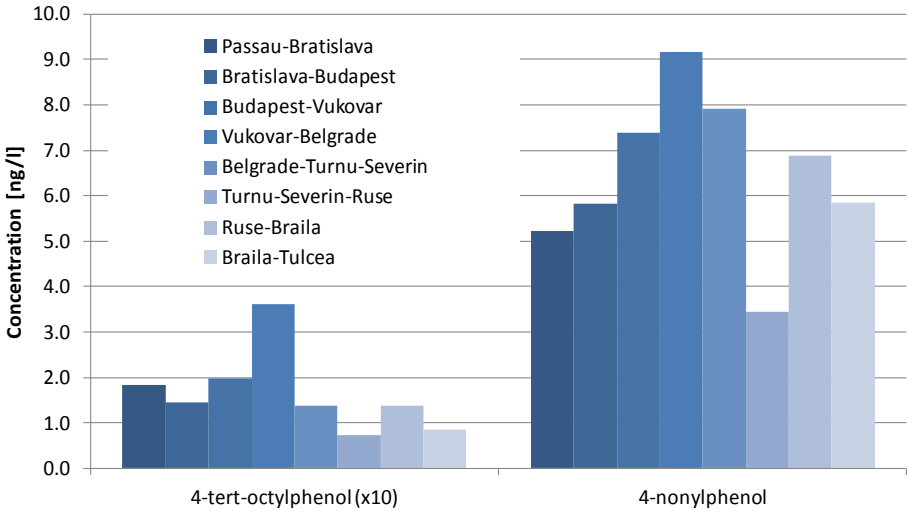

Figure 153: Free dissolved concentration of alkylphenols measured by SR samplers in 8 Danube stretches

29.3.2 Analysis of polar compounds – use of Empore disk samplers

29.3.2.1 Polar pesticides

A suite of 40 polar pesticides was analysed in extracts from the ED samplers. Results of analysis of five WFD priority pollutant polar pesticides, namely alachlor, atrazine, diuron, isoproturon and simazine are shown in Figure 154. Alachlor and diuron were present at concentrations less than or close to limit of quantification, which roughly corresponds to concentrations less than 100 pg l^{-1} in water. Estimated concentrations of atrazine, simazine and isoproturon in water were in the order of units of ng l^{-1} with the maxima of these pesticides in the stretch from Ruse to Braila. The results indicate that concentrations of the priority polar pesticides were far below their EQS values. It has to be noted that the main period of pesticide application is April-July and therefore the JDS results are not representative for the application season of these compounds.

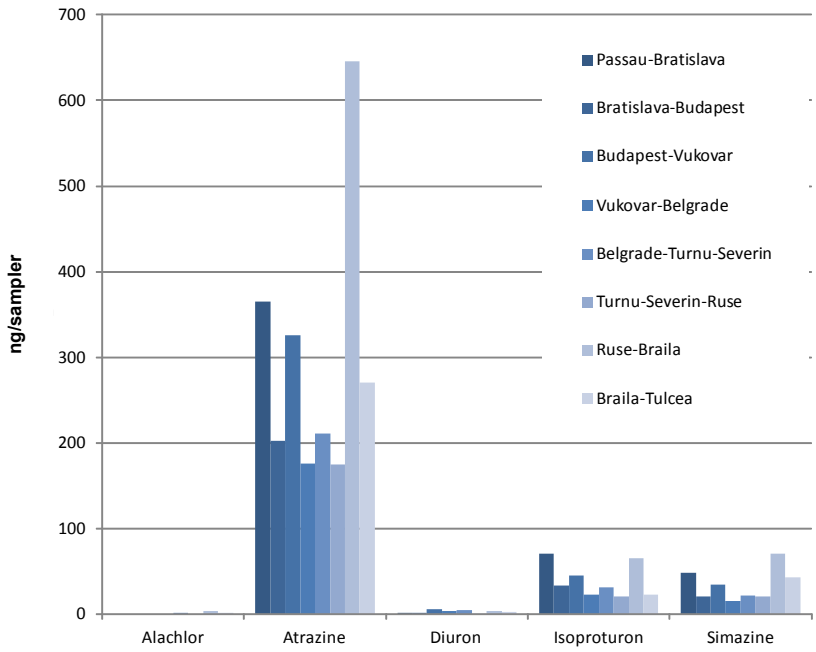

Figure 154: Spatial variability of WFD priority pollutant polar pesticides in the water column measured by ED samplers in 8 Danube stretches. Data is expressed as amount of compound taken up by an integrative sampler during an average sampler exposure (1.6 days)

29.3.2.2 Alkylphenols

The longitudinal relative concentration profile of alkylphenols in the Danube, measured by ED samplers (Figure 155), was similar to that reported by SR samplers. The highest concentrations of both 4-t-OP and 4-NP, but also of bisphenol A was measured in the stretch from Vukovar to Belgrade. In ED samplers concentration of 4-t-OP was on average 40 times lower than that of 4-NP.

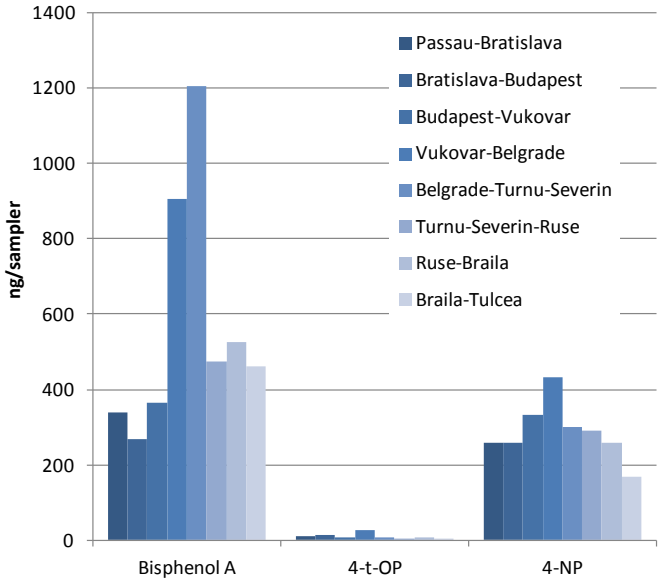

Figure 155: Spatial variability of alkylphenols in the water column measured by ED samplers in 8 Danube stretches. Data is expressed as amount of compound taken up by an integrative sampler during an average sampler exposure (1.6 days)

29.3.2.3 Pharmaceuticals

Results of analysis of caffeine and two pharmaceuticals, carbamazepine and diclofenac in extracts from the ED samplers are shown in Figure 156. The trend of caffeine concentration in the water column along the river was similar to that of bisphenol A. Estimated caffeine concentration levels were up to several tens of ng l-1 with the maximum observed concentration in the stretch from Vukovar to Belgrade. For comparison, analyses of caffeine in discrete spot samples taken collected the cruise and analysed by ELISA showed median concentration in Danube of 93 ng l-1 (Chapter 26). Estimated concentrations of carbamazepine along the river were in units of ng l-1 and less variable than that of caffeine. In agreement with the measurements made during JDS2 diclofenac was present at concentrations less than or close to limit of quantification, which can be explained by the biodegradability of this compound (Loos et al., 2008).

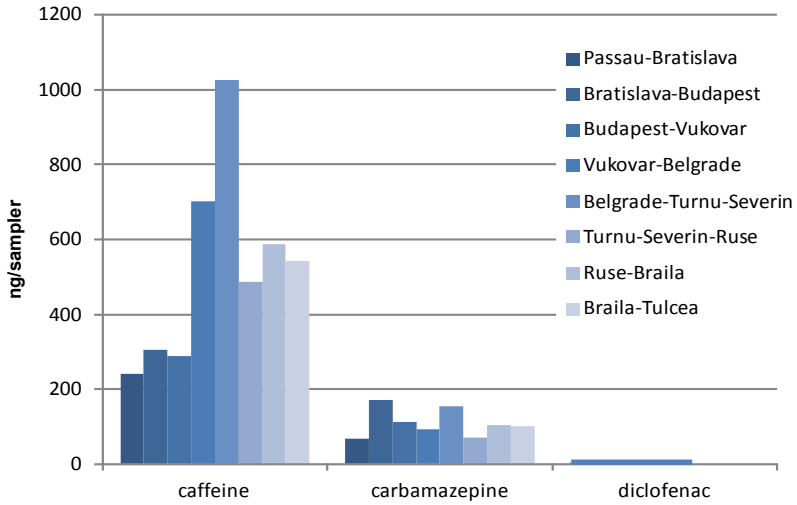

Figure 156: Spatial variability of caffeine and selected pharmaceuticals in the water column measured by ED samplers in 8 Danube stretches. Data is expressed as amount of compound taken up by an integrative sampler during an average sampler exposure (1.6 days)

29.3.3 Toxicological profiling

Selected toxic/bioactive potentials (see Table 90) of extracts of SR and ED passive samples are currently under evaluation. Preliminary results indicate that SR extracts contain significant amounts of dioxin-like compounds assessed by CAFLUX bioassay (Figure 157). Estimated toxic equivalents (bioTEQ) of samples recalculated for the sampled volume are between 6-10 pg l^{-1}. MELN bioassay has indicated estrogenic activity in SR samples. The specific estrogenic potential needs to be quantified yet. Available data from HG5LN-hPXR bioassay show that some SR extracts can significantly activate pregnane X receptor, but not the androgenic receptor. Negative results have been obtained in case of mutagenicity of SR extracts in Ames assay. Preliminary data indicate that at least some of the ED samples possess quantifiable estrogenic and PXR-related potential significantly higher than field blank samples.

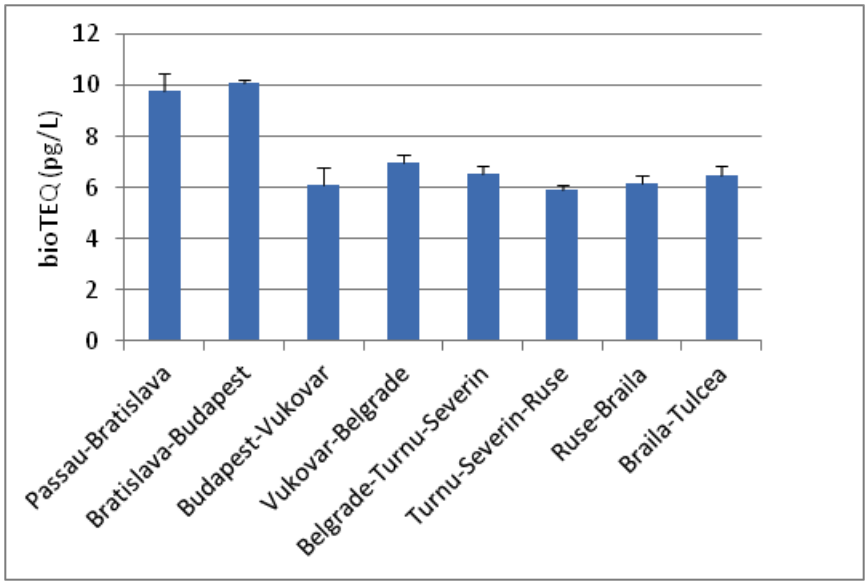

Figure 157: Estimate of toxic equivalent of TCDD in the water column measured by SR samplers in eight Danube stretches determined in CAFLUX bioassay

29.4 Conclusions

Despite the low or sub- ng l^{-1} concentrations of most organic pollutants present in the free dissolved phase, passive sampling enabled to clearly identify spatial gradients of a broad range of organic pollutants in the water column, including PCBs, OCs, PAHs, alkylphenols, selected polar pesticides and pharmaceuticals. In many cases, the integrative character of passive sampling allowed measurement of compounds down to pg l^{-1} levels where methods based on low volume spot sampling of water applied in the previous JDS2 survey failed to detect them (Sengl, 2008).

Passive samplers in most cases confirmed similar spatial distribution of pollutants along the river, as was observed in JDS2. The highest levels of PAHs, alkylphenols and caffeine in passive samplers were observed in the Danube stretches between Budapest and Belgrade. In agreement with JDS2, the downstream profile of PCBs and HCB showed a low variability and did not suggest particular emission maxima (Umlauf et al., 2008). In accordance with the findings during the JDS1 and JDS2, the downstream profile of β-HCH, DDT and its metabolites displays a sharp increase in the water column downstream Braila towards the Black Sea (Umlauf et al., 2008). The low percentage of p,p`-DDT of the total DDT concentration indicates that there was no current use of DDT in the area. The levels of priority pollutant polar pesticides alachlor, atrazine, diuron, isoproturon and simazine were comparable with the levels found in water samples during JDS2 and well below their respective EQS values (Loos et al., 2008).

Whereas data from spot sampling reflects the pollution at the individual JDS sampling sites at a single moment of time, passive samplers continuously sampled pollutants for several days, including river

stretches between individual JDS sampling sites. Thus, the information provided by spot sampling and passive sampling should be considered as complementary.

Finally, the combination of passive samplers with bioassays presents a very promising approach for detection of various trace organic pollutants and toxic potentials along the river and for identification of areas of concern for further investigation.

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29.6 Acknowledgments

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30 Non-target screening of organic pollutants

Draženka Stipaničev, Siniša Repec, Peter Oswald, Wolfgang Schulz, Manfred Sengl, Jaroslav Slobodnik

30.1 Introduction

Most of human activities (agricultural, industrial and domestic) lead to water contamination with numerous synthetic compounds of which most are not monitored in routine analyses. Although the majority of these compounds are present at low concentrations, many of them raise considerable (eco)toxicological concerns, particularly when present as components of complex mixtures. Largely unknown long-term effects on aquatic life and human health are caused by chemical pollution (Schwarzenbach et al., 2006; Kolpin et al., 2002; Richardson, 2007). The analyses of organic contaminants in different environmental compartments are predominantly based on chromatographic separations and mass spectrometric detection (Wille et al., 2012). To ensure that all contaminants with their degradation products and metabolites are detected a non-targeted approach is also required (Ferrer and Thurman, 2012). Considering the above, non-target and target screening was performed on the 68 JDS3 water samples collected from the Danube River and its tributaries. The prerequisite for non-target analysis is a mass spectrometer sufficiently sensitive to detect and identify the compound directly, recording the full spectrum rapidly and at the same time having high mass accuracy for components present at very low concentrations. According to Krauss et al. (2010) the aim of non-target analysis is to search for as many compounds in a sample as possible with the focus on compounds not previously known to be present. Another important feature of a non-target method is that the acquired full dataset of mass spectra enables retrospective analyses of the sample. An availability of comprehensive mass spectral libraries with accurate mass fragmentation information was shown to be of importance at confirmation of the identity of detected substances (Zedda and Zwiener, 2012). During the JDS3 ultra high performance liquid chromatography electrospray ionisation quadrupole-time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS), high performance liquid chromatography coupled with electrospray ionisation quadrupole-time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) and gas chromatography mass spectrometry (GC-MS) in three different laboratories were used for non-target screening. A specific statistical chemometric software was used to find pollution patterns of organic compounds acquired with the UHPLC-ESI-Q-TOF-MS.

30.2 Methods

30.2.1 Samples and sample preparation

Polycarbonate bottles containing 0,25 L (LC-MS) and 1 l (GC-MS) of surface water sample from all JDS3 sites were shipped to the laboratories each 3-4 days during the survey and stored cool until analysis. Sampling, quality control measures (field blanks) and the way of controlling the sample temperature during the transport are described in Chapter 2. Samples were filtrated through 0,2 µm PTFE filter prior to analysis. Ultrapure laboratory water samples were always processed in parallel with the environmental water samples.

A subset of 22 samples was obtained by large volume sampling of 500 l of water sample through a series of three solid phase extraction cartridges capturing a wide range of polarity (neutral, acidic, basic) substances (for details see Chapters 2 and 27).

30.2.2 UHPLC-ESI-Q-TOF-MS

30.2.2.1 Instrumentation

The samples were analysed in Central Water Management Laboratory of Croatian Waters in Zagreb, Croatia. Chromatographic separations were carried out with the 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA, USA) using a reversed phase ACQUITY UPLC HSS T3 analytical column (150 mm x 2.1 mm, 1.8 µm). The mobile phase gradient was from 100% water to 100% organic solvent in 20 min run and the sample injection volume was 100 µl. The temperature of the column chamber was set at 50°C. In positive electrospray ionisation (ESI+), the mobile phase was composed of solvent A (5 mM ammonium acetate/ HAc (pH=4.7) and B (100% MeOH). Gradient elution with a flow rate of 0.4 ml/min was used. The analytes were detected using an 6550 i-Funnel Q-TOF-MS (Agilent Technologies) providing 40,000 resolving power and < 2 ppm accuracy at 4 GHz detector rate.

30.2.2.2 MS only method

For MS screening method the acquisition rate in MS1 mode was 2 spectra/s (4100 transients per spectrum). The measured mass range was 100-1000 m/z in the centroid and profile mode. The capillary and fragmentor voltages were 3500 V and 400 V, respectively. The sheat gas flow was 11 l N2/min, flow of the drying gas was 18 l N2/min while nebulizer was kept at 30 psig. The resolution power for ESI+ was 52296 at 922.009798 m/z and 21801 at 118,086255 m/z. A correction for any possible drift in the mass axis during measurement was done automatically with lock 2 mass ion software.

30.2.2.3 AutoMSMS method

For auto MSMS mode screening method the acquisition rate in MS1 was 2 spectra/s (4100 transients per spectrum) and measured mass range was 100-1000 m/z in the centroid and profile mode. The acquisition rate in MS2 was 3 spectra/s (2650 transients per spectrum) and measured mass range was from 50 to 1000 m/z while the data were obtained at settings of narrow width isolation. Collision energies were fixed at 10, 20 and 40 eV.

30.2.2.4 Validation qual/quant method

Target screening method was developed for a mixture of 168 organic substances containing pesticides and pharmaceuticals such as antidepressants, anti-epileptic, neuroleptics, opioids, benzodiazepines/hypnotics, cardiovascular medial and hallucinogens/stimulants. Calibration curve was obtained by direct injecting, in triplicate standard solutions at seven concentration levels starting from 1 to 1000 ng/l. Correlation coefficients > 0.99 were used as linearity acceptance criterion. Accuracy and the precision was calculated by analyzing blank samples spiked at three concentration levels and were evaluated within-day in quintuplicate at each concentration level. Acceptance criteria were (i) recoveries of 70% and 110% for accuracy and (ii) RSD lower than 20% for precision. Once validated, the screening method has been applied to the analysis of different surface water to test its applicability.

30.2.2.5 Software for data analysis and PCDL databases

30.2.2.5.1 Software for data analysis

Analyses were conducted using the MassHunter Profinder Qualitative Analysis tools of the MassHunter Workstation Software (version B.06.00, Built 6.0.605.0, Agilent Technologies) with software tools: Molecular Formula Generator (MFG), Find by Ion, Find by Formula and Molecular Feature Extractor (MFE). Statistical analyses were conducted by using the Mass Profiler Professional software (MPP, Version 12.6.1, Agilent Technologies). Quality control in MPP was used for elimination of unreliably identified compounds or compounds not relevant for data evaluation. After quality control in MPP a differential analysis was performed. Chemometric statistical analysis with reliable peak-finding algorithm was applied at the non-target screening in order to reduce the false positives/negatives. Comparison of samples was based on compounds (entities) determined by their full scan data.

30.2.2.5.2 Personal Compound Database Library – PCDL databases

Forensic toxicology, Pesticide and Metlin metabolite PCDLs, all in total with more than 65000 compounds, were used to identify drugs of abuse, medical drugs, pesticides, alkaloids, toxic reagents, and their metabolites. Information obtained in PCDLs provides compounds' name, CAS number, molecular and structural formula, neutral mono-isotopic mass, isotope pattern, retention time (optional) and MS/MS spectra generated at CID energies of 10, 20 and 40 eV.

MassHunter Forensic Toxicology PCDL ver. 4.1 contains mass spectra of 7509 compounds and MS/MS library of more than 2500+ compounds; MassHunter Pesticide PCDL ver. 4.1 contains mass spectra of 1664 compounds and MS/MS library of more than 600 compounds and MassHunter METLIN metabolite PCDL ver. 5 contains mass spectra of 64092 compounds and MS/MS library of more than 8040 compounds. All MS/MS spectra were obtained at three collision energies (10, 20 and 40 eV).

30.2.2.6 Q-TOF-MS non-target screening workflow

After recording full scan acquisition in Q-TOF MS, all generated mass spectrometric data were sent to MassProfinder software (cf. Section 30.2.2.5.1 above) where untargeted data mining and batch recursive feature extraction was performed. Features (unprocessed information about the compounds) extracted with recursive analysis were subjected to compound alignment and statistical analyses using MPP. Molecular Feature Generator in MPP software was used for calculation of features' accurate masses accompanied with information on molecular formula, isotopic pattern, isotopic spacing, and the difference between the theoretical exact mass of the assigned formula and the acquired accurate mass for the feature. In the final list MPP features were divided into three groups: first was the PCDL match defined by presence of the compound in PCDL database (name of the substance assigned), second was unknown (molecular formula provided), and third was total unknown (only an accurate mass and retention time defined).

Results of MPP analysis were than exported to autoMSMS method for further identification and confirmation of compounds with accurate mass, fragmentation by MS/MS, and characteristic isotope signatures and fragments. In autoMSMS method Agilent MassHunter Qualitative software with MFE, MFG and PCDL accurate mass library were used.

Figure 158: Non-target workflow used for analyses by UHPLC-Q-TOF-MS

A presence of compound's mass spectrum found in autoMSMS also in PCDL led to the provisional identification of the compound. Characteristic fragments acquired in autoMSMS were considered as sufficient additional information to fully confirm identity of the substance. An injection of standard chemical would be needed for unequivocal confirmation in cases when compound's spectral data was not present in PCDL. A workflow used for identification of unknown compounds is presented in Figure 158.

30.2.3 HPLC-ESI-Q-TOF-MS

30.2.3.1 Instrumentation

The samples were analysed in Zweckverband Landeswasserversorgung (LW) Betriebs- und Forschungslabor in Langenau, Germany using high resolution LC-MS with duplicate direct injection of 100 µl water sample both in ESI+ and ESI- mode. The high performance liquid chromatography system Prominence LC20 Series (Shimadzu, Duisburg, Germany) coupled with the TripleTOF 5600 mass spectrometer (AB SCIEX, Concord (ON), Canada) was used. After electrospray ionization in positive and negative mode, the data were collected in full scan mode (m/z 100 – 1200 Da). The

HPLC column Zorbax Eclipse Plus C18, 2.1 x 150 mm (Agilent, Waldbronn, Germany) and the guard column AQ C18 2.0 x 4 mm (Phenomenex, Aschaffenburg, Germany) were used. Both eluents water (A) and acetonitrile (B) contained 0.1% formic acid, respectively. A multi-step gradient with the following parameters was applied in ESI+ and ESI-: 1 min at 2% B, within 1 min to 20% B, within 14.5 min to 100% B, hold for 5.5 min at 100% B, within 0.1 min back to 2% B and 4.9 min for equilibration at 2% B. The flow rate was constant 0.3 ml/min and the column temperature was 40°C. Nitrogen was used as drying and curtain gas. The source parameters were set to GAS 1 35 psi, GAS 2 45 psi, Curtain Gas 40 psi, temperature 550°C, ion source voltage 5500 V (-4500 V for ESI-), declustering potential of 100 V (-60 V for ESI-) and a collision energy of 10 eV (-10 eV for ESI-). In addition, an IDA-experiment (Information Dependent Acquisition) was used in which MS/MS-spectra of compounds that fulfill certain criteria were acquired (collision energy 40 eV). For instance, blank compounds as well as features which do not exceed a threshold of 100 cps were excluded. The mass spectrometer was calibrated using external calibration delivery system CDS and internal calibration with known contaminants. All systems, the HPLC and the mass spectrometer were controlled and data were acquired as well as processed by AnalystTF™ 1.6 software (AB Sciex, Concord (ON), Canada).

30.2.3.2 Data analysis

Data Analysis of target and suspected compounds were conducted using the qualitative analysis tool MasterView™ of the PeakView™ software (version 2.0, AB Sciex, Concord (ON), Canada). Comparisons of the Danube River samples with a blank injection and a multi component reference standard (about 315 substances) were performed. Compounds were designated as 'identified' if accurate mass, isotope pattern and retention time in the sample conformed to those of the reference standard. In cases where the IDA-experiments supplied reliable MS/MS-spectra, the data were additionally used for comparison.

30.2.4 Gas chromatography-mass spectrometry

30.2.4.1 Liquid-liquid extraction

Water samples (1000 ml) were placed into a glass separating funnel, spiked with 10 µl (10 ng/l) of methanolic perdeuterated phenanthrene and 10 µl (10 ng/l) of methanolic perdeuterated DDT internal standard solutions to give a final concentration of 1 µg/l and then extracted by two portions of dichloromethane (2 x 40 ml). After extraction the final combined extract was dried with anhydrous sodium sulphate and then evaporated to the final volume of 1 ml using vacuum rotary evaporator.
The GC-MS screening analysis was performed with Agilent 7890 gas chromatograph coupled to Agilent 5975 C mass spectrometric detector (MSD; Agilent Technologies, Little Falls, DE, USA). The system was equipped with the Agilent Multimode (MMI) Inlet allowing introduction of 50 µl of extract into the GC system in the solvent vent injection mode. The MMI was ramped from 70°C to 260°C (5 min) at a rate of 600°C/min. Capillary GC analysis was performed on a 30 m x 250 µm I.D., 1 µm d_f HP-5MS column (Agilent Technologies). The oven was programmed from 50°C (3 min) at 30°C/min to 200°C, at 5ºC/min to 280°C and finally at 30°C /min to 310°C (5 min). Hydrogen was used as a carrier gas. The MSD was operated in the electron impact (EI) full scan mode (m/z 50–600) for all samples. Identification of compounds was performed using mass spectrum libraries Wiley 7n and NIST11, followed by manual interpretation. Molecular masses of numerous detected compounds were additionally confirmed in the mode of positive chemical ionisation using methane as a reagent gas. A retention time index has been calculated for each detected substance based on the injection of the Kovats's mixture of alkanes for comparison with retention time indices in the NIST library and thus increasing the confidence in identification.

30.2.4.2 Direct analysis of large volume samples

An aliquot of 2 ml extract corresponding to 2 l water sample obtained by LVSPE (cf. Section 30.2.1) was used for GC-MS screening analyses after its reconstitution into organic solvent and spiking with methanolic perdeuterated phenanthrene at concentration level of 1µg/l. The system was equipped with the Agilent Multimode (MMI) Inlet allowing introduction of 125 µl of the extract to the GC system in

the solvent vent injection mode. The rest of the analysis conditions were identical to those described in Section 30.2.3.1.

30.2.4.3 Semi-quantitative assessment

An estimation of concentrations of compounds detected in the full scan EI mode was performed. Concentration values based on comparison of the signal (relative abundance) of an unknown compound to the signal generated by the known concentration of an internal standard were estimated (Slobodnik et al., 2012). In the procedure, a signal of the quantification ion of the deuterated internal standard (m/z 188 for phenanthrene-D_{10}) was compared with the signal of its overall mass spectrum (Total Ion Current; TIC), which resulted in estimation of its relative intensity (i.e., 34% from the TIC response, RSD = 0.93%, n = 6). The same procedure was applied to the unknown compound (selection of the most abundant ion; determination of its intensity relative to the overall intensity (TIC) of the whole mass spectrum). The ratio between signals of quantification ions of the unknown substance to that of the known internal standard was then corrected for their percentage representativeness of the TIC and the final concentration was calculated (e.g. IF signal of 10 ng/l internal standard phenanthrene-D_{10} is 100,000 (arbitrary units), TIC corrected signal is 34,000 AND TIC corrected signal of unknown substance is 17,000 THEN the estimated concentration of unknown substance is 5 ng/l). It should be made clear that the method provides only rough indicative estimations of actual concentrations. However, additional comparisons obtained with standard compounds for large proportion of the substances usually detected in surface water samples showed that the error is usually contained within one order of magnitude.

30.3 Results

30.3.1 UHPLC-ESI-Q-TOF-MS

30.3.1.1 Target analysis

Results of target screening of 68 JDS3 samples for a wide range of pharmaceuticals and illicit drugs are presented in Figure 159. A total of 154 out of 168 studied analytes were found to be present in at least one sample. Detailed information on the occurrence and concentrations of detected compounds per sampling site is presented in the full report on the attached CD-ROM.

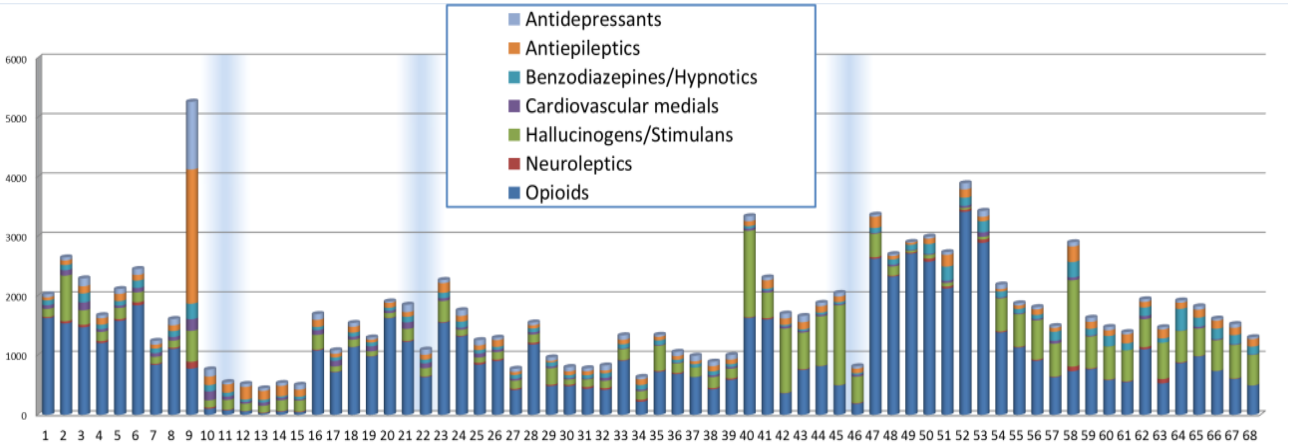

Figure 159: Occurrence profile of different groups of pharmaceuticals and illicit drugs in the 68 JDS3 samples; blue vertical lines are presenting rainy period, x-axis represents sampling stations and y-axis indicates cumulative concentrations of all determined substances (in ng/l) with a quantitative proportion of the particular group of substances (cf. different colours)

30.3.1.2 Non-target analysis

Initial quality control on acquired 16214 raw features in MPP with filtering by frequency, sample variability, flags, abundance, significance testing and fold change resulted in 7767 processed features that were detected in 68 JDS3 samples (Figure 160). Please, note that all target compounds (Section 30.3.1 above) were excluded from non-target analysis.

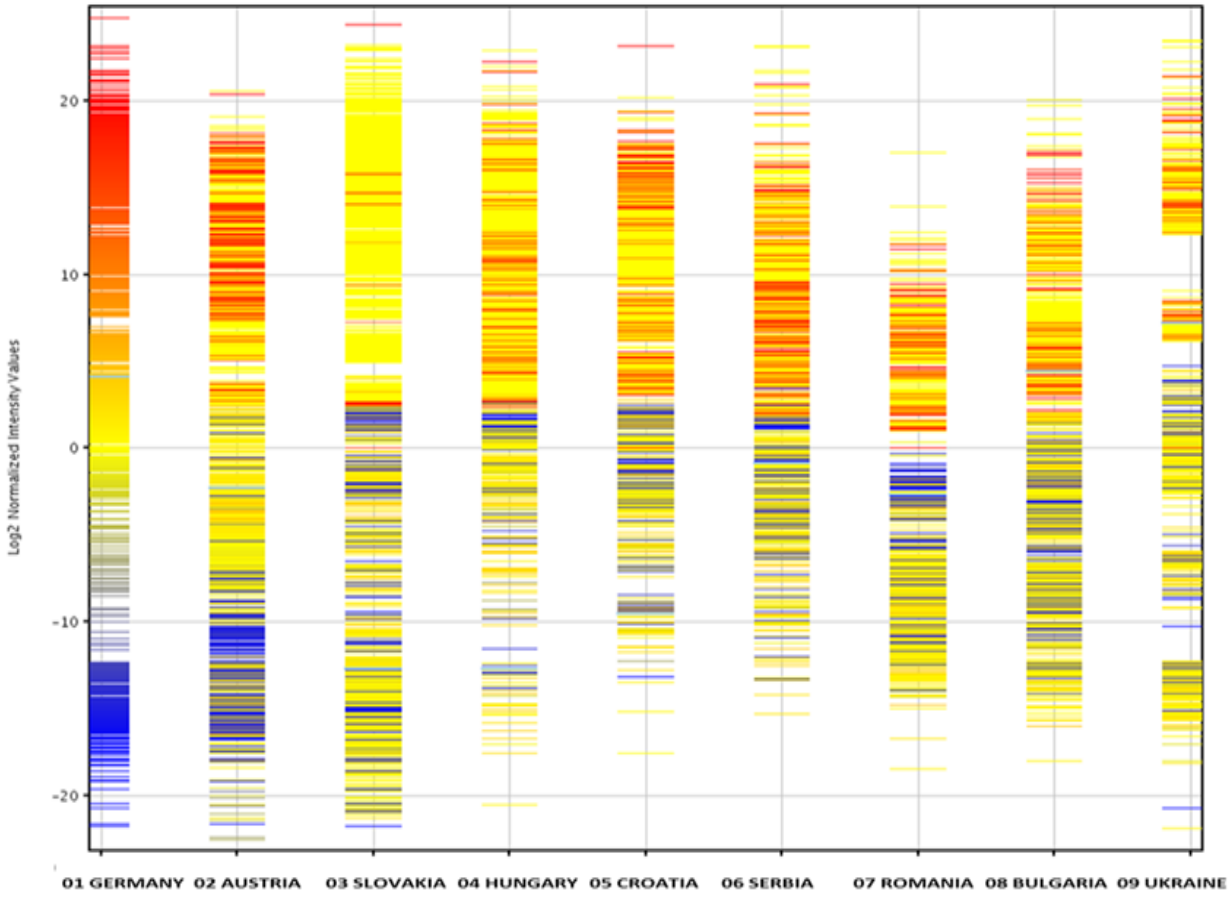

Figure 160: Distribution of 7767 different mass spectral processed features through the Danube river and its tributaries; Danube countries are shown on x-axis and normalised signal intensity values are represented on y-axis; each single feature/compound is represented by a horizontal bar at a fixed position on the chart (position given by a unique combination of retention time, accurate mass spectrum, name, molecular formula, etc.) and the intensity of signal increase is indicated by blue (low) to red (high) colour

The figure indicates that the highest number of different features (i.e. also chemical entities present in samples) with highest signal intensity was found in Germany and the least number of features/substances was identified in samples from Romania. From these 7767 processed features ID Browser recognised 3442 match compounds in the PCDL library which allowed for assigning the compounds with a defined name, accurate mass, molecular formula, retention time, CAS number and isotopic pattern. For 3370 (unknown) compounds a molecular formula was calculated and supplemented with accurate mass, retention time and isotopic pattern, and 955 (fully unknown) compounds were defined only with accurate mass and retention time (Figure 161). Detailed information on the occurrence of all features (PCDL match compounds, unknowns, total unknowns) determined by the MPP is presented in the full report on the CD-ROM.

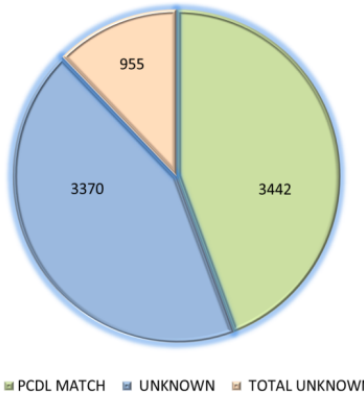

Figure 161: Overview of compounds' identification results; full scan mass chromatograms of all 68 JDS3 samples obtained by UHPLC-ESI-Q-TOF-MS were evaluated with the Mass Profiler Professional (MPP) software

The autoMSMS method was applied for all detected compounds from all 68 samples (7767 processed features resulting in assigning PCDL match compounds, unknowns and total unknowns (cf. text above), with focus on 5014 spectral data acquired with CE 10, 20 and 40 eV, which were matching those already stored in the available databases). This allowed to finally arrive to the reduced list of compounds recognised by name, high accurate mass and fragments. The autoMSMS evaluation of this large dataset is still on-going, however, the substances listed in Table 91 can already be considered as unequivocally identified, despite standard chemicals of these substances were not available for the final confirmation.

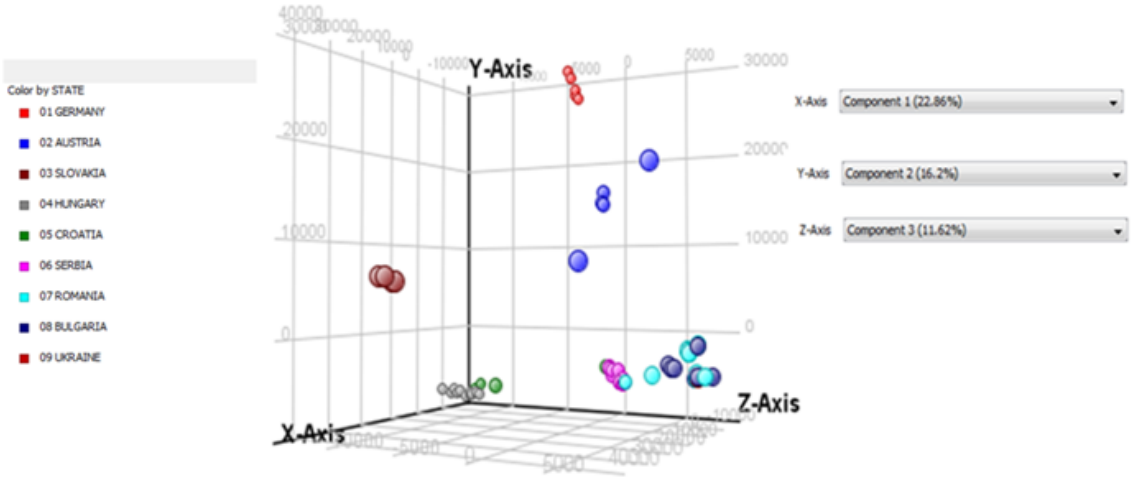

Figure 162: Similarity of pollution profiles among different Danube countries evaluated with the Principal Component Analysis (PCA) of JDS3 non-target screening data obtained with UHPLC-ESI-Q-TOF-MS

Principal Component Analysis (PCA) was performed with the MPP software on all data sets for detection of similarities and differences in the patterns of pollution between different Danube countries discriminated by the major trends in the data. Figure 162 shows that similarities in pollution pattern exist among Serbia, Romania, Bulgaria and Ukraine and between Croatia and Hungary whereas rather unique character of pollution can be seen in the upstream countries (Germany, Austria, Slovakia).

Table 91: List of selected non-target compounds unequivocally identified by UHPLC-QTOF-MS operated in autoMSMS mode

CAS	FORMULA	NAME	NOTES	FREQUENCY	m/z	H+	CE	fragment1	fragment2
2163-69-1	C11 H22 N2 O	Cycluron	Herbicide	68	198,1732	199,1810	20	72,0444	89,0709
134-62-3	C12 H17 N O	DEET / Diethyltoluamide	Insecticide	68	191,1310	192,1383	20	119,0491	91,0542
51235-04-2	C12 H20 N4 O2	Hexazinone	Herbicide	68	252,1586	253,1659	20	171,0877	
90-33-5	C10 H8 O3	Hymecromone	Choleretic;Insecticide	68	176,0473	177,0547	40	77,0386	68,9971
60142-96-3	C9 H17 N O2	Gabapentin	Anticonvulsant	67	171,1259	172,1332	40	67,0542	55,0178
56392-16-6	C15 H25 N O4	Hydroxymetoprolol	Beta-Blocker, metabolite	67	283,1784	284,1856	40	56,0495	74,0600
23103-98-2	C11 H18 N4 O2	Pirimicarb	Insecticide	66	238,1430	239,1503	20	72,0444	182,1288
37517-30-9	C18 H28 N2 O4	Acebutolol	Beta-Blocker	65	336,2049	337,2122	10	116,1070	98,0964
39809-25-1	C10 H15 N5 O3	Penciclovir	Antiviral	65	253,1175	254,1248	40	135,0301	110,0349
1593-77-7	C18 H35 N O	Dodemorph	Fungicide	64	281,2719	282,2791	40	98,0964	55,0542
34661-75-1	C20 H29 N5 O3	Urapidil	synthetic	64	387,2270	388,2355	40	190,1101	70,0651
33817-20-8	C22 H29 N3 O6 S	Pivampicillin	Antibiotic	63	463,1777	464,1850	10	274,1108	244,1002
298-81-7	C12 H8 O4	Ammoidin	Naturally occurring compound	60	216,0423	217,0495	40	174,0311	90,0464
13655-52-2	C15 H23 N O2	Alprenolol	Beta-Blocker	59	249,1729	250,1801	20	116,1070	72,0808
13912-80-6	C12 H17 N O3	Nicoboxil	Rubefacient	58	223,1208	224,1281	40	124,0393	78,0338
70-70-2	C9 H10 O2	Paroxypropione	Hormone	55	150,0681	151,0752	40	77,0386	
2382-79-8	C13 H15 N3 O2	Acetyltryptophanamide	Synthetic	54	245,1164	246,1237	20	159,0917	201,1022
827-61-2	C9 H15 N O2	Aceclidine	Parasympathomimetic	48	169,1103	170,1175	20	110,0964	
657-24-9	C4 H11 N5	Metformin	Antidiabetic	45	129,1014	130,1087	10	60,0556	71,0604
554-62-1	C18 H39 N O3	Phytosphingosine	PCPP, shampoo	42	317,2930	318,3003	20	60,0444	
1695-77-8	C14 H24 N2 O7	Spectinomycin	Antibiotic	40	332,1584	333,1671	10	98,0600	
633-47-6	C13 H24 N2 O2	Cropropamide	Stimulant	38	240,1838	241,1917	40	100,1121	69,0335
3485-14-1	C15 H23 N3 O4 S	Ciclacillin	Antibiotic	37	341,1409	342,1488	20	98,0964	
51338-27-3	C16 H14 Cl2 O4	Diclofop-methyl	Herbicide	37	340,0269	341,0336	20	123,0570	
99011-02-6	C14 H16 N4	Imiquimod	Immunomodulator, virustatic	35	240,1375	241,1449	20	185,0822	
1177865-17-6	C24 H35 N7	NSC 23766	Inhibitor	32	421,2954	422,3028	20	349,2135	
120162-55-2	C13 H16 N10 O5 S	Azimsulfuron (IN A8947)	Azimsulfuron-methyl	31	424,1026	425,1097	40	182,0560	139,0489
101622-51-9	C15 H18 N6 O	Olomoucine	Chemotherapeutic	28	298,1542	299,1623	40	91,0542	177,0883
1637-39-4	C10 H13 N5 O	trans-Zeatin	Naturally occurring compound	28	219,1120	220,1193	40	119,0352	136,0618
20380-58-9	C17 H23 N O2	Tilidine	Analgesic	26	273,1729	274,1809	40	155,0855	77,0386
103-33-3	C12 H10 N2	Azobenzene	Dye	25	182,0844	183,0917	40	77,0386	
75330-75-5	C24 H36 O5	Lovastatin	Anticholesteremic	25	404,2563	405,2636	10	199,1481	285,1849
224789-15-5	C23 H32 N6 O4 S	Vardenafil	Erectile Dysfunction Treatment	25	488,2206	489,2290	40	151,0853	312,1574
83-33-0	C9 H8 O	1-Indanone	Oxidation product	24	132,0575	133,0648	20	77,0386	105,0699
1704-28-5	C18 H37 N O	Aldimorph	Fungicide	24	283,2875	284,2950	40	57,0699	98,0946
15870-91-4	C14 H14 O4	Prenylamine	Vasodilatator	24	329,2143	330,2216	40	91,0542	
309-29-5	C24 H30 N2 O2	Doxapram	Stimulant	21	378,2307	379,2384	40	97,0886	129,0699
14028-44-5	C17 H16 Cl N O3	Amoxapine	Antidepressant	20	313,0982	314,1055	20	271,0633	70,0651
34866-47-2	C13 H21 N3 O3	Carbuterol	Bronchodilator	20	267,1583	268,1656	20	134,0600	177,0659
2430-27-5	C8 H17 N O	Valpromide	Anticonvulsant	20	143,1310	144,1383	20	57,0699	72,0444
30344-00-4	C18 H18 N4 O2	ADMA	Naturally occurring chemical	19	202,1430	203,1503	20	70,0651	88,0869
33629-47-9	C14 H21 N3 O4	Butralin (Sutralin)	Herbicide	19	295,1532	296,1605	40	57,0699	178,0737
6552-12-1	C10 H15 O4 PS	Fenthion-oxon	Insecticide Metabolite	19	262,0429	263,0501	20	231,0239	216,0005
5355-16-8	C13 H16 N4 O2	Diaveridin	Coccidiostatic	17	260,1273	261,1346	20	245,1033	123,0665
6452-71-7	C15 H23 NO3	1-(2-(allyloxy)phenoxy)-3-(isopropylamino)propan-2-ol	Beta-Blocker	16	265,1678	266,1751	10	72,0808	225,1359
57526-81-5	C12 H19 N O3	Prenalterol	Sympathomimetic	16	225,1365	226,1438	20	72,0808	56,0495
14556-46-8	C14 H22 Cl NO2	5-Carboxybupranolol	Beta-Blocker	15	301,1081	302,1154	20	246,0528	
70374-39-9	C13 H10 Cl N3 O4 S2	Lornoxicam	Non-steroidal antiphlogistic	14	370,9801	371,9874	20	95,0604	121,0415
865318-97-4	C15 H25 N5	Ametoctradin	Fungicide	13	275,2110	276,2174	40	176,0931	149,0822
59338-93-1	C16 H21 N5 O2	Alizapride	Antihistamine	11	315,1695	316,1768	20	124,1121	148,0393
57-68-1	C12 H14 N4 O2 S	Sulfadimidine	Chemotherapeutic	11	278,0837	279,0910	40	201,0441	92,0495
81-82-3	C19 H15 Cl O4	Coumachlor	Rodenticide	10	342,0659	343,0732	20	163,0390	285,0313
51276-47-2	C5 H12 N O4 P	Glufosinate	Alt CAS: 53369-07-6	10	181,0504	182,0577	20	56,0495	136,0522
543-82-8	C8 H19 N	Octodrine	Sympathomimetic	10	129,1517	130,1590	10	57,0699	71,0855
331830-20-7	C13 H8 N2 O3	1,4-DPCA	Inhibitor	5	240,0535	241,0608	10	223,0502	
525-82-6	C15 H10 O2	Flavone	Endogenous Metabolite	5	222,0681	223,0754	40	77,0386	65,0386

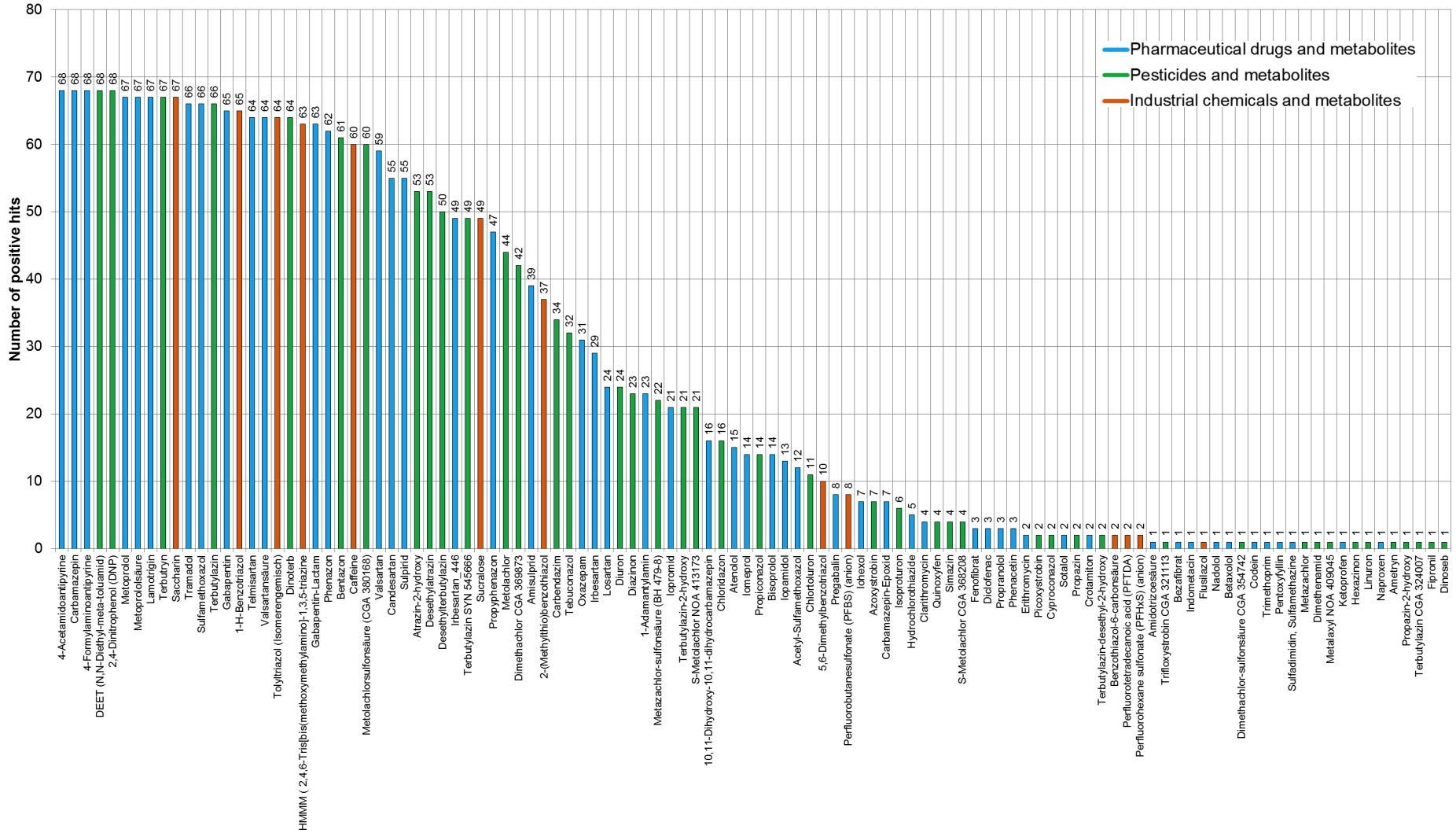

Figure 163: Frequency of appearance of 110 'identified' suspect pollutants (315 tested) in JDS3 surface water samples; results obtained from non-target screening workflow by HPLC-ESI-Q-TOF-MS operated in ESI$^+$ and ESI$^-$ modes

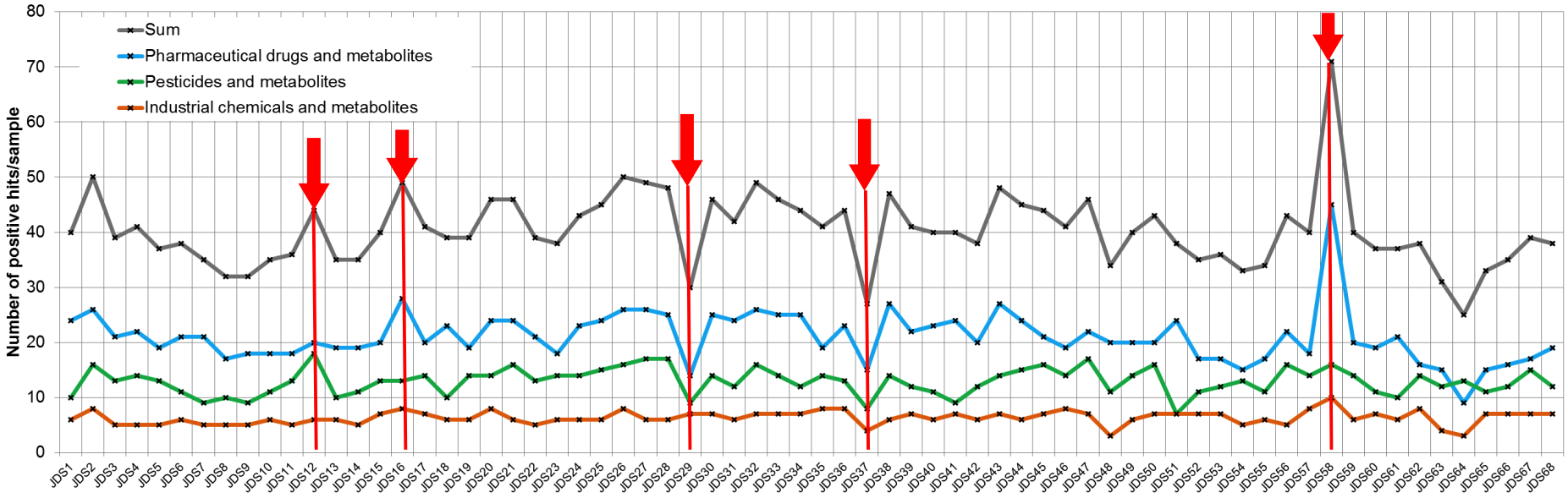

Figure 164: Frequency of appearance of 110 'identified' pollutants sorted by groups (Pharmaceutical drugs, pesticides and industrial chemicals as well as their metabolites, respectively) in JDS3 surface water samples.

30.3.2 HPLC-ESI-Q-TOF-MS

30.3.2.1 Target analysis and suspect screening

LW laboratory conducted screening of 315 suspected organic pollutants in all 68 JDS3 samples. The 'suspect screening' showed that 110 substances were detected in at least one sample (Figure 163). The substances 4-acetamidoantipyrine, carbamazepine, 4-formylaminoantipyrine, DEET and 2,4-dinitrophenol were detected in all 68 samples. Next to the evaluation of the relative signal intensities for each of the detected substances also a retrospectively obtained semi-quantitative results using a single-point calibration curve were provided for a subset of 110 compounds. Detailed results are presented in the full report on the CD-ROM.

In Figure 164 the frequency of appearance of these 'identified' pollutants is plotted for each single sample (JDS1 – JDS68). The pollutants were merged into three groups, namely Pharmaceutical drugs, pesticides and industrial chemicals (as well as their known metabolites). The grey line represents the sum of all detected substances. The grouping of the substances reveals interesting courses which are marked by the red arrows. For instance, the first red arrow highlights the fact that the "peak" in the sum function of sample JDS12 is mainly caused by Pesticides (green course) while Pharmaceutical drugs and Industrial chemicals show an inconspicuous course. Furthermore, the high number of positive hits in case of JDS58 is almost only related to Pharmaceutical drugs (and Metabolites). Further interesting courses are marked in the same manner. These finding might allow the assignment of different sources of pollution which are released into the aquatic environment. On the other hand, a decrease of the sum function (arrow three and four) could possibly indicate a dilution of the surface water by less influenced inflows.

30.3.3 GC-MS

All 68 JDS3 water samples were analysed by LLE/LVI-GC-MS (method 1) whereas 22 LVSPE extracts were reconstituted and injected directly into GC-MS (method 2; for details, see Sections 30.2.1 and 30.2.4.2). Based on the obtained spectral information, chemical structures of 298 analytes (method 1) and of 288 analytes (method 2) could be proposed (for a list, see full report attached on the CD-ROM). An additional ca. 29% (method 1) and 38% (method 2) detected compounds remained unidentified (Figure 165). For a comparison, screening of 98 water samples in the JDS1 revealed the presence of 96 provisionally identified analytes and screening of 124 water samples in the JDS2 revealed the presence of 158 provisionally identified analytes. The used LVSPE sampling and concentration technique seems to be superior to that of LLE in terms of extraction efficiency of wide polarity range compounds and sensitivity allowing determinations at ng/l levels. On the other hand LLE was more selective to non-polar and volatile compounds.

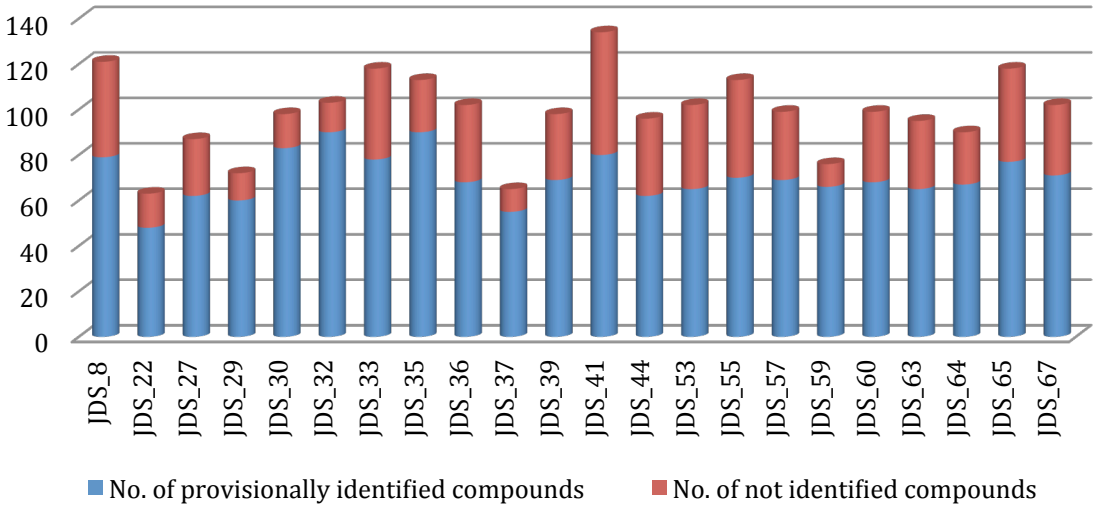

Figure 165: Number of compounds detected with LVI-GC-MS in the 22 JDS3 surface water samples obtained with the LVSPE sampling technique

The observed pollution is generally matching with the results of previous surveys (JDS1 and 2). Phthalates, alkylated polyaromatic hydrocarbons, alkylated phenols, alkanes and fatty acids belong to the most ubiquitous compounds detected (see Table 92).

Next to a wide variety of various substituted ethers, alcohols, esters, amines, amides, glycerols, tiols, aldehydes and ketones the following groups of substances were identified using the LVSPE samples: phthalates (diethyl phthalate, diisobutyl phthalate, dibutyl phthalate and DEHP), sun-screen agents (acetophenone, benzophenone, drometrizole, acetophenone), insect repellent DEET, flavour and fragrance agent ketoisophorone, food preservative benzoic acid, biocide triclosan, phosphorus flame retardants and phosphorus-based plasticisers (e.g. triphenylphosphine oxid and tris(2-chloro-1-methylethyl) phosphate), herbicides atrazine and terbuthylazine and fungicide spiroxamine. Remarkable was the widespread presence of p-toluenesulfonamide utilised as the starting material in the synthesis of biocide chloramin T. The most frequently identified pharmaceutical was gabapentin (anticonvulsant and analgesic drug), followed by carbamazepine (anticonvulsant and mood-stabilizing drug) and ibuprofen (nonsteroidal anti-inflammatory drug). Alkaloids and substances of daily use (caffeine, nicotine, cotinine) were revealed in the samples as well. Caprolactam – a precursor to Nylon 6 with an approximate annual production of 4.5 million t was detected at high concentrations in 20 samples. Metilox – an intermediate in the synthesis of phenolic antioxidants for polymers with a production rate of 23500 t (CIBA, 1992), has been detected in 21 samples.

In LLE samples substantial part of the identified substances were various derivatives of alkanes, alkenes, alkynes, esthers, aldhehydes, ketones, siloxanes, aromates and phthalates. The samples contained variety of emerging contaminants such as e.g. sun-screen agents (4-ethylbenzophenone, acetophenone and benzophenone), fragrances and musks (limonene, vanilin, isobornyl acetate, dihydro methyl jasmonate, galaxolide and ketoisophorone), herbicides (fenam), food additives (triacetin), phosphorus flame retardants (triphenylphosphate, tri(2-chloroethyl) phosphate, tributyl phosphate) and other cosmetic ingredients (glycols, tributyl acetylcitrate, linear alkyl benzenes (LABs) and ethylparaben). Pollution profiles by individual substances and discussion on the exceedance of available ecotoxicity threshold values (PNECs) is in the full report at the attached CD-ROM.

Table 92: List of twenty most frequently detected compounds provisionally identified in the surface water of the Danube river by the LVSPE/LVI-GC-MS and LLE/LVI-GC-MS methods

LVSPE/LVI-GC-MS		LLE/LVI-GC-MS	
Compound	Frequency of identification	Compound	Frequency of identification
DEHP	22/22	Dibutyl phthalate	42/68
Benzoic acid	22/22	Diethyl phthalate	41/68
Triphenylphosphine oxide	22/22	Naphthalene, X-methyl- (isomer)	39/68
Phenol, 2,4-bis(1,1-dimethylethyl)-	22/22	1-H-Indene, X-methyl (isomer)	36/68
Diethyl phthalate	22/22	1-H-Indene, X,X-dimethyl (isomer)	36/68
Acetophenone	21/22	X,X-Diisopropylnaphthalene	35/68
Caffeine	21/22	Indene	33/68
Metilox	21/22	1-Tetradecene	33/68
Ethanone, 1-[4-(1-methylethyl)phenyl]-	21/22	x-Xylene (isomer)	28/68
Diisobutyl phthalate	21/22	Caprolactam	28/68
Dibutyl phthalate	21/22	Ketoisophorone	28/68
Phthalimide	21/22	Caffeine	28/68
Cyclohexane, isocyanato-	20/22	Toluene	27/68
Ethanone, 1,1'-(1,4-phenylene)bis-	20/22	Phenol	27/68
Heptane, 3-[(ethenyloxy)methyl]-	20/22	Hexanoic acid	27/68
Caprolactam	20/22	Aniline	27/68
Heptane, 1-(1-butenyloxy)-	20/22	Phenol, x-methyl (isomer)	25/68
Phenol	19/22	Naphthalene, X,X-dimethyl- (isomer)	25/68
1,4-Benzenediamine, N-(1-methylethyl)-N'-phenyl-	18/22	Hexadecanoic acid, methylester-	24/68
Cyclohexane, isothiocyanato-	18/22	Linear alkyl benzene (LAB; isomer)	22/68

30.3.3.1 Retrospective analysis

Full scan EI mass chromatograms containing all spectral information from GC-MS screening of JDS3 samples was stored (digital sample banking) in order to allow for its retrospective analysis. The approach was tested with substances popping out from LC-MS analyses of the same samples, which were not detected using the routine GC-MS workflow. Here, only substances amenable to GC were considered and in the process all chromatograms were manually re-checked using specific ions of the suspect substances previously 'hidden' in the background.

The retrospective analysis of JDS3 chromatograms was surprisingly successful leading to identification of several compounds such as 1H-benzotriazole, p-toluenesulfonamide, carbamazepine, atrazine, diethyltoluamide (DEET), 2-(methylthio)benzothiazole, tetraglyme, triglyme, terbuthylazine, cotinine, triethylcitrate, triclosan and nicotine. An example of retrospective identification of biocide triclosan is shown in Figure 166.

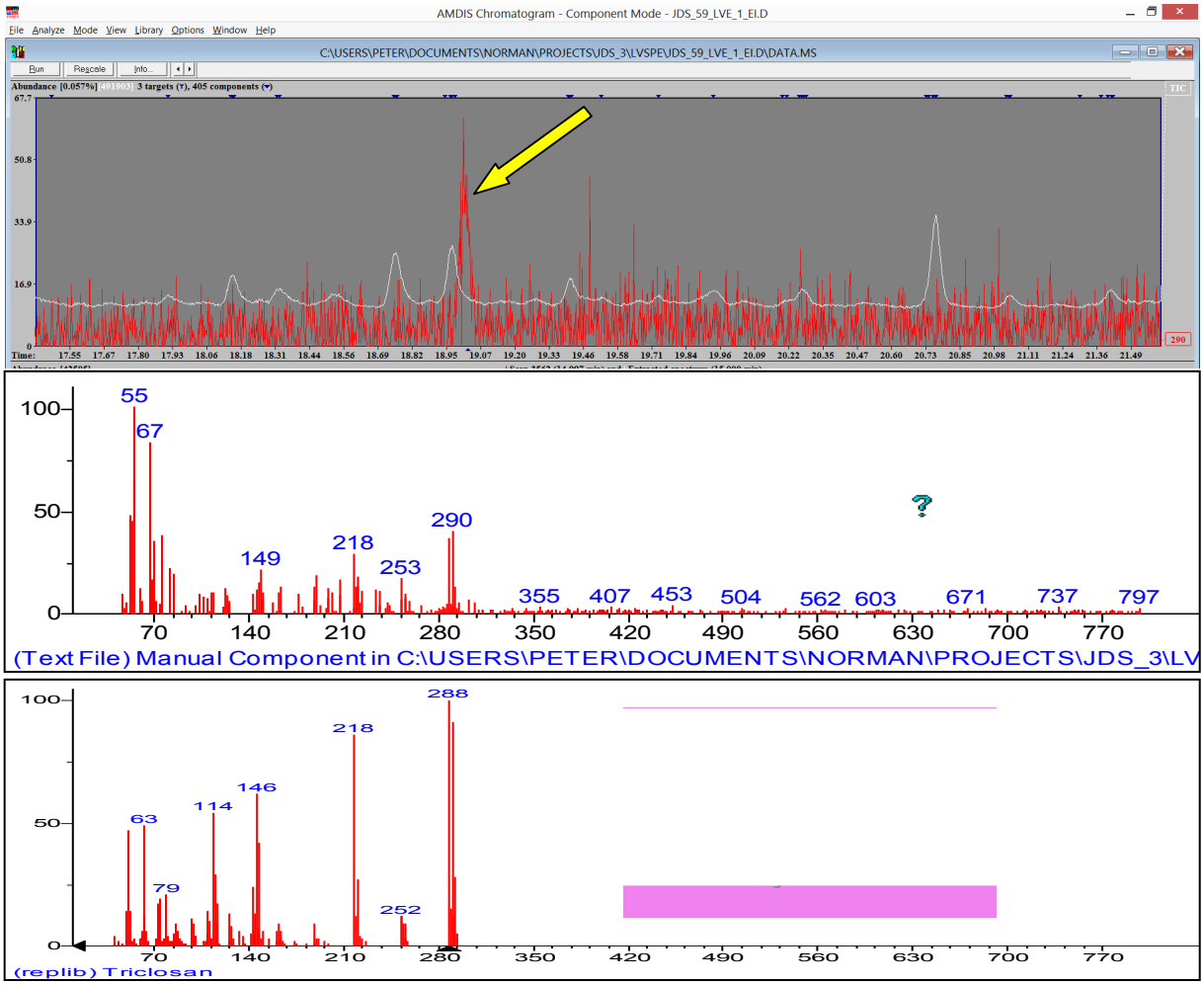

Figure 166: An example of retrospective analysis:
upper window – AMDIS software did not label a component marked with yellow arrow after manual deconvolution;
middle window - manually processed mass spectrum of the detected compound;
lower window - library mass spectrum of triclosan ($C_{12}H_7Cl_3O_2$)

30.4 Conclusions

Analysis of the Danube surface water samples at a basin-wide scale was conducted for the first time with two liquid chromatography-high resolution mass spectrometry instruments (UHPLC-QTOF-MS and LC-HR-MS). Target, suspect and non-target screening was performed with the major goal to search for as many compounds as possible while focusing on compounds not previously known to be present in the Danube river and its tributaries. Target analysis of 168 substances by UHPLC-Q-TOF-MS showed that 154 of the studied analytes were found to be present in at least one sample. Initial results from non-target screening by UHPLC-QTOF-MS revealed presence of more than 3370 different organic compounds listed by name (PCDL match). The follow up evaluations with autoMSMS method resulted in unequivocal identification of 56 substances dominated by pesticides, pharmaceuticals and personal care products. The rest of tentatively identified suspect compounds, unknowns (proposed molecular formula) and total unknowns (only accurate mass and retention time available) still need to be investigated and those results can be expected in the near future.

The 'suspect screening' by LC-HR-MS showed that 110 out of 315 'searched for' substances were determined in at least one sample and 50 compounds were present in more than 20 samples. A semi-quantitative analysis was performed for 110 analytes. Despite the lists of target/suspect substances in

two LC-MS laboratories differ, there is a good agreement on the overlapping compounds, e.g. DEET found by both laboratories in all 68 samples and gabapentin in 67 vs. 65 samples with LC-QTOF-MS and LC-HR-MS, respectively.

Both of the techniques could achieve low-ng/l detection limits of wide range substances with direct injection of the water sample, which is significantly reducing the need for laborious sample preparation. The statistical software at LC-QTOF-MS allowed for analysis of differing pollution patterns for the river stretches and countries within the basin. Combination of high resolution technique with different algorithms and the availability of comprehensive mass spectral libraries with accurate mass fragmentation information was shown to be important at the detected compounds' identification. A Danube river basin mass spectral library linked to/or being part of existing international databases equipped with various structure elucidation tools, such as NORMAN MassBank (Schulze et al., 2012, NORMAN Association, 2014), would be of great benefit for identification of present and future emerging substances.

The GC-MS results were complementary to those obtained by LC-MS. Chemical structures of 298 and of 288 substances in 68 and 22 samples collected by two different methods (LLE and LVSPE) could be proposed. Still, up to 38% detected substances remained unidentified. A rough estimation of the compounds' concentrations was made based on the comparison of their ion signal with that of the internal standard, which allowed for establishment of their pollution profiles across the basin and preliminary risk assessment by comparing the concentration data with available PNECs. A retrospective analysis of 'digital sample banking' GC-MS data proved to be successful. The presence of several pollutants, which would otherwise stay undetected, was revealed.

Obviously, spot sampling such as in the JDS3 does not allow for assessment of trends and variations in pollution pattern of the Danube river and its tributaries. Therefore additional one year sampling during four seasons would be recommended to register pollution by e.g. pesticides and their transformation products, virucides and antibiotics. A more intense sampling (e.g. one week; 24 h sample) at selected sites would be needed to capture pollution by e.g. illicit drugs used mainly during the weekend (Karolak et al. 2012).

Non-target screening is a powerful tool at the identification of the RBSPs. Present MS systems generate vast amounts of data and therefore there is a need for strategy to reduce the amount of detected (thousands of) substances in a single sample to 'workable' numbers (top 10 – 100 substances). One of the possible ways out is prioritisation of non-target screening data being currently developed by the NORMAN Working Group on Prioritisation (www.norman-network.net) using the principles outlined in the recent paper by Schymanski et al. (2014) and NORMAN prioritisation framework (2012). Presented results clearly indicate that for the assessment of the presence of organic compounds and for detection of environmental contamination in sufficiently early stage new sensitive quantitative target and non target analysis are needed. Detection of local environmental contamination in different environmental compartments at the right time prevents global spread of pollution and also a series of harmful effects that pollutants have on plant and animal organisms, including humans.

30.5 5 References

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31 Emerging organic substances in surface water

Jaroslav Slobodnik, Ildiko Ipolyi

31.1 Introduction

One of the main goals of the previous Joint Danube Surveys was that each of the determinands had been measured by one reference laboratory in order to get consistent data sets. However, the increased identification power of laboratory instrumentation and involvement of numerous external laboratories providing in-kind analyses in the JDS3 led to the situation that for the first time many parameters were analysed by several laboratories. Data on 719 target organic substances in water, sediment, SPM and biota were measured by 13 JDS3 laboratories. Out of these, 654 substances were analysed in surface water samples. All data (more than 47,000 data entries) are collected in the JDS3 specific Data Collection Templates and stored in an Access database developed by Environmental Institute to be later uploaded into the ICPDR Water Quality Database.

The analysis of the data measured by more than one laboratory often showed differing results. Considering that all laboratories involved are either accredited or highly experienced and well acquainted with the Danube samples, the aim of this chapter is to discuss if the differences observed are of relevance in the process of determining the Danube River Basin Specific Pollutants (DRBSPs).

Laboratories of UFZ Leipzig (UFZ), JRC Ispra (JRC), Umweltbundesamt Vienna (UBAAT), TZW Karlsruhe (TZW), UMEA University (UMEA), PM Brno (PM), WRI Bratislava (WRI) and LfU Augsburg (LfU) provided most of the target analyses data using long-term established quantitative methods. Unfortunately, the raw data from TZW were not available at the time of writing this report. Laboratories of Croatian Waters (CW) had been equipped with the state-of-the-art UHPLC-QTOF-MS(MS) analytical equipment in 2013 and provided quantitative data for 168 target substances, whereas laboratory of LW Langenau (LW) used semi-quantitative methods to estimate concentrations of 110 substances detected in the samples. For several parameters (e.g., caffeine and carbamazepine) as markers of urban waste water pollution a principally different method (ELISA) has been applied by BAM (Germany; in cooperation with UFZ). Laboratories of CW and Environmental Institute (EI) provided additional non-target screening analyses of all samples (cf. Chapter 30), however, these will not be used for additional comparisons in this chapter.

A preliminary prioritisation of these large datasets took place to find out which of the substances are exceeding the selected (eco)toxicity limit values in the Danube River Basin and at how many places (cf. Chapter 32). Additionally, authors of individual chapters dealing with organic substances suggested their 'top' list of pollutants based on various considerations (e.g. highest concentrations found, in how many samples a substance is present, link to ground water pollution, etc.).

A specific set of passive sampling analyses has been performed by RECETOX Brno (Czech Republic) in cooperation with NIVA (Norway) using a 'composite sample' of the Danube River collected within several days. Results of these analyses cannot be compared directly to the results of the 'spot' samples from individual JDS sites above, however, give a good indication which substances are of importance for a defined stretch of the Danube.

The aim of this chapter is to discuss the confidence in the results of the top ranking target substances which are not yet regulated at the EU level obtained by different JDS3 laboratories. Aspects of performance of analytical methodologies (e.g., limit of detection (LOD), limit of quantification (LOQ) vs. predicted no effect concentration/environmental quality standard (PNEC/EQS) values), occurrence

of pollutants in multiple samples detected by several laboratories etc. were considered. Four sampling sites (JDS33 – Downstream Novi Sad; JDS56 – Russenski Lom; JDS57 – Downstream Ruse/Giurgiu and JDS58 – Arges) were selected for this consideration. This selection was based on the preliminary evaluation of the entire JDS3 dataset indicating that the sites 58 (Arges) and 56 (Russenski Lom) provided the highest concentration values for numerous organic pollutants among Danube tributaries whereas the sites 33 (downstream Novi Sad) and 57 (downstream Giurgiu/Ruse) samples were analysed for all chemical parameters and related ecotoxicological parameters with all laboratories involved.

31.2 Results

31.2.1 Potential Danube river basin specific pollutants (DRBSPs - preliminary prioritisation)

A list of potential 15 DRBSPs (excluding five already regulated WFD priority substances discussed elsewhere) has been proposed in Chapter 32. A good analytical performance of all seven laboratories involved was documented by the LOQs of their methodologies at sub-µg/l and low-ng/l range (cf. Table 93). This holds also for LW claiming that their results are semi-quantitative due to using a single point calibration curve and therefore not providing method LOQs. Their findings match relatively well with the other laboratories and typically show results at low ng/l level. Highest LOQs were observed for bisphenol-A (0.1 and 0.01 µg/l; respectively). It should be noted that even these state-of-the-art methodologies were not able to reach detection limits imposed by the lowest PNEC values for 2,4-dinitrophenol (2,4-DNP), chloroxuron, desethylterbutylazine, PFNA, diazinon and 17-beta-estradiol (see Table 93; LOQs higher or equal to the respective lowest PNECs highlighted in yellow colour). Nevertheless, these substances were still detected at quite some sampling sites, thus exceeding the lowest PNEC values and scoring high in the prioritisation process. 'Pushing down' the LOQs closer to the lowest PNEC values might highly probably result in observation that especially diazinon and 17-beta-estradiol were present at environmentally relevant concentrations in more than the reported 21 and 8 sampling sites, respectively.

The top prioritised substance 2,4-DNP had been analysed by two laboratories, the semi-quantitative results by LW were ca. one order of magnitude higher than those by UFZ, however, matching clearly a trend of UFZ results in samples analysed by both laboratories (e.g., double increase in concentration between samples JDS57 and JDS33). Here, one may assume that despite the substance has been found at relevant concentrations in all 22 (LVSPE) samples analysed by UFZ, the LW results are probably reported as systematically higher also for other (46) sampling sites not analysed by UFZ and therefore the results of prioritisation might be slightly overestimated. The result of JDS57 by UFZ (value below reported LOQ) is probably related to the method LOD.

Chloroxuron has been detected by CW in samples JDS33 and JDS57. This finding has not been confirmed by UFZ whose method LOQ does not take into account used sample preparation procedure (LVSPE of 1000 l sample on cartridge filled with three different sorbents followed by freeze drying and elution by organic solvent; recovery rates estimated in comparison with another LC-MS-based UFZ method using direct injection of water samples). The reason might be reported loss in concentrations of some substances by the LVSPE procedure and thus systematic underestimation of reported results (cf. Chapter 27). CW methodology is also using direct injection of 100 µl water sample on-column and therefore any loss due to sample preparation step is eliminated. Similar discrepancy in results could be explained for PFOA detected in JDS33 and JDS57 samples by JRC whereas staying undetected by UFZ. The result of JDS57 by CW (value below reported LOQ) is probably related to the method LOD.

Desethylatrazine, despite being on the list of target substances of four laboratories, could be detected at concentrations above LOQ in the four discussed samples only by CW and LW. The results do not match very well, which might be due to the presence of the substance in these particular samples at very low-ng/l concentrations levels close to the LOQs of the methods applied.

Bisphenol-A has been detected by WRISK at high concentrations in JDS56 and JDS58 whereas two other laboratories (UFZ, CW) reported values lower than their respective LOQs. All laboratories reported 'less than LOQ' value in the other two samples. It has not been possible to clarify this issue at the time of writing this report, however, a detailed comparison of the used methodologies and consequently relevance of bisphenol-A as a DRBSP should be thoroughly verified.

A good match has been found between the results for diazinon by UFZ and LW. CW with its lowest method LOQ detected linuron in JDS57, the rest of the results 'less than LOQ' by the other two laboratories (UFZ, LW) is well matching. Metazachlor was below the method LOQs of three laboratories (CW, LW, UBAAT) in samples from the studied four sampling sites as it is obviously applied in different parts of the Danube river basin.

Diclofenac, notoriously present in environmental samples as one of the markers of pollution by urban waste water, could be considered as an example of the JDS3 laboratory performance. All results by five laboratories were in a very close range in all four discussed samples. Whenever a 'less than LOQ' value was reported it could be linked to its respective method LOQ (e.g. 4 ng/l reported only by JRC in JDS33 sample could indeed be obtained by the method with LOQ 0.4 ng/l). Bentazon results by UFZ, LW and UBAAT were matching nicely, the 'less than LOQ' values by UBAAT were justified by the higher reported LOQ.

Substances 2-hydroxy atrazine, PFNA, bromacil, dimefuron, amoxicillin and 17-beta-estradiol have been on the list of target substances only by one laboratory and therefore no critical intercomparison could be made.

31.2.2 Large volume sampling (UFZ)

LVSPE samples were collected at 22 sampling sites and subjected to target screening of 264 substances by UFZ. Out of this number, 91 compounds could be detected in at least one sample (cf. Chapter 27). To compare the results produced by various laboratories the top ten pollutants present at highest concentrations in the LVSPE samples were selected (cf. Table 94). Additionally included is also carbamazepine, which was thoroughly analysed and discussed as a marker of urban waste water pollution together with acesulfame and caffeine.

Six out of these 11 substances (metformin, enalapril, triphenylphosphine oxide, cyclamate, creatinine and 2-benzothiazolesulfonic acid) were determined only by a single method (UFZ) and therefore no comparison could be made.

Acesulfame was present in all 22 LVSPE samples and reported at extremely high concentrations of 1.22 and 1.05 µg/l in the Danube tributaries Rusenski Lom and Arges, respectively. Expectedly, slightly lower concentrations were determined downstream Novi Sad and downstream Giurgiu/Ruse (0.46 and 0.49 µg/l, respectively). Acesulfame was the compound with the highest concentrations observed also by TZW both in the Danube (1.1 µg/l) and its tributaries (2.9 µg/l) and in groundwater (0.45 µg/l). The results indicate systematically higher results by TZW by factor 2-3, which would be in line with reasoning on loss of some compounds during the LVSPE procedure (cf. above and Chapter 27). Unfortunately, the raw data allowing for more detailed comparison were not provided by TZW at the time of writing this report.

Sucralose concentrations in JDS33 and JDS57 were reported by two laboratories. The semi-quantitative results by LW were systematically higher by a factor of ca. 3 compared to those from UFZ.

Caffeine was analysed in JDS3 samples by five laboratories. Results of UFZ and BAM were systematically higher by a factor of 2-3 compared to concentrations reported by CW and LW in samples from JDS33 and JDS57. Despite the differences were at low ng/l levels, more harmonisation between the different methodologies (UFZ – LVSPE-LC-MS; BAM – ELISA; LW/CW – direct injection of water sample into LC-HR-MS system) might be needed. A good agreement among the results by all laboratories was found at highly polluted JDS56 and JDS58. The only outlying result, lower by factor of ca. 5-7 compared to the other laboratories, was obtained by ELISA method (BAM). This might indicate a need to extend the method's calibration range in case of expecting such high pollution levels.

Carbamazepine has been determined in all four discussed samples by six laboratories with a very good match of most of the results at both lower (JDS33, JDS56, JDS57) and higher (JDS58) pollution levels. Systematically underestimated concentrations by factor of ca. 2-4 in comparison to other laboratories were reported by CW. Nevertheless, the identical pollution trends across samples (ups, downs) were followed by results of each individual laboratory.

31.2.3 Emerging polar organic substances and benzodiazepines (JRC, UMEA)

Eight substances extensively discussed in the chapter on emerging polar organic substances by JRC were selected for a comparison with determinations of other laboratories (cf. Table 95). All of the pollutants were found in 93 to 100% of the analysed samples. The list does not include WFD priority substances and substances discussed in this chapter above (for a full list see Chapter 22). In addition, oxazepam has been selected as a representative of the benzodiazepine anxiolytic drugs by UMEA, which was found in 85% of the (68) analysed samples.

A very good match was observed between the results of JRC, UFZ and LW for 1-H-benzotriazole and methylbenzotriazole isomers (JRC and UFZ only). 2,4-D was found in the discussed samples only by JRC with the method being almost three orders of magnitude more sensitive than that of UFZ. CW and LW did not report LOQs, however, it seems obvious their methods also do not detect concentrations of 2,4-D below 0.02 µg/l. The same holds for another polar pesticide of the same class MCPA, where extremely low concentrations 1 ng/l in JDS33, JDS57 and 0.3 ng/l in JDS56 were reported. The only matching result from other laboratory (1 ng/l in JDS33 by UFZ; below the reported LOQ) seems to be related to their method LOD.

Results for 10,11-dihydro-10,11-dihydroxy-carbamazepine (metabolite of carbamazepine) were reported only by JRC. The concentrations were contained within the same order of magnitude as those determined for the parent compound (cf. Table 94).

A good match was obtained between results of JRC and UFZ for the insecticide repellent DEET despite its presence at low ng/l levels in samples from JDS57 and JDS33. The semi-quantitative results by LW seem to be overestimated by an order of magnitude at this low concentration range. However, in the mid-ng/l concentration range the results of JRC, LW ad UBAAT already match rather well (cf. Table 95 - JDS58).

The highest concentrations of metolachlor pesticide was recorded in the samples from JDS56 by four laboratories (JRC, UFZ, CW, LW) while LOQ of the method used by UBAAT was above the reported values. A good agreement between the values could be observed with JRC providing the highest and CW the lowest value. The same pattern could be observed also in the other three samples.

The concentrations in the obviously highest polluted sample JDS58 for the pharmaceutical sulfamethoxazole match well for JRC, UFZ and LW whereas CW seems to underestimate the results by ca. one order of magnitude. Similar pattern can be observed at the other three samples.

Methods for determination of oxazepam were available at UMEA, CW and LW. A good match was found between the results of UMEA and LW whereas the reported LOQ by CW seems to be slightly overestimated (concentrations above the LOQ of 3 ng/l not detected in several cases).

31.2.4 Surface water and ground water connection (TZW)

A set of 49 compounds was analysed by TZW according to standard routines which comprised benzotriazoles, artificial sweeteners, betablockers, lipid-lowering drugs, nonsteroidal anti-inflammatory drugs, cytostatic drugs and other pharmaceuticals, iodinated X-ray contrast media (X-RCM), the stimulant caffeine and the preservative salicyclic acid. Moreover, drug metabolites clofibric acid, 4-acetylaminoantipyrine and 4-formylaminoantipyrine (AAA and FAA) were included. For the first time the link between contamination of surface water and groundwater was explored. A number of emerging substances were detected during the JDS3 in the abstraction wells at bank filtration sites. This phenomenon can be expected for substances like amidotrizoic acid, iopamidol, acesulfame, benzotriazole or carbamazepine which are known to be quite persistent in the aquatic environment and which are mostly not completely retained by bank filtration.

Determination of acesulfame, sucralose, carbamazepine, diclofenac and benzotriazoles were discussed in the above text with information extracted from Chapter 25. It is expected that more detailed information in a form of raw data will be provided later allowing for more detailed comparison of results with other laboratories.

31.2.5 Passive sampling

Next to a number of WFD priority substances spatial variability of caffeine, carbamazepine and diclofenac in the water column were measured by the "active" passive sampling system samplers in 8 Danube stretches. Resulting data is expressed as freely dissolved amount of compound (in ng) taken up by an integrative sampler during a sampler exposure (1.2 - 2 days; cf. Chapter 29). The integrative character of passive sampling allowed measurement of compounds down to pg/l levels where methods based on low volume spot sampling of water may fail to detect them. Whereas data from spot sampling reflects the pollution at the individual JDS sampling sites at a single moment of time, passive samplers continuously sampled pollutants for several days, including river stretches between individual JDS sampling sites. Thus, the information provided by spot sampling and passive sampling should be considered as complementary and not to be compared.

31.3 Conclusions

Data on 654 target organic substances were analysed by 13 laboratories in JDS3 surface water samples. More than 47,000 data entries were collected in the JDS3 Data Collection Templates and stored in a provisional MS Access database developed by Environmental Institute to be later uploaded into the on-line ICPDR Water Quality Database (http://www.icpdr.org/wq-db/). An analysis of the data had shown that numerous organic substances were measured by more than one laboratory, often providing differing results. The differences in results were discussed if they are of relevance either in the process of determining the Danube River Basin Specific Pollutants or other ranking schemes. Four sampling sites (JDS33 – Downstream Novi Sad; JDS56 – Rusenski Lom; JDS57 – Downstream Ruse/Giurgiu and JDS8 – Arges) had been selected as a proxy to highlight the differences among the results.

As regards the list of 15 DRBSPs a good analytical performance of all seven laboratories involved in analyses was documented by limits of quantification (LOQ) of their methodologies at sub- µg/l and low-ng/l range (cf. Table 93). Substances 2-hydroxy atrazine, PFNA, bromacil, dimefuron, amoxicillin and 17-beta-estradiol have been on the list of target substances only by one laboratory and therefore no critical intercomparison could be made. Analysis showed that that even the state-of-the-art methodologies were not able to reach detection limits imposed by the lowest PNEC values for 2,4-dinitrophenol (2,4-DNP), chloroxuron, desethylterbutylazine, PFNA, diazinon and 17-beta-estradiol and the respective analytical methodologies should be improved. The results by various laboratories matched well and almost all differences could be explained on the basis of available methods' description. Data on bisphenol-A deserve more detailed analysis prior to including the compound among the DRBSPs.

Six out of the top 10 ranking substances from the list of LVSPE sampling (metformin, enalapril, triphenylphosphine oxide, cyclamate, creatinine and 2-benzothiazolesulfonic acid; for details see also Chapter 27) were determined only by a single method (UFZ) and therefore no comparison could be made. Acesulfame, sucralose, caffeine and carbamazepine (included additionally to the top ten compounds as a marker of municipal waste water pollution) showed nicely matching results among all laboratories. The differences were usually of a systemic nature, which suggests that a proficiency testing schemes for these substances should be run at the Danube River Basin or European scale to achieve uniform performance.

Eight of the emerging polar organic substances suggested as relevant for the Danube River Basin by JRC (cf. Table 95) were found overwhelmingly in 93 to 100% of the analysed samples. Similar to the discussion above differences in concentration were only of a systemic nature related to the method LOQs and construction of a calibration curve.

Valuable aggregate results were provided by TZW on the subset of 49 substances indicating possible transfer of pollutants from the Danube to the connected drinking water abstraction ground water bodies.. A good agreement on general trends of pollution has been found with a few outlying results (e.g.; caffeine at JDS58 by BAM), which should be clarified.

Concerning passive sampling analyses the information provided by spot sampling and passive sampling should be considered as complementary and cannot be compared directly.

No attempt has been made yet to support the target analyses data with the outcomes of non-target screening as the list of detected and tentatively identified substances is very large (possibly up to 16,000 entries/substances in all 68 JDS3 samples) and is still under investigation.

In general the majority of the top ranking emerging organic pollutants in the simplified 'proxy' set of four JDS3 samples were detected by more than one laboratory and the differences in their determinations were either negligible or explainable on the basis of reported methods' LOQs. Systematic differences (e.g. plus/minus order of magnitude in resulting concentrations) were usually contained in the low-ng/l range close to the state-of-the-art performance of the existing analytical methodologies. Here, good news is that the observed pattern of pollution (concentrations going up or down on different JDS3 sites) is followed by all involved laboratories which provides a high certainty about the presence of these pollutants in the analysed samples. The above indicates that once a final list of the 'important' Danube substances is established a proficiency testing scheme shall be applied to harmonise the performance of all laboratories for each individual substance. In general there is also a strong need to improve analytical methods for the described substances of concern.

Table 93: Comparison of concentrations (in µg/l) of 17 proposed Danube River Basin Specific Pollutants in samples from JDS33 (Downstream Novi Sad), JDS56 (Rusenski Lom), JDS57 (Downstream Giurgiu/Ruse) and JDS58 (Arges) sites analysed by several laboratories

No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Substance		2,4-Dinitrophenol (DNP)	Chloroxuron	PFOA (Perfluorooctanoate)	Desethylterbutylazine	Atrazine-2-hydroxy	PFNA (Perfluorononanoate)	Bromacil	Dimefuron	Bisphenol A	Diazinon	Linuron	Amoxicillin	Metazachlor	17beta-estradiol	Diclofenac	Bentazon	Fipronil
No. of sites substance detected[1]		68	65	66	54	53	52	31	58	30	21	32	33	30	8	51	61	1
Lowest PNEC or EQS		**0.001**	**0.002**	**0.0029**	**0.0024**	**0.002**	**0.00039**	**0.01**	**0.008**	**0.1**	**0.001**	**0.26**	**0.078**	**0.019**	**0.0004**	**0.05**	**0.06**	**0.012**
LOQ	UFZ	0.006	0.003	0.006	0.005					0.01	0.003	0.003				0.02	0.01	0.003
	JRC			0.001			0.0007									9E-04		
	CW		0.002		0.002			0.001	0.003	0.005		0.002	0.003	0.004	0.003			
	LW[2]	*			*	*					*	*		*		*	*	*
	UBAAT				0.05									0.05		0.05	0.05	
	TZW															*		
	WRISK									0.1								
JDS58	UFZ				<LOD						0.009					0.32	0.08	
	JRC			0.0026			0.0011									0.25		
	CW		0.006		0.003			<LOQ	<LOQ	<LOQ			<LOQ		<LOQ			
	LW	0.01			<LOQ	0.06					0.007					0.2	0.1	0.02
	UBAAT				<LOD											0.24	0.09	
	TZW															0.28		
	WRISK									0.27								
JDS57	UFZ	0.004	<LOQ	<LOQ	<LOQ					<LOQ	<LOQ			<LOQ		<LOQ	0.001	<LOQ
	JRC			0.004			<LOQ									<LOQ		
	CW		0.001		<LOQ			<LOQ	0.006	<LOQ		0.006	<LOQ	<LOQ	<LOQ			
	LW	0.02			0.006	0.01					<LOQ	<LOQ		<LOQ		<LOQ	0.004	<LOQ
	UBAAT				<LOD									<LOD		<LOQ	<LOD	
	WRISK									<LOQ								

JDS56	UFZ				<LOQ						<LOQ	<LOQ		<LOQ		0.05	0.02	
	JRC			0.0032			<LOQ									0.07		
	CW		0.009		<LOQ			<LOQ	0.01	<LOQ		<LOQ	<LOQ		<LOQ			
	LW	0.01			<LOQ	0.02					<LOQ	<LOQ		<LOQ		0.02	0.02	<LOQ
	UBAAT				<LOD									<LOD		<LOD	<LOD	
	TZW															0.05		
	WRISK									0.51								
JDS33	UFZ	0.008	<LOQ	<LOQ	<LOQ					<LOQ	<LOQ	<LOQ		<LOQ		<LOQ	0.001	<LOQ
	JRC			0.012			0.0007									0.004		
	CW		0.008		<LOQ			<LOQ	0.018	<LOQ		<LOQ	<LOQ	<LOQ	<LOQ			
	LW	0.04			0.009	0.01					<LOQ	<LOQ		<LOQ		<LOQ	0.005	<LOQ
	UBAAT				<LOD											<LOD	<LOD	
	WRISK									<LOQ								

[1]LVSPE samples were collected only at 22 (out of 68) sampling sites.
[2]LW Langenau did not report method LOQs due to using a semi-quantitative 'single-calibration-point' quantification.

Table 94: Comparison of concentrations (in µg/l) of top listed pollutants in LVSPE samples from JDS33 (Downstream Novi Sad), JDS56 (Rusenski Lom), JDS57 (Downstream Giurgiu/Ruse) and JDS58 (Arges) sites analysed by several laboratories

No.		1	2	3	4	5	6	7	8	9	10	11
Substance		Metformin	Acesulfame	Enalapril	Triphenylphosphine oxide	Cyclamate	Caffeine	N-acetyl-4-aminoantipyrine	Creatinine	Sucralose	2-Benzothiazolesulfonic acid	Carbamazepine
No. of sites substance detected[1]		21	22	21	22	21	12	21	12	21	22	22
LOQ	UFZ	0.045	0.02	0.029	0.0038	0.03	0.08	0.01	0.025	0.03	0.003	0.001
	JRC											0.00015
	CW						0.003					0.0016
	LW						*	*		*		*
	BAM						0.03					0.02
	TZW[2]		*				*			*		*
JDS58	UFZ		1.05					1.5				0.14
	JRC											0.07
	CW						1.2					0.03
	LW						1.8	1.3		0.4		0.10
	BAM						0.25					0.12
	TZW[2]		*				1.7			*		0.14
JDS57	UFZ	0.19	0.49	0.097	0.019	0.029	0.17	0.14	<LOD	0.07	0.045	0.043
	JRC											0.018
	CW						0.06					0.009
	LW						0.06	0.08		0.2		0.040
	BAM						0.1					0.025
	TZW[2]		*				*			*		*
JDS56	UFZ		1.22					0.6				0.07
	JRC											0.04
	CW						0.3					0.02
	LW						0.5	0.5		<LOQ		0.06
	BAM						0.8					0.06
	TZW[2]		*				0.8			*		*
JDS33	UFZ	0.13	0.46	0.047	0.1	0.049	0.18	0.07	0.02	0.08	0.067	0.034
	JRC											0.035
	CW						0.06					0.009
	LW						0.05	0.1		0.2		0.060
	BAM						0.12					0.036
	TZW[2]		*				*			*		*

[1]No. of sites with substance detected in LVSPE samples; altogether 22 samples out of 68 JDS3 investigated sites; for a list, see Chapter 27.

[2]List of substances analysed and results extracted from the text of the Chapter 27; raw data allowing for thorough comparison not available.

Table 95: Comparison of concentrations (in µg/l) of top listed pollutants by JRC and UMEA in water samples from JDS33 (Downstream Novi Sad), JDS56 (Rusenski Lom), JDS57 (Downstream Giurgiu/Ruse) and JDS58 (Arges) sites analysed by several laboratories

No.		1	2	3	4	5	6	7	8	9
Substance		1-H-Benzotriazole	Methylbenzotriazoles	2,4-D (2,4-Dichlorophenoxyacetic acid)	10,11-Dihydro-10,11-dihydroxy-carbamazepine (CBZ-metabolite)	DEET (N,N-Diethyl-m-toluamide)	MCPA (2-Methyl-4-chlorophenoxyacetic acid)	Metolachlor	Sulfamethoxazole	Oxazepam
Percentage of sites substance detected[1]		100%	100%	96%	100%	100%	93%	99%	100%	85%
LOQ	UFZ	0.05	0.01	0.1		0.003	0.003	0.01	0.01	
	JRC	0.0007	0.0005	0.0002	0.0003	0.002	0.00015	0.002	0.0001	
	CW							0.003	0.003	0.003
	LW	*				*		*	*	*
	UBAAT					0.05		0.05		
	UMEA									0.0005
JDS58	UFZ	0.07	0.35	<LOQ			<LOQ	<LOQ	0.21	
	JRC	0.3	0.29	0.008	0.16	0.04	<LOQ	0.003	0.14	
	CW			<LOQ				0.0004	0.001	<LOQ
	LW	0.4				0.1		0.003	0.2	0.008
	UBAAT					0.08		<LOD		
	UMEA									0.011
JDS57	UFZ	0.32	0.22	<LOQ		0.002	<LOQ	0.0009	0.005	
	JRC	0.24	0.12	0.001	0.03	0.008	0.001	0.0047	0.016	
	CW			<LOQ				0.0012	0.0007	
	LW	0.4				0.02		<LOD	0.03	
	UBAAT					<LOD		<LOD		
	UMEA									
JDS56	UFZ	0.08	0.012	<LOQ			<LOQ	0.017	0.019	
	JRC	0.1	0.025	0.02	0.09	0.008	0.0003	0.039	0.047	
	CW			<LOQ				0.006	0.0008	<LOQ
	LW	<LOQ				0.02		0.01	0.04	<LOQ
	UBAAT					<LOD		<LOD		
	UMEA									<LOQ
JDS33	UFZ	0.22	0.12	<LOQ		0.003	0.001	0.001	0.006	
	JRC	0.3	0.07	0.001	0.05	0.002	0.001	0.01	0.013	
	CW			<LOQ				0.0004	0.0007	<LOQ
	LW	0.8				0.03		0.005	0.03	0.01
	UBAAT					<LOD		<LOD		
	UMEA									0.005

[1]Precentage of samples analysed by JRC.

32 Prioritisation and identification of Danube River Basin Specific Pollutants

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32.1 Introduction

Given the vast number of chemicals which may be released into the environment and existing time and budget constraints of all involved parties to deal with thousands of potential pollutants, there is a need to prioritise chemicals for their regulatory risk assessment and monitoring. Article 16 of the WFD sets out the strategy to reduce the chemical pollution of European waters (EU 2000). Thereby, the chemical status assessment is used alongside the ecological status assessment to determine the overall status of a water body and to define management measures. The recently updated Directive 2013/39/EU (EU 2013) establishes environmental quality standards (EQS), expressed as both annual average (AA) concentrations and maximum allowable concentrations (MACs) for 45 priority substances. Compliance with AA-EQSs and MAC-EQSs sets the chemical status of the water body as ''good''. Under the WFD, Member States must set quality standards (according to Annex V, 1.2.6) for ''river basin specific pollutants'' (RBSPs; listed in Annex VIII, 1–9) that are ''discharged in significant quantities'' and take action to meet those quality standards by 2015 as part of ecological status (Article 4, 11, and Annex V, 1.3 (EU 2000). EQSs are therefore key tools in assessing and classifying both chemical and ecological status. Whether a compound is ''discharged in significant quantities'' is commonly decided based on the substance's exposure level, referred to as Predicted Environmental Concentration (PEC). This, in turn is compared to an ecological safety threshold expressed as Predicted No-effect Concentration (PNEC). PEC/ PNEC risk ratios above 1 would trigger the substance's consideration as RBSP and its inclusion in the routine monitoring and the derivation of a legally-binding EQS.

Despite majority of the Danube countries have already defined their national RBSPs and related EQSs, there is no recent update of the Danube river basin-wide list of specific pollutants. The currently valid list includes only arsenic, chromium, copper and zinc without specifying their EQSs. A prioritisation methodology to select RBSPs in a wider European context, including the data from the Danube river basin, was introduced by von der Ohe et al. (von der Ohe 2011). It was based on the methodology developed by the prioritisation working group of the NORMAN network (Dulio 2013). The approach has more recently been applied for the prioritisation of the monitoring data from the Slovak Republic (Slobodnik 2012). All of the prioritisation efforts run so far either at the EU, river basin or national level concluded that there is a need for more occurrence and ecotoxicity data of high quality. This has been understood also at the design of the JDS3 and one of the specific goals of the survey was to provide a complex dataset allowing for selection of the Danube RBSPs.

The aim of this study was to prioritise among the large number of substances detected in the surface water samples during the JDS3, using the simplified NORMAN prioritisation approach (von der Ohe 2011, Slobodnik 2012).

32.2 Methods

32.2.1 Prioritisation methodology

The NORMAN prioritisation methodology uses a decision tree that first classifies chemicals into six categories depending on the information available. That allows water managers to focus on the next steps to be taken, e.g. (not exhaustive): (1) derivation of EQS for substances already well investigated with sufficient amount of data on their occurrence and toxicity; (2) improvement of analytical methods for substances monitored whose limits of quantification (LOQs) are higher than PNEC values; (3) additional screening when more occurrence data are needed to confirm a basin wide thread; and, (4) discontinue with monitoring of substances that are already well investigated and proved not to represent a threat to the environment. The priority within each category is then evaluated based on several indicators, including exposure (e.g. frequency of observations above LOQs of used methods, annual usage, use pattern, etc.), hazard (e.g. Persistence, Bioaccumulation, Toxicity (PBT), Endocrine Disruption (ED) and Carcinogenicity, Mutagenicity and Reprotoxicity (CMR) properties) and risk (cf. text below).

Considering the specifics of the JDS3 dataset, no categorisation was run and only two risk indicators were proposed for the prioritisation of target analytes detected in surface water samples, namely the *Frequency of Exceedance (FoE)* and the *Extent of Exceedance (EoE)*, that are subsequently added to a final ranking score (RS). Both indicators make use of the maximum environmental concentration (MEC) at each site, but assess them in two ways to address both the spatial and temporal variation in the exposure. These two indicators are based on MECs, rather than the commonly used statistically based averages (PECs), and compared to the lowest acute-based ($PNEC_{acute}$) or chronic-based ($PNEC_{chronic}$) thresholds. The surface water samples from the 68 monitoring sites have been analysed by different laboratories, using various analytical methods. Hence, multiple entries for the same site/compound combination exist. In order to aggregate them to a single measure of exposure for each sampling site, the maximum concentration from all measurements was used. The reason for this was not to bias towards substances, which have been analysed only by one laboratory.

32.2.1.1 Frequency of Exceedance

The first indicator considers the spatial distribution of potential effects of a certain compound, i.e. the frequency of sites with observations above the lowest PNEC. For the calculation of this indicator, the maximum observed concentration at each site (MEC_{site}) is compared to the lowest PNEC. In the JDS3 case, quite often several measurements of a single compound were performed by different laboratories at the same sample using different methodologies. The maximum concentrations per compound per site were directly used to compare them with the lowest PNEC. Subsequently, the number of sites where the threshold was exceeded was divided by the total number of sites, where the respective compound was measured. Please note that the total number of 68 sites was used for all prioritised substances despite some of the substances were not determined in all samples for some analytical methods (e.g. Large Volume SPE samples were taken only from 22 sites; cf. Chapter 27). The resulting values lie within 0 and 1 and can directly be used as input for the ranking score.

To give an example of the calculation, a hypothetical dataset consist of 20 sites with one sample each. In total, compound A was found 18 times, while compound B was found 12 times. The maximum concentrations of compound A exceeded the lowest PNEC at ten sites, while the maximum concentrations of compound B exceed the lowest PNEC only at 5 sites. The RS for the indicator "*Frequency of Exceedance*" calculates as follows:

Compound A: FoE = 10 sites exceeding lowest PNEC / 20 sites = 0.50

Compound B: FoE = 5 sites exceeding lowest PNEC / 20 sites = 0.25

Hence, compound B has a lower risk as compared to compound A.

32.2.1.2 Extent of Exceedance

The second indicator considers the extent of local effects. For the calculation of this indicator, again all raw data is used. All concentration data above the Limit of Quantification (LOQ) is pooled and

used to calculate a MEC_{95}. The MEC_{95} is the 95th percentile of the measured concentrations, separately for each compound. It is recommended to have at least 20 monitoring sites to get a reliable statistical result. For the calculation, the Excel formula "QUANTIL" can be used. The MEC_{95} is then divided by the lowest PNEC to derive the "*Extent of Exceedance*". This value can consist of values below 1 and up to several thousands. In case of a value below 1, no risk is assumed (as the lowest PNEC is considered to protect the environment from any harm) and no points for the RS are given. In case of a value above 1, RS values are given depending on the extent of the exceedance. Exceedances greater than 1 up to 10 are assigned 0.1 points, while exceedances of 10 up to100 were assigned 0.2 points. Substances with MEC_{95} exceeding the lowest PNEC by a factor of more than 100 up to 1000 were assigned 0.5 points, while substances exceeding greater 1000 received the maximum of 1 point.

For the example above, we assume that the MEC_{95} of compound A is 2µg/l, while the MEC95 of compound B is 20µg/l, due to generally higher concentrations. If the lowest PNEC in this example is 1µg/L for both substances, the "*Extent of Exceedance*" calculates as follows:

Compound A: EoE = MEC_{95} of 2µg/l / lowest PNEC of 1 µg/l = 2

Compound B: EoE = MEC_{95} of 25µg/l / lowest PNEC of 1 µg/l = 25

The RS score for compound A is then 0.1 (EoE < 10), while compound B has a higher score of 0.2 for the second indicator.

32.2.1.3 Final Ranking Score

The final ranking score RS is then calculated by simply adding both scores. Please note that the maximum score is therefore a RS value of 2. In our example, the RS calculates as follows:

Compound A: RS 1 of 0.50 + RS 2 of 0.1 = 0.60

Compound B: RS 1 of 0.25 + RS 2 of 0.2 = 0.45

In this example, compound A has a higher priority than compound B, although both compounds had a highest score in one of the two indicators. However, the relatively large distribution of compound A (50% of sites exceeded the lowest PNEC) lead to the overall higher priority.

32.2.2 Data for prioritisation

Data on 719 target organic substances in water, sediment, SPM and biota were measured by 13 JDS3 laboratories. Out of these, 654 substances were analysed in surface water samples. All data (more than 47,000 data entries) were collected in the JDS3 specific Data Collection Templates and first stored in a JDS3 Access database developed by Environmental Institute to be later uploaded into the ICPDR Water Quality Database. The prioritisation dataset also included semi-quantitative results from target suspect screening by LW Langenau. The prioritisation at this stage did not consider substances determined in sediments, SPM and biota matrices. It also did not take into account findings from passive sampling.

The ecotoxicity threshold (PNEC) values were either taken from the NORMAN Working Group on Prioritisation or newly derived for 189 out of 277 JDS3 substances actually determined in the samples above their respective LOQs. Substances not provided with PNEC and thus not included into the prioritisation were for the time being not considered of prior importance based on the expert judgement, which had to be applied due to the lack of time to collect all needed information. It is planned to continue with deriving PNECs for all JDS3 target substances and re-run the prioritisation when completed.

32.3 Results

32.3.1 Prioritisation

First results of the prioritisation are presented in Table 96. Altogether 20 substances exceeded lowest PNEC value at more than 1% of the investigated (68) sites. Considering that benzo(a)pyrene together with other polyaromatic hydrocarbons (benzo(g,h,i)perylene and indeno(1,2,3-c,d)pyrene),

fluoranthene and PFOS are already regulated (and thus will have to be monitored by all Danube countries) the list is showing additional 17 pollutants of potential basin-wide concern. 2,4-dinitrophenol, chloroxuron, bromacil, dimefuron, diazinon, linuron, metazachlor and bentazon represent a general class of pesticides causing exceedances of ecotoxicological limit values across the basin. Transformation products of pesticides atrazine (2-hydroxy atrazine) and terbutylazine (desethylterbutylazine) exceeded the lowest PNEC value at 76 and 79% of the investigated sites, respectively. Amoxicillin, 17beta-estradiol and diclofenac were among the pharmaceuticals to be considered of importance. The latter two substances were already included in the proposal for update of the EQS Directive (CEC 2011) and finally not considered for inclusion among the WFD priority substances with the justification that more evidence on their occurrence in Europe is needed. Both substances are on the EU Watch List of substances to be included in the national monitoring programmes (EU 2013). The widely discussed plasticiser bisphenol-A was found in surface water samples from 30 sites of which the newly proposed lowest PNEC of 0.1 µg/l was exceeded at ten sites (e.g. 1.94 µg/l downstream Olt; JDS52). A new class of biocides represents fipronil, which exceeded the PNEC value at the JDS58 (Arges).

Table 96: Results of the prioritisation of pollutants determined in the JDS3 surface water samples

No.	Substance	CAS No.	No. of sites substance detected	C_{max}[1]	MEC_{95}[2]	Lowest PNEC/EQS	Key study	Type	EoE[3]	EoE score	FoE[4]	Final score
1	2,4-Dinitrophenol (DNP)	51-28-5	68	0.06	0.04	0.001	RIVM 2014	EQS chronic water[5]	40	0.2	1.00	1.20
2	PFOS (Perfluorooctansulfonate)	1763-23-1	63	0.026	0.02	0.00065	EU 2013	EQS chronic water[5]	31	0.2	0.93	1.13
3	Chloroxuron	1982-47-4	65	0.04	0.02	0.0024	James et al. 2009	PNEC acute	8.3	0.1	0.93	1.03
4	Desethylterbutylazine	30125-63-4	54	0.028	0.01	0.0024	RIVM 2014	EQS chronic water[5]	4.2	0.1	0.79	0.89
5	2-hydroxy atrazine	2163-68-0	53	0.06	0.02	0.002	Ecostat 2013	EQS chronic water[5]	10	0.1	0.76	0.86
6	Bromacil	314-40-9	31	0.19	0.14	0.01	INERIS 2013	EQS chronic water[5]	14	0.2	0.46	0.66
7	Dimefuron	34205-21-5	58	0.041	0.04	0.008	Oekotoxzentrum 2014	EQS chronic water[5]	5.0	0.1	0.56	0.66
8	Bisphenol A	80-05-7	30	1.94	1.03	0.1	Nendza 2003	EQS chronic water[5]	10	0.2	0.16	0.36
9	Benzo(g,h,i)perylene	191-24-2	65	0.029	0.003	0.002	CEC 2008	EQS chronic water[5]	1.5	0.1	0.26	0.36
10	Diazinon	333-41-5	21	0.009	0.01	0.001	Management Team PPDB 2009	PNEC acute	10	0.1	0.12	0.22
11	Indeno(1,2,3-c,d)pyrene	193-39-5	15	0.005		0.002	CEC 2008	EQS chronic water[5]			0.19	0.19
12	Linuron	330-55-2	32	1.42	1.12	0.26	Oekotoxzentrum 2014	EQS chronic water[5]	4.3	0.1	0.07	0.17
13	Amoxicillin	26787-78-0	33	0.28	0.08	0.078	van der Aa et al. 2011	PNEC chronic	1.0	0.1	0.03	0.13
14	Metazachlor	67129-08-2	30	0.03	0.02	0.019	INERIS 2014	EQS chronic water[5]	1.1	0.1	0.03	0.13
15	17beta-estradiol	50-28-2	8	0.029		0.0004	CEC 2011	EQS chronic water[5]			0.12	0.12
16	Benzo(a)pyrene	50-32-8	3	0.002		0.00017	EU 2013	EQS chronic water[5]			0.04	0.04
17	Diclofenac	15307-79-6	51	0.318	0.036	0.05	Oekotoxzentrum 2014	EQS chronic water[5]			0.04	0.04
18	Bentazon	25057-89-0	61	0.1	0.02	0.06	USEPA 2008	PNEC acute			0.01	0.01
19	Fipronil	120068-37-3	1	0.02		0.012	EU 2011	EQS chronic water[5]			0.01	0.01
20	Fluoranthene	206-44-0	58	0.02	0.006	0.0063	EU 2013	EQS chronic water[5]			0.01	0.01

1 C_{max} – Maximum concentration in µg/L reported in case the substance has been measured by several JDS3 laboratories

2 MEC_{95} – 95th percentile of the Maximum Environmental Concentration in µg/L; calculated only if the substance has been found above LOQ at minimum 20 sites

3 EoE – Extent of Exceedance

4 FoE – Frequency of Exceedance

5 Equal to Annual Average EQS (AA-EQS)

32.4 Conclusions

A list of 20 substances relevant for the Danube river basin has been compiled based on the results of the JDS3 target screening of 654 substances in the Danube water samples by 13 laboratories. PNEC values were available for 189 out of 277 JDS3 substances actually determined in the samples. The cut off criteria to include a compound in the list was its exceedance of the ecotoxicological threshold value (PNEC or EQS) at minimum of one JDS3 site. It should be noted that 16 of these substances were found at more than 20 (out of 68) sites (cf. Table 96). The list contains five WFD priority substances (three PAHs, fluorathene and PFOS) and two EU Watch List candidate compounds (17beta-estradiol, diclofenac). The 'top ten' substances are dominated by (i) the pesticides 2,4-dinitrophenol (exceeding the limit value at all sites), chloroxuron, bromacil, dimefuron, diazinon and transformation products of widely used atrazine and terbuthylazine, (ii) polyfluorinated substance PFOS, (iii) the plasticiser bisphenol A and polyaromatic hydrocarbon benzo(g,h,i)perylene.

More investigation is needed to find additional evidence whether these substances are indeed candidates for the Danube RBSPs or not. Derivation of PNEC and P-PNEC values for all substances found in the samples, collection of usage, PBT, vPvB, ED, CMR, etc. data will be carried out within the SOLUTIONS project. In addition a separate prioritisation of hundreds of substances tentatively identified by GC-MS and LC-HR-MS techniques will be carried out too. Ultimately, it is planned in a short term to pool all available data on organic pollutants in the Danube river basin and prioritise them using the NORMAN prioritisation framework (Dulio, 2013).

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32.6 Acknowledgments

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33 The $^{87}Sr/^{86}Sr$ river water isoscape of the Danube catchment

Andreas Zitek, Anastassiya Tchaikovsky, Johanna Irrgeher, Herwig Waidbacher, Thomas Prohaska

33.1 Introduction

Isoscapes are spatial maps of the distribution of isotopes on Earth. As a basis for ecological studies such as long distance migrations of animals, the study of environmental fluxes or for determining the origin and provenance of e.g. plants, food or other goods these tools have been developed on a global and local level. Isoscape models of different quality are available for the stable H, C, N and O isotopes on a global and local range. Especially the δ^2H and $\delta^{18}O$ values vary significantly due to physical fractionation on a continental scale what makes them efficient large scale environmental tracers, while $\delta^{13}C$ values primarily reflect effects related to the transformation of carbon from organic material (Bowen 2010). The spatial variation of N isotopes in terrestrial and aquatic ecosystems can be related to climatic controls on N cycle fluxes (global-scale) (Bowen 2010) or to anthropogenic influences (local to catchment scale) (Lake *et al.* 2001, Borderelle *et al.* 2009, Karube *et al.* 2010), and has been used successfully for ecological (Harrington *et al.* 1998) and traceability (Fox & Papanicolaou 2008) studies. On a catchment level δ^2H and $\delta^{18}O$ show significant time variation on seasonal and interannual scales (Gibson *et al.* 2002, Rank *et al.* 2009) which limit their applicability to specific ecological studies like migration and dispersal on this spatial level.

Due to its relative local and temporal stability over time, the $^{87}Sr/^{86}Sr$ isotope ratio is increasingly recognized as important eco-geochemical tracer in many fields of science like ecology (Capo *et al.* 1998), anthropology (Price *et al.* 2002, Prohaska *et al.* 2002), food science (Kelly *et al.* 2005, Swoboda *et al.* 2008, Voerkelius *et al.* 2010) and forensics (Beard & Johnson 2000, Muynck & Winne 2012).

The $^{87}Sr/^{86}Sr$ isotope ratio varies naturally in the environment as a result of the underlying geology (Faure & Mensing 2005). The reason for its local variation is the constant radioactive β-decay of ^{87}Rb into ^{87}Sr over geological time scales (half-life = 48.8×10^9 years, Holden (1990)) while the absolute amount of ^{86}Sr remains stable over time, which leads to higher $^{87}Sr/^{86}Sr$ isotope ratios in older rocks or rocks with higher Rb/Sr ratio (Faure & Mensing 2005) (Figure 167). By weathering, Sr is released from the rocks influencing the local ^{87}Sr concentration in soils and water and is incorporated into living organisms according to its availability without any further fractionation (Graustein 1989, Capo *et al.* 1998, Blum *et al.* 2000) via the food chain.

As far as variations in the isotopic distribution in a studied area exist, the isotopic composition bears the potential to be used as natural tracer e.g. for ecological questions concerning e.g. provenance and migration, but also to study physical processes like erosion, and the determination of material sources and sinks. Therefore, as central basis for ecosystem studies, isoscapes reflecting the spatial distribution of the $^{87}Sr/^{86}Sr$ isotope ratio are being increasingly developed for terrestrial (Evans *et al.* 2010, Bataille & Bowen 2012, Willmes *et al.* 2014) but also for aquatic (Muhlfeld *et al.* 2012) systems. For large river systems, because of their specific features, the $^{87}Sr/^{86}Sr$ isotope ratio has been recognized as important research and management tool (Gibson *et al.* 2002, Zitek *et al.* 2011). For example, as the local $^{87}Sr/^{86}Sr$ isotope ratio is incorporated in fish hard parts like otoliths, it offers the unique potential to trace fish migrations between zones of different isotopic composition (Kennedy *et al.* 2000).

Within the JDS3, the $^{87}Sr/^{86}Sr$ isotope ratio pattern within the Danube catchment from the source to the Delta was mapped for the first time.

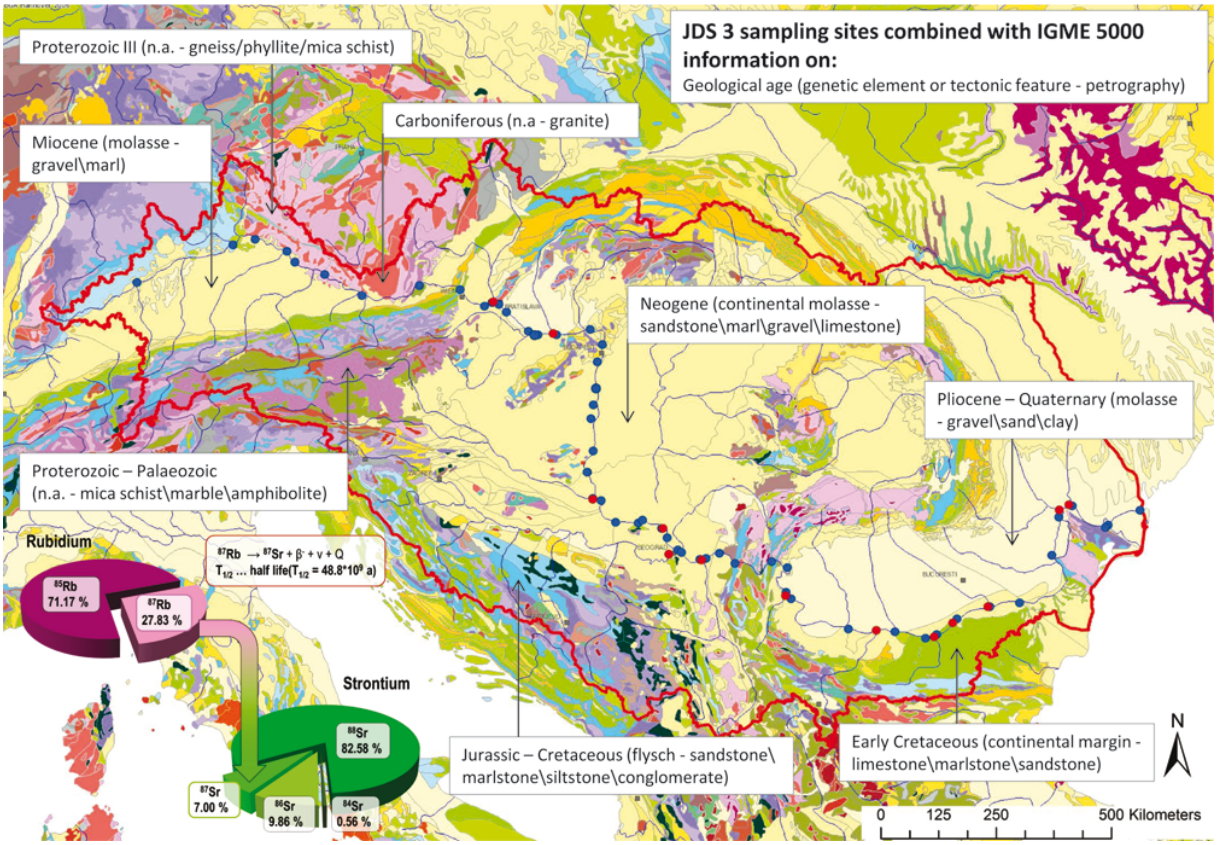

Figure 167: JDS3 sampling sites (Danube river sampling sites - blue circles; tributary sampling sites - red circles), selected information on geological formations (age, genetic element/tectonic feature and petrography) and the Rb/Sr isotope systems (Berglund & Wieser 2011) with the radioactive ^{87}Rb to ^{87}Sr β-decay (Holden 1990) (Copyrights: Danube catchment by WISE River Basin Districts version 1.3, European Environment Agency (EEA); Data source of geological information and rivers: IGME5000, copyright by BGR Hannover, 2007)

33.2 Methods

During the JDS3, water samples from 68 sampling sites in the Danube and in the major tributaries were collected (Figure 167). Triplicate water samples were taken at each site along the Danube at about 10 cm below the water surface in pre-cleaned and pre-labelled PE-bottles (3*100 ml) individually sealed in LDPE-bags. (Cleaning was accomplished by acid-washing (in 10% (*m*/*m*) HNO_3 followed by a bath in 1% (*m*/*m*) HNO_3 for 24 hours and rinsing by purified water (18 MΩ cm) (TKA Wasseraufbereitungssysteme GmbH 'Part of Thermo Fischer Scientific', Niederelbert, Germany). Bottles were filled up to ¾, and kept frozen at -20°C until further processing in the laboratory.

Water samples were defrosted, acidified to 2% (*v*/*v*) HNO_3 (double subboiled from p.A. grade acid, Merck, Darmstadt, Germany) and filtered using a cellulose acetate filter membrane (Minisart 0.45 µm syringe filter units, Minisart, Sartorius, Göttingen, Germany) prior to analysis.

In a first step, quantification of the Sr mass fraction in water was performed using an inductively coupled plasma quadrupole mass spectrometer (ICP-QMS) (NexION 300D, Perkin Elmer, Waltham, MA, USA). External calibration using the ICP Multi-Element Standard Solution VI (CertiPur, suprapure, Merck KGaA, Darmstadt, Germany) and internal normalisation using the Indium ICP Standard, 1000 mg l^{-1} (CertiPur, suprapure, Merck KGaA, Darmstadt, Germany) at a mass fraction of 1 ng g^{-1} were performed.

After this step, samples were further processed for isotopic analysis by accomplishing Rb/Sr separation performed using a Sr-specific resin (EIChroM Industries, Inc., Darien, IL, USA) based on established protocols according to Swoboda *et al.* (2008).

The Sr isotope ratios of the water samples were measured with a double-focusing sector field multiple collector inductively coupled plasma mass spectrometer (MC ICP-MS) (Nu Plasma HR, Nu Instruments, Wrexham, UK) equipped with a desolvating membrane nebuliser (DSN 100, Nu Instruments, Wrexham, UK). Calibration was performed following an external intra-elemental strategy (*aka* sample-standard bracketing) using the NIST SRM 987 (NIST, Gaithersburg, MD, USA), which is a certified reference material for the natural Sr isotopic composition.

Blank correction was done on-peak by aspirating a 2% HNO_3 blank solution. After blank correction data was mathematically corrected for interferences (remaining Rb). Instrumental isotopic fractionation was corrected for using the above mentioned calibration. Finally, combined standard uncertainties were calculated following EURACHEM/GUM guidelines.

33.3 Results

The $^{87}Sr/^{86}Sr$ isotope ratio along the course of the Danube showed only slight variations. $^{87}Sr/^{86}Sr$ isotope ratios varied around 0.709, with some significant lower values between 0.7084-0.7086 at some upstream sites (sampling sites JDS1, JDS2, JDS5) (Figure 168).

Significant differences of $^{87}Sr/^{86}Sr$ isotope ratio were mainly found between the Danube and most of the tributaries. $^{87}Sr/^{86}Sr$ isotope ratios in Morava, Drava, Tisa, Timok, Iskar, Jantra, Russenski Lom, Arges and Prut differed significantly from the adjacent Danube sections.

Morava showed the highest $^{87}Sr/^{86}Sr$ isotope ratio (0.7111) and Timok the lowest (0.7068). The mean value along the Danube was 0.7091 (± 0.0002 SD).

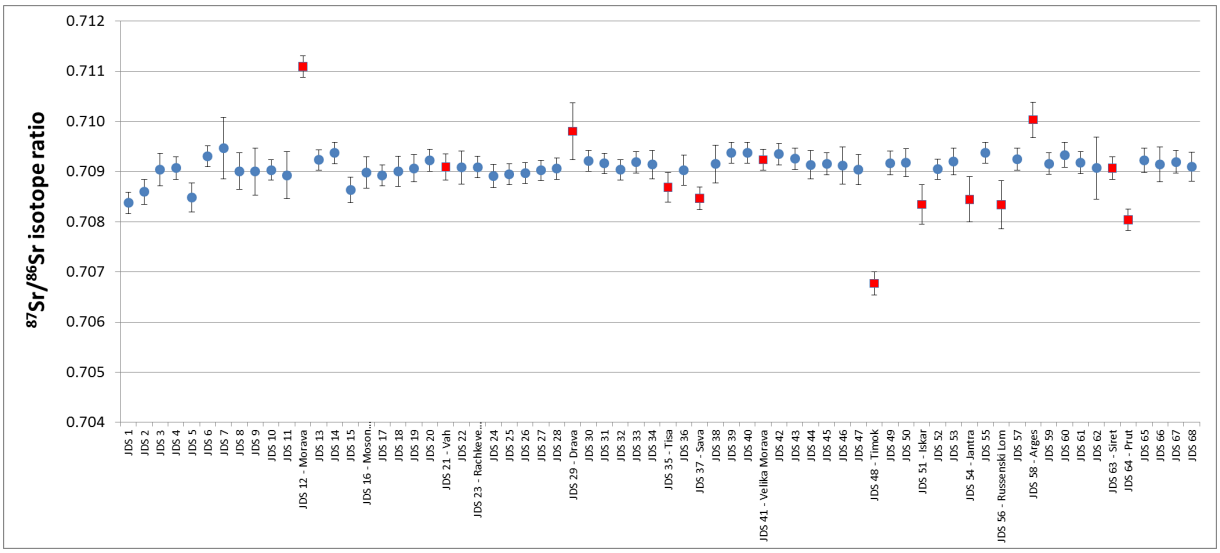

Figure 168: $^{87}Sr/^{86}Sr$ isotope ratios along the course of the river Danube with blue circles representing Danube river sampling sites, and red squares tributary sampling sites; mean values of all sampling sites are based on triplicate samples, except for JDS5, JDS10, JDS11, JDS43, JDS60, JDS62 which are based on two samples; error bars represent combined standard uncertainties (u_c, k=1)

33.4 Discussion

Analyses of mid water samples along the course of the Danube yielded relative small variations in the $^{87}Sr/^{86}Sr$ isotope ratios along the main course of the Danube itself. Except for some upstream sampling sites, $^{87}Sr/^{86}Sr$ isotope ratios in the Danube itself varied around 0.709, with a mean value along its full

course of 0.7091 (± 0.0002 SD). This is in accordance with a $^{87}Sr/^{86}Sr$ isotope ratio reported by Palmer and Edmond (1989) for the Danube of 0.7089 (with no information on the location of the sampling site). Pawellek *et al.* (2002) reported lower $^{87}Sr/^{86}Sr$ isotope ratio for the first 400 km of the Danube downstream of the source as well, with values around 0.709 for the following river section from km 400 to km 1100 downstream to Kamenice.

Within JDS3, most sampled tributaries showed a significant difference of the $^{87}Sr/^{86}Sr$ isotope ratio from the Danube itself. These local variations between the tributaries and the Danube itself indicate the influence of varying geological compositions in these subcatchments. Pawellek *et al.* (2002) reported significantly higher $^{87}Sr/^{86}Sr$ isotope ratio values for the silicate-dominated subcatchments in the upper Danube, and the percentage of igneous rocks in subcatchments was found to be positively correlated with the $^{87}Sr/^{86}Sr$ ratio for the Austrian section of the Danube and its tributaries (Zitek *et al.* 2011) (see also Figure 167 with regard to the distribution of different geological formations according to their composition and age).

Especially the documented differences in the $^{87}Sr/^{86}Sr$ isotope ratios between the tributaries and the Danube itself bear the potential to be applied as tool to study natural migration phenomena of fish in the Danube catchment. As Pawellek *et al.* (2002) and Zitek *et al.* (2011) showed for the upper Danube catchment, significant small scale differences in $^{87}Sr/^{86}Sr$ isotope ratios between the Danube and its tributaries exist, allowing the application of $^{87}Sr/^{86}Sr$ isotope ratios to fish ecological questions even at relatively small spatial scales. In addition to the $^{87}Sr/^{86}Sr$ isotope ratio, the Sr/Ca ratio is able to serve as an important additional tracer to discriminate fish from different sites in the Danube catchment (Zitek *et al.* 2010).

Future efforts will focus on combining the data of the JDS3 with existing data on $^{87}Sr/^{86}Sr$ isotope ratios along the Austrian part of the Danube catchment, modelling the $^{87}Sr/^{86}Sr$ isotope ratio in river water in relation to the geology (Hegg *et al.* 2013), and finally linking the information to fish ecological questions in the Danube catchment.

33.5 Conclusion

The documented differences of the $^{87}Sr/^{86}Sr$ isotope ratios between the Danube and most of its tributaries bear the potential to be applied as tool for studying fish migrations and fish dispersal especially in Danube-tributary systems. The combination with other relevant natural chemical tracers like the Sr/Ca ratio will further enhance the possibilities for reconstructing migrations fish based on otolith chemistry in the Danube catchment.

33.6 Outlook

The spatially distinct data on $^{87}Sr/^{86}Sr$ isotope ratios along the course of the Danube collected during the JDS3 in combination with existing data mainly from the upper section of the Danube will be used to develop the aquatic $^{87}Sr/^{86}Sr$ isoscape of the Danube catchment. In combination with other chemical tracers, e.g. like the Sr/Ca ratio, the Danube catchment isoscape will serve as an important input for studying e.g. migrations of aquatic animals like fish and weathering and erosion processes in the Danube catchment.

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34 Conclusions and lessons learned

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The EU Water Framework Directive provides a coherent approach to determining the status of waters and to organizing this information for political decisions. The overall logic of the Directive is used by the countries in a cooperative way to organize the data that they have and to produce the information still not available. A central element of such cooperation under the ICPDR has been focused on collecting reliable and organized information on water quality. The countries of the region have been actively engaged in activities that are needed to ensure mutual understanding and cooperation. In particular, a yearly status of water quality has been published since 1996 based upon the Transnational Monitoring Network developed by the countries in response to the Danube River Protection Convention. This monitoring activity provided the necessary basis for harmonized water quality assessment throughout the whole basin, which not only gave an overview of water quality trends in the basin and of loads of substances discharged into the Black Sea but it fostered achieving of compatibility between water assessment approaches in the Danube countries.

The yearly assessment of water quality has been supplemented by periodic surveys of the status of the water carried out under the banner of the Joint Danube Survey. The Joint Danube Surveys (JDS1 organized in 2001 and JDS2 in 2007) provided an organized set of data for the main stem of the Danube that was comparable and agreed among the countries. The scientific contribution of these special monitoring exercises was immense but similarly important were the aspects of training and methodological harmonization as well as public awareness rising. The success of the first two surveys initiated a clear commitment of the ICPDR Contracting Parties to organize the third Joint Danube Survey (JDS3) in 2013.

During JDS3 altogether 68 sites were sampled by the Core Team of experts along a 2581 km stretch of the Danube, 15 of which were located in the mouths of tributaries or side arms. Sampling at the JDS3 stations included five different sample types – surface water, biological quality elements, sediment, suspended particulate matter (SPM) and biota for chemical analysis (fish and mussels) - each with a different determinand list. Following the survey's completion in September 2013, the collected data were analyzed in laboratories and scientific institutes across Europe, which produced the data that served as the basis for preparing this report.

The authors of this report cover a wide area of expertise on aquatic chemistry, biology, microbiology and hydromorphology and their findings create a comprehensive knowledge base for further assessment of water quality in the Danube River Basin and beyond.

The findings of JDS3 are supportive to the implementation of EU WFD as they provide an extensive homogeneous dataset production of which was mainly based on WFD compliant methods commonly used by the Danube experts. Even though these data have no ambition of replacing the national data used for the assessment of the ecological and chemical status they are an excellent reference database serving for future efforts of method harmonization in the Danube River Basin, especially concerning the development of a concerted type-specific approach to the status assessment of large rivers, and of the prioritization of the Danube river basin specific pollutants.

34.1 Hydromorphology

The WFD-3digit analysis of the entire Danube indicated the general alteration (prevailing classes 3-5). Out of the 241 analyzed 10 rkm segments 13% fall for morphology in class 2 (slightly modified), 39% in class 3 (moderately modified), 31% in class 4 (extensively modified) as well as 17% in class five (severely modified). For hydrology/flow regime and the continuity only the classes 1, 3 and 5 were assessed. For hydrology only 16% fall in the first class whereas class 3 with 50% and class 5 with 34% prevail. Regarding continuity, dams are located in 8% of segments (in total 18 dams, two dams with functioning fish passes and partial sediment management fall in class 3, the rest in class 5).

The CEN overall hydromorphological analysis indicates that about 60% of the analyzed Danube stretch falls below class 3 (21% in the second class „slightly modified“ and 39% in the third class „moderately modified“), 40% fall in the two worse classes four (26%) and five (14%).

During JDS3 information on hydromorphological conditions was significantly improved as in-situ measurements of hydrological, morphological and hydraulic characteristics were for the first time performed on the entire Danube and tributaries (JDS3-sites).

The assessment results confirm the main findings of JDS2 in 2007 however the increased resolution allowed a more precise assessment.

The survey reconfirmed the importance and strong impact of existing dams in particularly on the sediment balance upstream and downstream of dams, and on the hydrological changes (e.g. due to potential flow regulations). This issue should be matter of further basin-wide investigations (sediment balance up- and in particular downstream of dams, detailed hydrological analysis downstream of dams).

34.1.1 Bird survey

In this survey for the first time birds as non-aquatic species were monitored for a better characterization of the bank habitats. The results of the monitoring of indicator bird species correlate significantly with the results of the hydromorphological assessment and show the added value of such an interdisciplinary approach. The monitoring showed the absence of Sand Martin and the low density of Little Ringed Plover along the Upper Danube which indicates the alteration of hydromorphological processes. However the high number of territories of Little Ringed Plover on the last remaining free flowing sections of the Upper Danube indicates the high relevance of river restoration projects along the Upper Danube. Both along the Middle and Lower Danube much higher mean abundances of bird population were found. The monitoring of indicator bird species stresses the high ecological value of river sections which are only slightly modified (class 2) or even in a better hydromorphological status. Stronger hydromorphological alterations reduce the ecological value: already in class 3 (moderately modified) the probability of occurrence of one of the two species is reduced to about 65%, and the probability is dramatically reduced to about 30% in class 4 (extensively modified).

34.2 Biology

Appropriate and harmonized biological monitoring is essential to securing the proper assessment of the ecological status of surface water bodies. Variability of methods currently in use in the Danube countries for the assessment of large rivers necessitates coordinated efforts towards a concerted approach. In the frame of JDS3 biological monitoring exercise different methods were applied in parallel for the evaluation of several biological quality elements which sometimes resulted in delivery of differing results. The major reason was that these (mostly national) methods were tailored to specific Danube reaches but the results obtained clearly demonstrate the need for further development and harmonization of type-specific methodologies which would be applicable for the whole Danube River for the evaluation of biological quality elements necessary for the assessment of the ecological status according to WFD.

34.2.1 Macrozoobenthos

During JDS3 three different sampling methods were applied: Multi Habitat Sampling (MHS) and Kick and Sweep (K&S) for wadeable and riparian areas and Deep Water Sampling with a dredge (DWS) for deeper areas of the river. Altogether 460 macroinvertebrate taxa were identified. Insects, with 319 taxa, were the dominant component of the communities. Higher abundances of EPT- Taxa (Ephemeroptera, Plecoptera and Trichoptera) were restricted to the upper stretch, whereas Trichoptera showed the highest abundances within these sensitive groups.

Saprobic Indices and the respective water quality status class per site are comparable to the JDS2 data: 73% of 55 sampled sites in 2013 can be classified as "indication of good ecological status", 15% of the sites as "indication of moderate ecological status" and 4% actually as "high ecological status" according to the WFD. Serious organic pollution was identified upstream Novi-Sad (bad status). Poor status was indicated in Jochenstein, upstream Drava, downstream Velika Morava and at Vrbica/Simjan in the Irongate reservoir.

On the basis of the Slovak assessment method for general degradation (Multimetric Index) for large rivers, the morphologically high degraded sites (channelized or impounded, with rip-rap dominating at the shore zones) in the Upper Danube reach indicate moderate status, while hydromorphologically more natural sites at the Upper and Middle Danube reach indicate generally good status. The compatibility of this method in the Lower Danube reach has to be further tested as substrate composition differs considerably from the Middle Danube, for which the method was designed.

34.2.2 Phytobenthos

The Danube phytobenthos was mainly composed of diatoms and cyanobacteria, with the former prevailing in the Upper Danube. The algal biomass showed to increase in the Upper and Lower Danube and was most significantly influenced by phosphates and suspended solids. Altogether 68 non-diatom taxa and 318 diatom taxa were identified during JDS3. Both diatoms and non-diatoms in the Danube indicated that there is a strong environmental longitudinal gradient in the Danube profile related to natural changes in the river typology as well as to increasing anthropogenic disturbance. Both species composition of diatoms and non-diatoms as well as the diatom metrics changed gradually downstream reflecting a distinct longitudinal pattern in environmental conditions in the Danube. The algal assemblages in the upper reaches were most significantly influenced by velocity, slope oxygen content, pH and nitrates. The assemblages in the middle and lower Danube reacted mainly on phosphates, potassium, DOC and suspended solids indicating the increasing pressures on aquatic environment. All diatom indices tested decreased gradually and significantly downstream reflecting the increase of general degradation of aquatic environment and natural longitudinal changes. The IPS-based indication of the ecological status assessment of the Danube showed that the ecological status of the Upper Danube (sites down to Gabčíkovo reservoir at 1852 rkm) varied between high to good. Sites downstream Budapest (after the 1852 rkm) appeared consistently below the good/moderate boundary indicating that the ecological status of the middle and lower Danube is moderate and worse. Nevertheless, the assessment method applied (even though having been intercalibrated) does not fully take into account the Danube typology and the results should be therefore considered only as indicative.

34.2.3 Macrophytes

A total of 198 macrophyte taxa were identified during JDS3 belonging to bryophytes (35 taxa), ferns (4 taxa), angiosperms (150 taxa), charophytes (1 taxon) and other macroalgae (8 taxa). The Slovak and Austrian assessment systems applicable for large rivers were used for data evaluation and indicated a decrease in ecological status from the source to the mouth of the Danube. These findings however cannot be justified by the typical pressure data macrophytes are regarded to be indicative for. Neither the nutrient concentrations nor hydromorphological impairments show a significant increase along the Danube stretch. Thus these results demonstrate clearly that the indicative value of species, especially concerning trophic conditions, changes within different regions and river-types and underline the necessity for developing and applying type-specific assessment systems.

34.2.4 Phytoplankton

The distribution of phytoplankton chlorophyll-a and biomass along the river corridor was significantly different from previous JDS investigations. From the findings during JDS1 and JDS2 three river sections were defined: An upstream section with low values, a middle section where values increased to a maximum and a downstream section with generally low values. During the 2013 survey, this distinct sections were somewhat replaced by alternating sections of low and high concentrations. As previously, the highest chlorophyll and biomass concentrations occurred in the middle section of the river between km 1481 (Baja) and 1159 (downstream Sava). Different from earlier observations however, chlorophyll-a and biomass concentrations exceeded threshold values between Klosterneuburg (km 1942) and upstream of Budapest (km 1660). These high values most likely were a reflection of the heat wave preceding the investigation period and low discharge associated with.

According to the TNMN quality classification most chlorophyll-a concentrations in the Danube belonged to water quality class I. The type specific WFD criteria for large rivers using the metrics total phosphorus (TP) and chlorophyll-a (chl-a) for trophy assessment were also applied and chl-a indicated high to good status (water quality class 1-2) in most of the upper and the lower reach of the Danube. Moderate status was assigned to the river section from rkm 1384, upstream Drava to rkm 1216, upstream Tisa. The 15 investigated tributaries were in high to good status except Morava in bad state and Vah in poor status.

34.2.5 Fish

In total 139.866 individuals representing 67 fish taxa were caught during JDS3. The electrified benthic frame trawl proved to be a great additional sampling method, detecting species not caught by littoral sampling. The Danube fish fauna is heavily influenced by non-native species which can be found in all habitats, even close to the river bottom and partly in remarkable densities. It appears that the dominance of *Neogobius* species in the Upper Danube has dramatically increased since JDS2, especially in altered littoral structures as rip rap.

In the upper course of the Danube the fish fauna mainly reflects hydromorphological alterations and damming as most important human impacts, but also the lack of connectivity along the whole river stretch. The excessive use of hydropower in the upper Danube, which consequently leads to an impoverishment of aquatic habitats can be detected easily by the absence of sensitive species and certain age classes and is clearly indicated by the applied national WFD assessment indices FIA and FIS. The lower course of the Danube seems to be influenced by professional & recreational fishery and poaching.

The three applied national WFD assessment indices of JDS3 indicate a call for action as 50% of the sites according to FIA, 72,1% (EFI) and 94,7% (FIS) respectively show a value worse than “good” and do not meet the requirements of the WFD.

34.2.6 Zooplankton

149 zooplankton taxa have been discovered, out of which 107 Rotifera, 33 Cladocera and 9 Copepoda have been registered. There are tychoplanktonic elements among the planktonic community, coming from aquatic plant stocks, the sediment, dead arms and side arms. The composition of the dominant species was the same as in former investigations but the density of zooplankton was in general higher than in 2007 (JDS2). The maximum individual number was registered in the Serbian reach, where the most eutrophic-polytrophic environment was found. There was no increased abundance or species number observed in reservoir sections and in the Danube Delta.

During the previous surveys the rotifera species Brachionus forficula was found only in some sections of the Danube, but during JDS3 this species was found almost in the whole longitudinal profile of the Danube. This is a warm stenotherm organism and its stabile presence may refer as well to increasing temperature. The tributaries did not have a significant effect neither on the quantity nor on the composition of Danube zooplankton.

34.2.7 Invasive Alien Species

Based on the results of JDS3, the Danube River is significantly exposed to non-native species. 25 neophytes (4 aquatic), 34 non-native aquatic macroinvertebrates and 12 non-native fish species were recorded during the JDS3.

The level of biocontamination of the Danube River was estimated as moderate to high, with higher levels for the Upper (high to severe biocontamination) and Middle Danube (moderate to high biocontamination), in comparison to the Lower Danube (low biocontamination).

Comparison with the results of previous Danube Surveys clearly showed a constant impact of invasive alien species on native biota and a considerable increase of the number of non-native aquatic macroinvertebrate species. As a specific example the allochthonous *Neogobius* fish species can be given which were found in high or even dominating abundance along the rip-rap protected banks in the upper and middle course of the Danube.

34.3 Microbiology

Even though the microbiological contamination does not determine the status of surface waters according to the EU WFD, it is relevant for the assessment of drinking water and bathing water quality in line with the respective EU Directives. Moreover, the microbiological communities in surface waters are integral part of the aquatic ecosystems having its influence on the WFD biological quality elements. They also indicate the sources of organic pollution. Therefore a thorough analysis of river microbiology has been always on the scientific programme of the Joint Danube Surveys bringing an added value to the survey findings.

34.3.1 Bacterial Faecal Indicators

Escherichia coli and intestinal enterococci are used worldwide as sensitive indicators for the assessment of faecal pollution in the aquatic environment. Faecal indicators are excreted by humans and warm blooded animals in high concentrations and survive for a certain time in aquatic systems. Faecal pollution can be caused by point sources like discharges of sewage from human sources or livestock enterprises and by non-point sources like pasture, urban and agricultural run-off or water fowl. Faeces frequently contain pathogenic microorganisms like bacteria, viruses and parasites. Therefore intestinal indicator bacteria like *E. coli* and enterococci indicate the potential presence of pathogens and are especially well appropriate to indicate faecal pollution in surface waters.

Fourty-two JDS sampling points (35 Danube samples and 7 tributaries/branches) out of 186 were classified as critically (34), strongly (5) or excessively (3) polluted by Bacterial Faecal Indicators. As hot spots of excessive pollution the tributaries Arges and the Russenski Lom were identified. Surprisingly, the highest contamination in the Danube with excessive pollution levels was measured at Kelheim (DE), in the uppermost stretch, with otherwise little to moderate faecal pollution levels. Other hot-spots of faecal pollution in the Danube (strong pollution or high critical pollution levels) were the stretch between Novi Sad and downstream Belgrade (SRB), downstream Budapest (HU, right side) and Dunaföldvar (HU, midstream), downstream Zimnicea (RO, left side) and downstream Arges (RO, left side). A comparison with data from JDS2 revealed very similar median values for both faecal indicators *E.coli* and Enterococci. Although a slight tendency towards lower values was observed in the Danube, an improvement of the microbiological water quality cannot be deduced from this snap-shot data as it would require long-term observations.

34.3.2 Microbial source tracking

The results of the microbial source tracking investigation of JDS3 samples demonstrate quite clearly that human faecal impact is the main driver for faecal pollution levels in the Danube and its major tributaries. Human-associated genetic faecal marker levels could be predicted by the bacterial standard indicator variations, such as *E.coli*, to a high extent.

34.3.3 Antibiotic resistance

Antibiotic resistant bacteria are known almost since the use of antibiotics has started. But in recent years the spread of multi-resistance, outside the hospital environment, enhanced this problem. One possible transmission route is via waste water and the water environment. More than 50% of the *Escherichia coli* isolated during JDS3 showed a modified resistance pattern, but most of them (47 isolates) were only resistant against one or two tested antibiotics. Hence, multi-resistant isolates (with resistance in three or more antibiotic classes) were rare. The frequency of multi-resistance was elevated at the downstream sampling points, (including isolates with resistance against up to seven tested antibiotics). This may reflect the more problematic resistance situation in clinical settings in the downstream countries or could also refer to a cumulative effect. All *Escherichia coli* isolates were susceptible to last-line antibiotics.

34.3.4 Microbial ecology

Heterotrophic bacterial production rates and the concentration of large bacterial cells, representing the active part of the bacterial community were significantly inter-correlated and followed a similar trend. The inflow of polluted tributaries and wastewater from point sources was partly reflected in the Danube at the respective river sides and partly contributed to the observed trends. The patterns of bacterial production observed in 2013 were similar to the ones observed for JDS2 in 2007. However, with the exception of only a few samples, heterotrophic production rates in 2007 were lower than in 2013. In many smaller tributaries and branches (Morava, Moson Danube, Vah, Rackeve-Soroksar, Velika Morava, Iskar and Arges) both the concentration of large bacterial cells and bacterial production rates were markedly higher than in the Danube at the respective merging site. Such an observation was already made during JDS2 in 2007. The highest cell concentration and production rate were observed in the tributary Arges, most probably due to the enormous wastewater input from Bucharest in this river.

34.3.5 Microbial metagenomics

The microbial communities present in the water at four sites were investigated using a novel metagenomics approach (without cultivation) and the microbial composition was recorded.

34.4 Chemistry

Water temperature measured in the Danube River and in selected major tributaries followed the typical pattern for the timing of the survey (August – September), with larger variation range in tributaries than in the Danube. The longitudinal distribution of conductivity in the Danube River showed a strong decrease in the upper stretch, followed by a constant profile towards the middle and lower stretches. The dilution effect along the Danube was demonstrated by the significant correlation coefficient of conductivity with water discharge values.

pH and dissolved oxygen content demonstrated a good balance between primary production and decomposition of organic matter, with most of the oxygen saturation levels situated around the equilibrium value. Several local depletions were found in specific areas (dammed Rackeve-Soroksar side arm, the Iron Gates reservoir) and two tributaries (Tisa and Velika Morava).

Total Nitrogen presented a strong decreasing profile from upper to lower stretch of the Danube, and it was significantly negatively correlated with water discharge. The typical lower profile was noticed in the Iron Gates reservoir, due to the denitrification process from this area. Most of the tributaries presented levels similar to those in the Danube, but elevated concentrations were found in the Timok, Russenski Lom and Arges. No systematic trend in Total Phosphorous concentrations along the Danube River was found; still, a slight decreasing line appeared in the lower stretch, more pronounced in the Iron Gates reservoir area, due to the retention of the suspended material on which this nutrient form is adsorbed. The Total Nitrogen and Phosphorous levels measured in the three arms of the Danube Delta come in good agreement to previous findings which showed that the contribution of the Danube Delta in nutrients retention is negligible, because most of the Danube water passes directly to the Black Sea, almost not reaching the Delta itself. N-ammonium and N-nitrites showed levels below the limit of

quantification in most of the sampling sites. Compared with JDS1 and JDS2 results, Total Nitrogen and Total Phosphorous concentrations measured in the Danube River during JDS3 were lower.

The ecological indication given by the general physico-chemical quality elements was assessed based on the intervals for high/good and good/moderate ecological classes as resulted from the environmental quality standards/guiding values reported by the Danube countries. The general view is that most of the sampling sites located on the Danube River belongs to either ”high” or ”good” class, except for the dammed side arm Rackeve-Soroksar and the Iron Gates reservoir area, which fall in ”moderate” class due to the oxygen depletion. ”Moderate” class is also present in several tributaries (Morava, Tisa, Velika Morava, Jantra, Russenski Lom and Arges), caused by low oxygen saturation and dissolved nutrients forms.

34.4.1 Heavy metals and arsenic

In general, the concentrations of heavy metals and arsenic in water, and the contents of metals and metalloids in suspended particulate matter and bottom sediments estimated during JDS3 were similar to those observed in the JDS1 and JDS2 samples. Comparison of results in water with WFD environmental quality standards showed occasional and scattered non-conformity primarily for Ni and Pb. For mercury and arsenic there were no violations of limits at all. For heavy metals and arsenic in suspended particulate matter (SPM) the quality standards applied in the past for JDS were used also during JDS3 and they were not exceeded for Cd, Cr, Hg and Pb. The target value for As in SPM was not met at one site, for Cu at three sites, for Ni at 20 sites and for Zn at seven sites. In sediment the German targets for metals were with one exception met at all sites for all elements. Only copper at JDS48 exceeded the quality target value of 160mg/kg by a factor of 3.3.

At six sites Hg was also determined in dried fish tissue. Results ranged between 0,11 to 0,35mg/kg wet weight. In addition three fish samples taken during JDS2 in 2007 and properly stored in between were also analyzed. Results were comparable with those of 2013 giving Hg contents of 0,21 – 0,44mg/kg wet weight. All these results were clearly above the EQS set by 2008/105/EC and 2013/39/EU exceeding it by factors between 5 and 18.

34.4.2 Dissolved Organic Matter (DOM)

An indication of severe organic pollution was not found from the DOM analysis for the Danube River. The Dissolved organic carbon (DOC) concentrations were in the lower range typical for large, intensely used rivers. There is evidence that besides terrestrial inputs, algal based sources significantly contribute to the overall carbon pool (as shown for the Upper Danube sections and this is in agreement with other findings as shown in the phytoplankton report). The results point to the low substrate availability and humic content in the upper section and more importance of terrestrial inputs in the Lower Danube reach.

34.4.3 Organic compounds

The challenge for the JDS3 was not only to review the occurrence of the priority substances which were found relevant during previous surveys but also to focus on the new priority substances and on the emerging pollutants which are not covered by legislation but are frequently detected in European rivers. Priority substances with known concentrations well below the current EQS (e.g. DDT) from other Danube surveys were not analyzed. Thanks to cooperation of a numerous European laboratories the largest search ever on the Danube for the unknown pollutants has been carried out.

It must be stressed that EQS in water for priority substances are defined by the WFD for an average value of 12 measurements within one year, while the JDS3 only provided a single sample from August/September.

DEHP in water was present in all samples significantly below the AA-EQS of 1.3 µg/l whereas during JDS2 in 44% of the water samples DEHP concentrations were above the AA-EQS. For the first time C10-C13-chloroalkanes could be analysed. All measured concentrations in water were below the AA-EQS of 0.4 µg/l but C10-C13-chloroalkanes were found in SPM in concentrations up to 79 µg/kg dry mass. Concentrations of PFOS exceeded the AA-EQS of 0.00065 µg/l at 94% of the sampling sites.

Also the biota EQS limit value for PFOS was exceeded in fish liver in all cases. For PAH and tributyltin the AA-EQS for water was exceeded only at few sampling sites. Concentrations for PAH in SPM and sediments were comparable to JDS2 results. Only low concentrations of analysed pesticides were detected due to the fact that sampling was carried out in August/September which is not the main season for pesticide application. The positive data observed for terbutryn show its predominant use as a biocide. AMPA (metabolite of the widely used herbicide glyphosate) was found in all water samples in concentrations around 0.25 µg/l in the Danube and higher in some tributaries. The biocide cybutryne was analysed in all water samples for the first time detecting only very low concentrations well below the AA-EQS. For HBCDD all biota sampling sites showed values below the EQS. Dicofol and heptachlor/heptachlorepoxide could not be found in biota samples.

For the organic compounds investigated in SPM the spatial patterns for PCDD/F and PCBs are similar in 2007 and 2013, while for BDE-209 the concentration maximum from 2007 shifted from the middle stretch more downstream. From the downstream concentration profile, there is no indication of relevant point sources. Concentrations in SPM are stable since 2007 except for BDE-209, displaying a 30% decrease in concentration. The observed concentrations of PCDD/Fs, PCBs and BDE-209 in SPM ranged between half- and more than one order of magnitude lower compared to the River Elbe. The concentrations in fish show a decreasing trend since 2007, PCDD/Fs decreased by about 20%, PCBs, both dioxin-like and the sum of 6 marker PCBs and BDE-209 by approximately 50%.

The concentrations of PCDD/Fs and DL-PCBs found during JDS3 generally fit into the low end of the ranges reported for the rivers Elbe, Rhine and their tributaries. For the EC-6 PCBs no data for bream on a wet weight basis were found. However, with the DL-PCBs low, the marker PCBs are supposed to follow this trend. The few BDE-209 data available suggest that the concentrations in Danube bream are similar to the River Elbe. Since most other organic pollutants appear up to one order of magnitude lower in the Danube-Elbe comparison, this could be an indication for a higher relative relevance of the brominated flame retardants in the Danube.

For PCDD/F and PCBs none of the existing EQS values for aquatic biota and SPM/sediments, and none of the EU food limits concerned were exceeded.

Among the investigated organophosphorus compounds (OPCs) in water, TCPP clearly dominates, both in the Danube and in the tributaries. Looking into their toxicities, the concentrations for OPCs are several orders of magnitude below their effect levels for aquatic biota. Regarding toxicity, TMPP and TPhP, although lower in concentration, deserve further attention regarding their temporal trends.

Multi-component target-analysis of water using different sample preparation techniques in combination with LC-MS/MS methods performed by different laboratories provided data for some hundreds of anthropogenic trace compounds. The most important groups of compounds were pharmaceuticals, biocides, artificial sweeteners, industrial chemicals and metabolites of these substances when available as analytical standards. Limits of quantification in the low ng/l-range and sometimes below yielded positive results for many substances. As the target lists of the laboratories involved were somehow overlapping, parallel results for many substances are available for cross-checking. The JDS3 data set for water is the most comprehensive investigation ever done in a single river basin. It offers the opportunity to identify emerging substances relevant for the whole Danube to be monitored in future.

In general a large number of emerging polar organic substances were found in very small concentrations. The pharmaceuticals occurred mostly in concentrations below 40 ng/L. Pollutants with generally higher concentration levels were the metamizol metabolites FAA and AAA, the artificial sweeteners acesulfame, cyclamate and sucralose, metformin, enalapril, triphenylphosphinoxide, 2-benzothiazolesulfonic acid, benzotriazoles, iodinated X-ray contrast media and the stimulant caffeine. Overall, concentration levels of most of these substances slightly decreased downstream the Danube to the Black Sea.

As regards the hot-spots there was an impact detected of municipal wastewater released from major cities like Belgrade or Bucharest. However due to the relatively very small discharge of most tributaries receiving the contaminated wastewaters the Danube itself hardly showed higher concentrations after their inflow. Occurrence of elevated concentrations of rather easily biodegradable

compounds like caffeine, cyclamate and saccharine in surface water could also indicate a release of significant portions of untreated wastewater into the surface waters.

The concentrations for most of the contaminants were lower in 2013 compared to JDS2 in 2007.

During JDS3 several new analytical techniques and strategies were applied:

- **Effect-based screening** could be an important prerequisite for a holistic and risk-based river basin management to support the WFD. To explore the presence of non-regulated organic substances in the Danube a newly developed mobile **large-volume extraction** device (LVSPE) was used to concentrate water samples of up to 1000 litres on-site during the JDS3. The extracts were then analysed for 264 water phase relevant organic compounds using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) in support of the effect-based screening with a set of different in vitro and in vivo bioassays. Despite the overall low concentrations of organic compounds, all extracts were effective in one or more bioassays with the endpoints mutagenicity, dioxin-like and PXR mediated activity, oxidative stress responses and estrogenicity as well as growth inhibition and photosystem II inhibition of green algae. Extract from site JDS33 (downstream Novi Sad) and JDS63 (tributary Siret) were among the samples showing the most toxic potential, which were effective in almost all bioassays. Even though the bioassays are not fully evaluated und thus the toxicological potential might be over- or underestimated and the more complex analyses will still follow, these preliminary results indicate that the attention should be given to the presence of the organic compounds in the Danube beyond those listed in the regulatory documents.
- **Non-target screening** was performed at a basin-wide scale based on UHPLC-QTOF-MS and LC-HR-MS techniques with the major goal to search for as many compounds as possible. Initial results from non-target screening by UHPLC-QTOF-MS revealed presence of more than 3370 different organic compounds listed by name (PCDL match). The follow up evaluations with autoMSMS method resulted in unequivocal identification of 56 substances dominated by pesticides, pharmaceuticals and personal care products. The rest of tentatively identified suspect compounds, unknowns (proposed molecular formula) and total unknowns (only accurate mass and retention time available) still need to be investigated and those results can be expected in the future. The ‘suspect screening’ by LC-HR-MS showed that 110 out of 315 ‘searched for’ substances were determined in at least one sample and 50 compounds were present in more than 20 samples. A semi-quantitative analysis was performed for 110 analytes. Despite the lists of target/suspect substances in two laboratories carrying out this exercise differ, there is a good agreement on the overlapping compounds, e.g. DEET found by both laboratories in all 68 samples and gabapentin in 67 vs. 65 samples with LC-QTOF-MS and LC-HR-MS, respectively.
- An alternative sampling approach to detect the trace concentrations of organic substances was tested during JDS3. The **passive samplers** were exposed to the Danube water for a period of up to two days to adsorb the dissolved pollutants. Despite the low or sub- $ng.l^{-1}$ concentrations of most organic pollutants present in the free dissolved phase, passive sampling enabled to clearly identify spatial gradients of a broad range of organic pollutants in the water column, including PCBs, organochlorine compounds, PAHs, alkylphenols, selected polar pesticides and pharmaceuticals. In many cases, the integrative character of passive sampling allowed measurement of compounds down to $pg.l^{-1}$ levels where methods based on low volume spot sampling of water applied in the previous JDS2 survey failed to detect them.
- A specific **biomarkers based assay** was used **detecting the genotoxic pollution** by comet assay in haemolymph of mussels and in peripheral erythrocytes of fish species. The highest levels of DNA damages were observed in specimens collected in section between Baja and Velika Morava (comet assay) and in sections between Bazias and Orsova and between Guirgeni and Reni (micronucleus assay). The metropolitan region of Bucharest/Ruse showed significant highest values of micronucleus formation in erythrocytes of A.alburnus. Lower values in the comet assay at these sites may indicate different genotoxic modes of action.

For the first time the link between contamination of surface water and groundwater was explored. A number of emerging substances were detected during JDS3 in the abstraction wells at bank filtration sites. This phenomenon can be expected for substances like amidotrizoic acid, iopamidol, acesulfame, benzotriazole or carbamazepine which are known to be quite persistent in the aquatic environment and which are mostly not completely retained by bank filtration. However, due to the relatively low concentration levels in the Danube, concentrations in the abstraction wells were mostly below 0.1 µg/L for most substances. An exception was the artificial sweetener acesulfame which occurred in concentrations up to 1.1 µg/L in the Danube and was detected in most of the abstraction wells with a maximum concentration of 0.45 µg/L. Acesulfame is used as a food additive and the observed concentrations are not considered to be harmful for humans. However, acesulfame can act as an example for a more or less persistent and very mobile substance which is consumed in large quantities.

The analysis of a large amount of organic substances during JDS3 enabled to provide suggestions for the update of the Danube river basin-wide list of specific pollutants. The prioritization methodology which was based on the approach developed by the prioritization working group of the NORMAN network produced a list of 22 substances suggested as relevant for the Danube river basin based on the results of the JDS3 target screening of 654 substances in the Danube water samples by 13 laboratories. PNEC values were available for 189 out of 277 JDS3 substances actually determined in the samples. The cut off criteria to include a compound in the list was its exceedance of the ecotoxicological threshold value (PNEC or EQS) at minimum of one JDS3 site. The list contains five WFD priority substances (three PAHs, fluorathene and PFOS) and two EU Watch List candidate compounds (17beta-estradiol, diclofenac). The 'top ten' substances are dominated by (i) the pesticides 2,4-dinitrophenol (exceeding the limit value at all sites), chloroxuron, bromacil, dimefuron and transformation products of widely used atrazine and terbuthylazine, (ii) three polyfluorinated substances (PFOS, PFOA, PFNA) and (iii) the plasticiser bisphenol A determined at 30 sampling sites.

The parallel investigation of a large amount of organic substances by several laboratories enabled comparison of the data produced by the state-of-the-art analytical techniques. In general the majority of the top ranking emerging organic pollutants were detected by more than one laboratory and the differences in their determinations were either negligible or explainable on the basis of reported methods' LOQs. Systematic differences were usually contained in the low-ng/l range close to the state-of-the-art performance of the existing analytical methodologies. It is recommended that once a final list of the 'important' Danube substances is established a proficiency testing scheme shall be applied to harmonise the performance of all laboratories for each individual substance. In general there is also a strong need to improve analytical methods for the described substances of concern.

34.4.4 $^{87}Sr/^{86}Sr$ river water isoscape

The spatially distinct data on $^{87}Sr/^{86}Sr$ isotope ratios along the course of the Danube collected during the JDS3 in combination with existing data mainly from the upper section of the Danube will be used to develop the aquatic $^{87}Sr/^{86}Sr$ isoscape of the Danube catchment. In combination with other chemical tracers, e.g. like the Sr/Ca ratio, the Danube catchment isoscape will serve as an important input for studying e.g. migrations of aquatic animals like fish and weathering and erosion processes in the Danube catchment.

34.5 LESSONS LEARNED

The application of novel methodologies and approaches in water biology, chemistry and microbiology at the basin-wide scale during JDS3 increased our knowledge remarkably and the results received helped to better understand the complex processes that determine the status of the Danube River.

The findings of the survey brought not only new data about the Danube water status and beyond but they also provided an instrumental support to the progress and harmonization of the water quality assessment approaches in the Danube River Basin. The recommendations provided below will help in ensuring a proper planning and design of the ICPDR monitoring activities in future.

34.5.1 Hydromorphology

During future surveys the sites for the hydromorphological assessment should be selected in close cooperation with monitoring and biological experts to select the most representative river sections. There is an increasing need to improve the "descriptive" method of hydromorphological assessments in particular for large rivers as it should be more "physical process"- based. Also the link between hydromorphological parameters and biological response and the related monitoring efficiency should be improved. The first steps in this direction were already done by performing in-situ measurements during JDS3. The future monitoring shall take fully into consideration the type-specific conditions in line with WFD requirements.

34.5.1.1 Bird survey

The water conditions in 2013 confirmed how important it is to conduct a monitoring over several years. The results of 2013 give a good impression of the short-time effects of flooding on the populations of the monitored bird species, however, further analysis concerning the hydromorphological situation in the side arms and tributaries would be still needed. A follow-up survey for these bird indicator species is recommended to enable Danube-wide analysis based on population size. The more detailed analysis of the correlation between biological indicators and hydromorphological alteration could be a step towards the formulation of the biological criteria for a good hydromorphological situation on rivers.

34.5.2 Biology

34.5.2.1 Macrozoobenthos

The involved sampling methods were found to complement each other: MHS method is especially applicable for ecological status assessment of large rivers at low water periods due to its standardized, stressor-specific and habitat-oriented approach. K&S can provide additional information particularly on mussel populations inhabiting deeper zones next to the bank. DWS is not affected by water level and discharge and therefore is appropriate for data collection from all of deep parts and habitats of a large river.

The different methodological approaches produce different datasets leading to different assessment results. While Saprobic Indices from riparian habitats (K&S and MHS) are largely comparable, DWS collates lotic fauna associated with lower Saprobic Indices resulting in a better ecological status. To incorporate this spatial diversity a worst-case approach of deep water and riparian sampling was applied. For the investigation of just saprobic water quality the MHS would be sufficient.

At present the different national methods for assessment of the ecological status with the biological quality element macrozoobenthos are not intercalibrated. The experiences from the JDS show that there is a strong need for the harmonization and type-specific adjustment of saprobic index values for species and for the development of type specific multi-metric indices for the assessment of general degradation.

34.5.2.2 Phytobenthos

The results confirm that despite the methodological limitations related to phytobenthos in large rivers diatoms are valuable indicators of water quality and of general degradation of the Danube and can be reliably applied to the assessment of its ecological status. Not only the diatom indices, but also the diatom guilds proved to provide a reliable reflection of the environmental conditions and supply an additional insight to the aquatic ecosystem functioning.

34.5.2.3 Macrophytes

The results of the macrophyte study clearly demonstrate that a macrophyte-based quality assessment of large rivers is possible. A conclusion can be made that the assessment systems deliver plausible results only for the river-types or regions they were developed for. For enabling an assessment on a larger scale in all systems tested river-type and region-specific adaptations would have to be performed. In this context the findings of dissimilarities and similarities between river-sections can support the necessary region- and river-type-specific adaptions when dealing with ecological quality assessment. As a further outcome of the macrophyte study the importance of including helophytes and selected bank-vegetation in a macrophyte-based quality assessment could be demonstrated, especially with regard to hydromorphology.

34.5.2.4 Phytoplankton

Both the concentrations of chlorophyll-a and the phytoplankton biomass were higher compared to JDS2 in 2007 particularly in the section between Vienna and Budapest. It must be emphasized however, that direct comparison of chemical and biological concentrations of the two investigation periods might be inconclusive because of different hydrological discharge situations. The smaller concentrations during JDS2 can partly be a reflection of dilution due to higher run-off.

However comparison of phytoplankton results is difficult in general because a reliable assessment would need to involve several sampling dates within a year.

34.5.2.5 Fish

The electrified benthic frame trawl indicated the commonness of specific benthic species along the Danube and added valuable information which would have remained hidden using only shoreline surveys. It revealed the common occurrence and relatively high abundance of *Zingel* species, especially of *Zingel streber* which occurred at 16 sampling sites with 127 individuals (cf. with all the other methods only 84 individuals were caught at 8 sites).

The applied national fish indices (FIA, FIS, EFI) deliver inconsistent results for the whole river course and indicate, that they react on different stressors (hydromorphology vs. water quality) and are hence applicable for restricted river stretches only. Especially in the Lower Danube additional sampling methods (e.g. trammel nets) are required to complete the data set. It must be pointed out that the harmonisation of fish assessment methods for large European rivers is an essential task to meet the goals of the WFD.

34.5.2.6 Zooplankton

The further investigation of veligera larvae in zooplankton is suggested along the River Danube, due to the importance of invasive alien Bivalvia species.

34.5.2.7 IAS

Further work has to be done in the field of collecting of basic information on the distribution of invasive alien species and their influence on native biota, of developing effective tools for the assessment of the level of pressures caused by the bioinvasions, as well as of designing the appropriate mitigation measures.

It is important to evaluate accurately and rationally the real pressure of each invader to native ecosystems, because its influence on the native biota should not be considered a priori as negative.

34.5.3 Microbiology

34.5.3.1 Bacterial Faecal Indicators

Both, the Colilert system for E.coli detection and the ISO microtiter plate technique with two dilutions for the enumeration of enterococci were appropriate and robust microbiological methods for the enumeration of faecal indicator bacteria. Sampling at the left, middle and right river sides enabled a much deeper view into the microbial faecal pollution patterns of the Danube. At many JDS sampling sites the influence of a wastewater input (from a point source or a tributary) could only be detected at one of the two river sides, most prominently at Kelheim (DE), downstream Russenski Lom (BG) and downstream Arges (RO), but also at Oberloiben and Vienna (AT), downstream Vah (HU) or after the Iron gates at Vrbica/Simijan (RS/RO). Thus sampling at both river sides in addition to the midstream is a prerequisite for assessing the microbiological-faecal status of the river.

34.5.3.2 Microbial source tracking

For the first time human-associated faecal pollution detection was complemented with animal-associated MST markers, making it possible to contrast the potentially most relevant pollution sources against each other. In contrast to human faecal pollution, ruminant and pig faecal pollution could very infrequently be detected and showing very low levels (close to the detection limit of the method). One valuable addition in the future would be the application of genetic faecal markers for bird faecal pollution, but unfortunately up to date there are no such methods available that have been tested in the Central European region. The MST results of JDS3 are in good accordance with the results from JDS1 and JDS2. Although different markers for human-associated pollution have been used (HF183II and BacHum in contrast to BacH) the dominance of human faecal impact stayed evident.

34.5.4 Chemistry

The results of Total Nitrogen and Total Phosphorous showed high comparability with the time-corresponding data (August – September) from long-term ICPDR surveillance monitoring (TNMN results from 2001 – 2011). This outcome clearly demonstrates that one set of homogenous data produced by a single sampling procedure and laboratory analysis carried out by selected laboratory soundly confirms the on-going harmonisation and improvement of operational activity of the of National Reference Laboratories network and the effectiveness of the Analytical Quality Control (AQC) programme organised by the ICPDR at the basin wide level.

The analytical methods applied during JDS3 were ambitious, but for some parameters as benzo(a)pyrene, quinoxyfen, bifenox, heptachlor and dichlorvos the required limits of quantification of the analytical methods could not be reached to assess the new EQS. For these parameters further analytical efforts in future are required.

Non-target screening has been found a powerful tool for the identification of the river basin specific pollutants. Present mass spectrometry systems generate vast amounts of data and therefore there is a need for strategy to reduce the amount of detected (thousands of) substances in a single sample to ‘workable’ numbers (top 10 – 100 substances). One of the possible ways out is prioritisation of non-target screening data being currently developed by the NORMAN Working Group on Prioritisation (www.norman-network.net).

JDS3 also demonstrated the feasibility of an effect-based screening in a river basin wide scale using on-site LVSPE even under conditions of high dilution such as in Danube River. Similarly the combination of passive samplers with bioassays presents a very promising approach for detection of various trace organic pollutants and toxic potentials along the river and for identification of areas of concern for further investigation.

34.6 OVERALL CONCLUSIONS

JDS3 provided a unique opportunity to assess the water quality in the whole Danube and provided the largest ever amount of knowledge about the Danube water pollution collected within a single scientific exercise.

The key findings of the biological assessment show that:

- 77% of sites could be classified according to the most widely used Saprobic Index of Macrozoobenthos as good or high however the hot-spots indicating significant organic pollution were detected on the whole Danube;
- The results for Phytobenthos and macrophytes indicated decrease of ecological status downstream the Danube, this however has to be confirmed by type-specific assessment;
- The phytoplankton chlorophyll-a indicated high to good status in most of the upper and the lower reach of the Danube while the moderate status was mostly found in the Middle Danube;
- High fish species diversity was found in the Danube (over 139 000 fish of 67 species were sampled) but due to existing pressures (hydropower, poaching and fishery) about 50 to 90% sites (based on the method applied) did not meet the requirements of the WFD.
- Comparison with the results of previous Danube Surveys clearly showed a constant impact of invasive alien species on native biota (fish, macrozoobenthos and macrophytes).

During JDS3 information on hydromorphological conditions was significantly improved as in-situ measurements of hydrological, morphological and hydraulic characteristics were for the first time performed on the entire Danube and tributaries. Hydromorphological survey confirmed the main findings of JDS2 in 2007 however the increased resolution allowed a more precise assessment. The WFD-3digit analysis of the entire Danube indicated the general alteration (prevailing classes 3-5). The CEN overall hydromorphological analysis indicated that about 60% of the analyzed Danube stretch falls below class 3.

Altogether JDS3 reconfirmed that the Danube flora and fauna shows a high degree of biodiversity. The biological results obtained clearly demonstrate the need for further development and harmonization of type-specific methodologies applicable for the whole Danube River for the evaluation of biological quality elements necessary for the assessment of the ecological status according to WFD.

The chemical analysis showed that:

- Compared with JDS1 and JDS2 results, Total Nitrogen and Total Phosphorous concentrations measured in the Danube River during JDS3 were lower and for general physico-chemical quality elements most of the sampling sites belong to either ”high” or ”good” class – this could indicate a positive impact of improved municipal wastewater treatment in the basin on the Danube water quality;
- The contents of metals in water, suspended particulate matter and bottom sediments were similar to those observed during the JDS1 and JDS2 but the concentrations of mercury in all analyzed fish samples exceeded the EQS significantly;
- Most of the analyzed WFD Priority Substances were found below the newly set environmental quality standards (EQS). Concentrations of PFOS exceeded EQS at 94% of the sampling sites. For PAH and tributyl-tin the AA-EQS for water was exceeded only at few sampling sites;
- As regards the persistent organics PCDD/F and PCBs concentrations were similar to JDS2, and mostly the concentrations observed were between half- and more than one order of magnitude lower compared to the River Elbe;

- Large number of emerging polar organic substances was found but they were at very small concentrations; the concentrations for most of the contaminants were lower in 2013 compared to JDS2 in 2007;
- During JDS3 several new analytical techniques and strategies were applied targeting hundreds of organic substances and resulting in the most comprehensive information ever acquired on this topic for the Danube.
- The analysis of such a large amount of organic substances enabled to provide suggestions for the update and prioritization of the Danube river basin specific pollutants.

A number of emerging substances were detected during JDS3 in the abstraction wells at bank filtration sites. They were below the quality standards however their presence indicated vulnerability of groundwater being an important source of drinking water. All the results and findings of JDS3 provide an exceptional database for the Danube countries which can be used for the river basin management planning at the national level not only because of the large amount and unique character of the produced data but especially due to their homogeneity enabling a good transboundary intercomparison.

JDS3 data confirmed that there is still a need for appropriate measures such as:

- Preventing or limiting to minimum fresh bank revetments and reinforcement;
- Restoration of floodplains to meet the objectives of EU Water framework Directive and Floods Directive;
- Management of the sediment balance at Danube basin-wide scale;
- Further construction and upgrade of wastewater treatment plants especially in the Middle and Lower Danube area;
- Comprehensive and detailed investigation of the occurrence of mercury in fish in the Danube River Basin;
- Implementation of effective policies addressing the reduction of emissions of hazardous substances;
- Further research needs on occurrence of invasive alien species and on development of type-specific methods for the evaluation of WFD biological quality elements;
- Attention given to protection of bank-filtered water wells used for drinking water production.