Ecology and bioindicator potential of benthic macroinvertebrates in a Mediterranean salt wedge estuary: the Ebro River case

Alfonso Nebra Costas

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2015

The Ebro Delta-Estuary complex is one of the largest wetland areas in the western Mediterranean, and it is considered one of the most important estuarine zones in Europe. In 2013, the Ebro watershed, including its deltaic plain, was declared World Biosphere Reserve by the UNESCO. Due to its singularity, a total of 7.736 ha of Delta are protected under the Spanish Natural Park figure (including coastal lagoons, freshwater springs, bays and adjacent coastline), which stands out by its faunal (mainly, ornithological and ichthyological) and its halophilic floral composition. The Ebro River flows into the Mediterranean Sea and forms a salt wedge or highly stratified estuary, a unique type only found in microtidal coasts worldwide. Diverse human activities occur in this area such as tourism, shooting, commercial fishing and agriculture. As a consequence, the entire Delta-Estuary complex is under permanent anthropogenic pressures threatening its ecological integrity; therefore, its conservation should be a priority task.

The main objective of the present PhD thesis is to analyze the ecology of the benthic macroinvertebrate community from the Ebro Estuary in order to evaluate its potential use as biological indicator of highly stratified Mediterranean estuaries. To achieve this goal, the macroinvertebrate community was studied at a high level of taxonomic resolution, and its spatiotemporal dynamics, in relation to the estuarine environmental gradients, was assessed.

Alfonso Nebra Costas 2015

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Programa de Ecosistemas Acuáticos

Centro Sant Carles de la Ràpita

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Ecología de los macroinvertebrados bentónicos y su potencial como bioindicador en un estuario meditarráneo de cuña salina: el caso del río Ebro

TESIS DOCTORAL

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Memoria presentada por

Alfonso Nebra Costas
Para optar al grado de

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A mi abuela

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABREVIATIONS and ACRONYMS

ACA	Catalan Water Agency
AENOR	Standardization and Certification Spanish Association
AIC	Akaike Information Criterion
AMBI	AZTI's Marine Biotic Index
ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
BI	Biotic Index
BOPA	Benthic Opportunistic Polychaetes Amphipods Index
BQE	Biological Quality Element
ca.	circa (around)
CCA	Canonical Correspondence Analysis
CHE	Ebro River Basin Authority
CTZ	Critical Transition Zone
CWA	Clean Water Act (33 U.S.C. §1251 et seq. 1972)
d	Margalef Index
D	Density
DCA	Detrended Correspondence Analysis
DF	Deposit Feeders
DO	Dissolved Oxygen
e.g	exempli gratia (for example)
E_h	Oxidation/Reduction Potential
EMAP	Environmental Monitoring and Assessment Program
EMAP-E	Environmental Monitoring and Assessment Program -Estuaries
EN	European Norm

EP	Ephemeroptera and Plecoptera
EPA	United States Environmental Protection Agency
EPT	Ephemeroptera, Plecoptera and Trichoptera
EPTCBO	Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, Bivalvia and Odonata
EQR	Ecological Quality Ratio
ES	Ecological Status
et al.	et alii (and others)
EUMS	European Union Member States
FA	Field of Application
GAM	Generalized Additive Models
G	Grazers
H'	Shannon-Wiener's diversity Index
IBMWP	Iberian Monitoring Working Party
i.e	id est (that is)
IF	Impact Factor
IRTA	Research, Technology, Food and Agriculture Institute
ISI	Institute for Scientific Information
JCR	Journal Citation Report
J'	Pielou's Evenness Index
KMO	Kaiser-Meyer-Olkin Test
LE	Lower Estuary
LSD	Least Significant Difference
MDS	Non-Metric Multi-Dimensional Scaling
MEDDOC	Mediterranean Occidental index
MSFD	Marine Strategy Framework Directive-2008/56/EC
MSP	Methodology and Sample Processing
M-AMBI	Multivariate AZTI's Marine Biotic Index
N	Total Abundance
NEP	National Estuary Program

O	Omnivores
OSS	Organic Suspended Solids
PCA	Principal Components Analysis
pH	Pondus Hydrogenii (power of hydrogen)
PRIP	Predicted Response to Increasing Perturbation
RC	Reference Condition
S	Richness
SCI	Science Citation Index
S_0	Cross-section Averaged Salinity (see Hansen and Rattray, 1966)
SF	Suspension Feeders
SIMPER	Similarity Percentage Analysis
SW	Salt Wedge's Tip or Null point
TOM	Total Organic Matter
TR	Taxonomic Resolution
TSS	Total Suspended Solids
TW	Transitional Water
UE	Upper Estuary
UN	United Nations
UNE	Spanish Norm
UNESCO	United Nations Educational, Scientific and Cultural Organization
U_f	Sectional averaged flow (see Hansen and Rattray (1966)
u_s	Surface flow-speed (see Hansen and Rattray (1966) diagram)
WB	Water Body
WFD	Water Framework Directive-2000/60/EC
WOS	Web of Science
1-λ'	Simpson dominance Index
δ**S**	Top to bottom salinity difference (see Hansen and Rattray, 1966)

1 DIRECTOR'S REPORT

Report of the directors of the Ph.D. thesis in reference to its derived publications and the student's contribution to them

Dr. Nuno Caiola, researcher of the Aquatic Ecosystems program, Institute of Agrifood Research and Technology (IRTA), as supervisor

and,

Dr. Carles Ibáñez i Martí, Director of the Aquatic Ecosystems program, IRTA, as co-supervisor

of the Ph.D. thesis authored by Alfonso Nebra Costas and entitled: '*Ecology and bioindicator potential of benthic macroinvertebrates in a Mediterranean salt wedge estuary: the Ebro River case'*

INFORM

That the results and conclusions achieved in the research developed by Alfonso Nebra Costas as part of his Ph.D. thesis have been organized in 3 chapters which correspond to 3 scientific papers (2 already published in SCI journals and 1 manuscript currently submitted).

The list of publications and manuscripts is shown, indicating the journal Impact Factor (IF) (according to SCI of ISI Web of Science, Journal Citation Report-2014) as well as the median IF of the main subject categories and the position of the journal within the corresponding category.

I) Nebra A., Caiola N., Ibáñez C. **Community structure of benthic macroinvertebrates inhabiting a highly stratified Mediterranean estuary.** *Scientia Marina*, 75(3): 577-584 (2011).

Impact factor: 1.144

This journal is reported in Quartile 3 of "Marine and Freshwater Biology" subject category, being in the 63th position of the 102 journals included, which have a median IF value of 1.448

II) Nebra A., Alcaraz C., Caiola N., Muñoz-Camarillo G., Ibáñez C. **Benthic macrofaunal dynamics and environmental stress, across a riverine-marine boundary, in a salt wedge Mediterranean estuary.** *Marine Environmental Research* (submitted and currently under review).

Impact factor: 2.762

This journal is reported in the Quartile 1 of "Marine and Freshwater Biology" subject category being the 12th out of 102 journals; and in the Quartile 2 of the "Environmental Sciences" and "Toxicology" categories, being in the 56th and 34th position of the 221 and 87 journals included in both categories, respectively. "Marine and Freshwater Biology" category has a median IF of 1.448; whereas, the IF for "Environmental Sciences" and "Toxicology" categories is 1.641 and 2.377, respectively.

III) Nebra A., Caiola N.A., Muñoz-Camarillo G., Rodríguez-Climent S., Ibáñez C. **Towards a suitable ecological status assessment of salt wedge Mediterranean estuaries: a comparison of benthic invertebrate fauna indices.** *Ecological Indicators*, 46 (2014) 177-187

Impact factor: 3.444

This journal is reported in Quartile 1 of "Environmental Sciences" subject category, being in the 34th position of the 221 journals included in this category, which have a median IF value of 1.641.

and CERTIFY

that Alfonso Nebra Costas contribution has been very active, as it is demonstrated by his first coauthoring of all the manuscripts that conform this Ph.D. thesis. In particular, his participation included the following tasks:

- Sampling design and field work, including water, macroinvertebrate samples collection, and in situ physico-chemical measurements.
- Sediment grain size and organic matter content analysis.
- Sorting, counting and identification of macroinvertebrate and microcrustaceans species.
- Data analysis and interpretation of results.
- Tables and Figures design and preparation.
- Definition of the objectives and focus of the research and its derived manuscripts.
- Main writing of the manuscripts, and contact person for the reviewing and editing process.
- Writing of this PH.D Thesis manuscript

Barcelona, Novenber 23th 2015

Dr. Nuno Caiola　　　　Dr. Carles Ibáñez i Martí

2 GENERAL INTRODUCTION

2.1 Estuaries: Definition and Classification

The term estuary is derived from the Latin word '*aestus*' meaning tide or billowing movement, more specifically the word '*aestuarium*' means marsh or channel (McLusky and Elliot, 2004). The most widely accepted definition of an estuary was proposed by Cameron and Pritchard (1963). According to his definition, an estuary is "*A coastal semienclosed body of water, with free connection to the open sea, and within which sea water comes diluted by fresh water derived from land drainage*". Although, this classical definition does not mention tide as the main mixing driver in estuaries, seawater dilution implies the action of this mixing agent. At the same time, this definition implies fresh water input entering into a semienclosed basin.

The above definition of an estuary deals with temperate tidal estuaries but is unspecific for arid and semiarid basins (scarce freshwater input in dry periods) (Potter *et al*., 2010) and for non-tidal or microtidal seas (no mixing effect due to low tide power) (Valle-Levinson, 2010; Day *et al*., 2012), which is the case of salt wedge estuaries (Muñoz, 1990; Ibáñez, 1993; Ibáñez *et al*., 1997). In order to address with limitations of Cameron and Pritchard's definition some attempts to redefine estuaries have been done. Fairbridge (1980) coined the next definition: "*An estuary is an inlet of the sea reaching into a river valley as far as the upper limit of tidal rise, usually being divisible into three sectors: (i) a marine or lower estuary, in free connection with the open sea; (ii) a middle estuary subject to strong salt and fresh water mixing; and (iii) an upper or fluvial estuary, characterized by fresh water but subject to daily tidal action*". Fairbridge's definition still excludes some estuarine systems, *e.g*. non-tidal estuaries or bar-built estuaries that become separated from sea during bar formation periods. Day (1981) adapted Cameron and Pritchard's definition to accommodate South African estuaries to the following: "*An estuary is a partially enclosed coastal body of water which is either permanently or periodically open to the sea and within which there is a measurable variation of salinity due to the mixture of sea water with fresh water derived from land drainage*". However, this definition is also incomplete, excluding estuaries from important microtidal regions of the world such as Mediterranean Sea or Gulf of Mexico.

In recent years, the controversy about estuaries' definition continues (*e.g*. Elliott and McLusky, 2002; Tagliapietra *et al*., 2009; Potter *et al*., 2010) and nowadays, no consensus has been found. One of the most 'broad range' definition of an estuary was proposed by Potter *et al*. (2010): "*An estuary is a partially enclosed coastal body of water that is either permanently or periodically open to the sea and which receives at least periodic discharge from a river(s); and thus, while its salinity is typically less than that of natural sea water and varies temporally and along its length, it can become hypersaline in regions when evaporative water loss is high and freshwater and tidal inputs are negligible*". This definition was proposed with the aim to incorporate hypersaline estuaries found in arid climate from south-western Australia and southern Africa coasts. The basis of this new definition falls on the shared characteristics (biological, functional and structural) with typical temperate estuaries (Potter *et al*., 2010). However, this definition seems to exclude microtidal estuaries which receive important annual mean freshwater inputs.

Tidal estuaries are widespread and abundant in most of oceanic coasts. On the contrary, microtidal systems are restricted to semienclosed seas, such as the Mediterranean, the Black and the Baltic seas, and the Gulf of Mexico. Rivers flowing into microtidal seas form a special estuary type, the salt wedge estuaries. In spite of being more frequent than other estuary types (*e.g*. tectonic type estuaries) and sharing characteristics (biological, functional and structural) with other estuaries, they are still not contemplated in most accepted estuary definitions (classical or current). Salt wedge estuaries are well-represented along microtidal coasts worldwide *e.g.* Ebro River (Spain), southwest Pass of Mississippi River (USA), Columbia River (USA), Río de la Plata (Argentina), Vellar River (India), Pánuco River (Mexico) and Itajaí-Açu River (Brazil), among others. All the above definitions were developed under the scope of a wide variety of disciplines such as, hydrology or physical oceanography. Different definitions have been termed in each of the many disciplines studying estuaries, and sometimes the contradictions between them are evident (Perillo, 1995). An accurate estuary definition should include different components, geomorphological, hydrological, ecological, biological and chemical components (Perillo, 1995). Nevertheless, current available definitions do not reflect all these criteria and none of them consider ecological functioning premises. Consequently, the difficulties of including particular estuarine systems such as hypersaline or microtidal in those definitions are evident. The lack of a suitable definition including this unique estuary types, *e.g.* microtidal estuaries, is a good starting point to introduce the different classification systems for estuaries. In contrast with previous definitions, several widely accepted classification systems include microtidal estuaries. To better understand this special kind of estuaries, a relation of classification systems based in different approaches is present below.

The classifications of estuaries, likewise definition attempts, were addressed in different ways based on the discipline of each author. At the beginning, estuaries were mostly classified by geologists and physical oceanographers who centered their classifications in geomorphological features and salinity stratification (Perillo, 1995; Day *et al.*, 2012). Thus, Cameron and Pritchard (1963) from a geomorphological standpoint, divided estuaries into four categories: **coastal plains** or **drowned river valleys**, **fjords**, **bar-built estuaries** and **tectonic origin estuaries** (Fig 2.1). **Coastal plains** are the classical estuaries for physical geographers, formed in the Pleistocene (~15,000 years ago) as a consequence of sea-level rise; as they were originally rivers, they show a typical river valley shape with several kilometers wide and relatively shallow. **Fjords** are associated with high latitudes where erosive glacial activity is intense; they are characterized by a deep elongated U-shape deep channel (several hundreds of meters), with a sill (a shallow lip) at their mouths as a consequence of moraine transport-deposit activity. **Bar-built estuaries** were embayments that became semienclosed as a consequence of littoral drift, causing the formation of a sand bar or spit (broken by one or more inlets) between the coast and the ocean; originally, they were river valleys drowned by sea-level rise; thus, this type of estuary is considered a composite system. The inlets are relatively small compared to the dimensions of the sound within the barrier; tidal action is weak, and these estuaries are usually shallow systems where the wind is the main mixing mechanism. Finally, **tectonic estuaries** are a consequence of geological events such as earthquakes, fractures, subsidence, creases or faults that generated deformations on the Earth's crust in adjacent regions to the ocean, forming a hollow basin; an estuary is formed when this sink is drowned by the ocean.

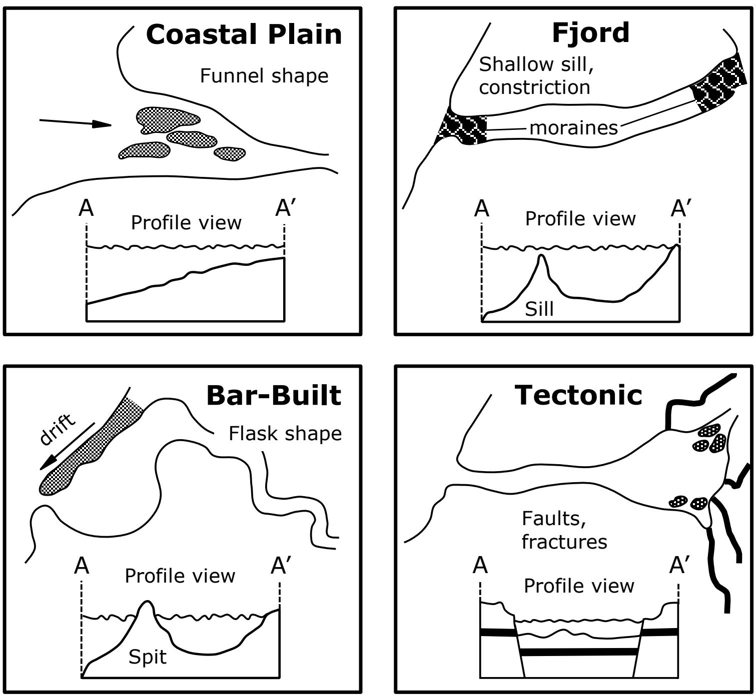

Figure 2.1. Classification of estuaries based on geomorphological features (A-A': cross-section area).

Pritchard (1955) and Cameron and Pritchard (1963) presented another approach to the classification of estuaries, considering the vertical structure of salinity or estuarine circulation. According to water column stratification estuaries can be classified as **salt wedge**, **strongly stratified**, **weakly stratified** or **vertically mixed** (Fig. 2.2). There are three basic processes that produce motion and mixing in an estuary: the wind, the tide and the inflow of fresh water (Pritchard, 1967). In a wind-dominated estuary (such as bar-built estuaries), the wind provides all the energy for motion and mixing of the water. On the other hand, in a tide-dominated estuary the main driver for sea and freshwater mixing is the turbulence associated to tidal currents. In a river-dominated estuary (those from microtidal seas) the mixing is mostly produced by the breaking of unstable interfacial waves at the upper boundary of the salt wedge (halocline). This classification mainly considers competition between river discharge and tidal action. **Salt wedge** estuaries are the result of weak tidal force and large river discharge. In such systems, the water column is stratified due to the density difference between fresh and sea water layers (Fig. 2.2). **Strongly stratified** estuaries are the result of moderate to large river discharge and weak to moderate tidal force; these estuaries have a similar stratification profile to salt wedge estuaries, but the stratification remains stable during the whole tidal cycle; fjords and other deep estuaries are included in this category. **Weakly stratified** estuaries result from moderate to strong tidal action and weak to moderate river discharge; their mean salinity and density profile show a weak cline or continuous stratification from surface to bottom except for a near bottom mixed layer. Strong tidal forcing and weak river discharge result in **vertically mixed** estuaries (minimal vertical stratification); salinity profiles in mixed estuaries are practically uniform and mean flows are unidirectional with depth; in wide (and shallow) estuaries, inflows may develop on one side across the estuary and outflow on the other side, especially during the dry season.

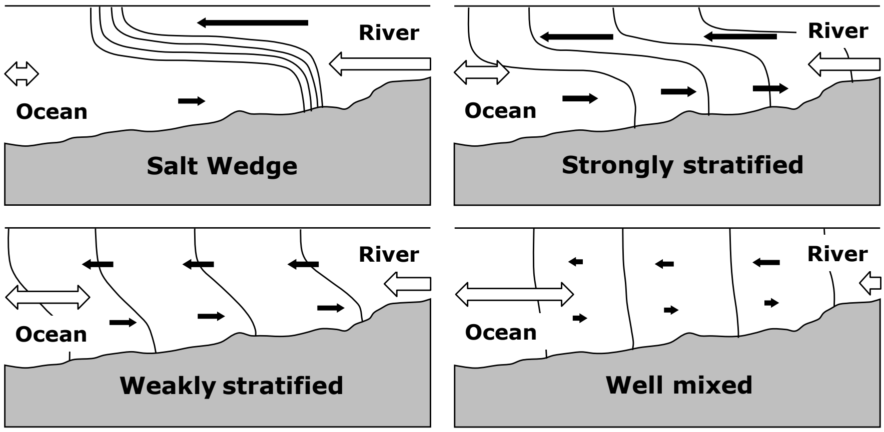

Figure 2.2. Classification of estuaries based on the vertical structure of salinity (Cameron and Pritchard, 1963).

The range of tidal amplitude varies in a constant pattern in the seas of the world. For this reason, Hayes (1975) proposed a classification of estuaries based on the tidal range that led to three different types of estuaries: **microtidal**, **mesotidal** and **macrotidal**. More recently, McLusky and Elliott (2004) added one category to this classification; hence, the final classification ended in **microtidal**, tidal amplitude less than 2 meters; **mesotidal**, tidal amplitude between 2 and 4 meters; **macrotidal**, tidal amplitude between 4 and 6 meters and **hypertidal**, tidal amplitude higher than 6 meters. The importance of tidal amplitude relies on the power of wave action, and therefore, on the mixing of waters and vertical profile of salinity. In addition, the tidal amplitude may considerably influence the mudflats and vegetation of an estuary (Ibáñez *et al.*, 2012c). While **microtidal** estuaries have only limited intertidal areas, **mesotidal** estuaries often develop extensive intertidal areas, which are covered with vegetation, such as *Spartina*; whereas **macrotidal** and **hypertidal** estuaries typically have bare mudflats without large plants (McLusky and Elliot, 2004, Ibáñez *et al.*, 2012c).

Regarding estuarine hydrodynamics, one of the most accepted classifications was the diagram proposed by Hansen and Rattray (1966). This classification is based on two hydrodynamic parameters (stratification and circulation); in particular, by plotting the stratification versus the estuarine circulation (Fig. 2.3). These two parameters refer to tidal averaged (salinities are first averaged over one or more complete tidal cycle) and flow cross-sectional averaged (to smooth lateral circulation). In this way, the stratification parameter is the ratio of the salinity difference between surface and bottom (δS) and the cross-section averaged salinity (S_0). The circulation is simply the ratio between net surface flow-speed (u_s) and freshwater sectional averaged flow (U_f). Hansen and Rattray (1966) found that most estuaries could be grouped into four main regions on their diagram (Fig. 2.3). **Type 1** estuaries are lagoons or bar-built estuaries; **subtype 1a** estuaries are vertically mixed or slightly-stratified, whereas **subtype 1b** estuaries show 'appreciable' vertical stratification. Both subtypes (1a and 1b) have scarce gravitational circulation and they are mainly dominated by diffusive processes. In general terms, **type 1** estuaries describe well-mixed estuaries with net seawards flows (outflows) and no vertical structure. **Type 2** includes most temperate estuaries; these systems are characterized by a reasonable well-developed gravitational and longitudinal circulation, with contribution of adventive and diffusive processes to landwards salt transfer. Again this type is subdivided in analogous subtypes like **Type 1**, well-mixed or weakly-stratified (**Subtype 2a**) and stratified estuaries (**Subtype 2b**). **Subtype 2a** are well mixed or weakly-stratified estuaries; whereas, **Subtype 2b** estuaries are strongly stratified. **Type 3** is distinguished from **Type 2**, primarily by the dominance of advection (well-developed gravitational circulation) accounting for over 99% of the upstream salt transfer. This type of estuaries corresponds to fjords (deep basins with strong surface outflow and very small depth-averaged flows). **Subtype 3a** estuaries are moderately stratified and **Subtype 3b** estuaries are highly stratified. In **Type 4** (salt wedge or highly stratified) the stratification is still greater than that for **Type 3** estuaries; as the freshwater flow grades from a thick layer (upstream) to a narrow surface layer (close to the river mouth), the salt water flows under the freshwater layer and change from a thin upstream layer to a deep lower layer (at river mouth). Vertical mixing is limited and the gravitational circulation is weak or nonexistent. The influence between layers is scarce and restricted to a thin contact layer called halocline or pycnocline, where salt transfer by advection and diffusion processes is limited to.

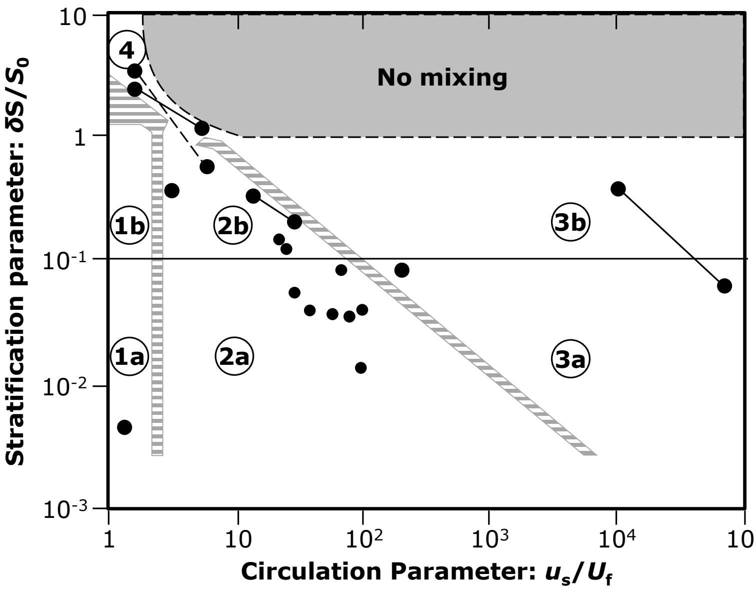

Figure 2.3. Estuarine classification diagram (redrawn from Hansen and Rattray, 1966) according to non-dimensional stratification and circulation parameters. Black dots represent examples of estuaries (label names omitted).

Finally, there is a classification based on water balance. Therefore, estuaries can be classified as **positive**, **inverse** or **negative** and **low-inflow** (Valle-Levinson, 2010) (Fig. 2.4). **Positive** estuaries are those in which freshwater inputs from land drainage and rain exceed freshwater losses from evaporation; these estuaries show a well-established longitudinal density gradient, with net surface outflow seawards due to freshwater contribution. **Negative** estuaries are typically found in arid regions where there is a scarce river discharge into the system because freshwater losses from evaporation exceed freshwater inputs, for this reason, they are called **negative**; because the longitudinal density and salinity gradient increases landwards. Water renewal rate in **negative** estuaries is very low; thus, they are prone to water quality problems. **Low-inflow** estuaries also occur in arid regions, but show a small influence from river discharge (in the order of a few m^3s^{-1}). During dry periods, evaporation processes may cause a salinity maximum zone or 'salt plug' within the estuary. Seawards of the salt plug, the salinity decreases, as in **negative** estuaries; whereas, upstream the plug the salinity decreases as in **positive** estuaries. The salt plug (maximum density zone) acts as a barrier avoiding marine intrusion landwards and river discharge seawards; because of this, **low-inflow** estuaries are also prone to water quality problems.

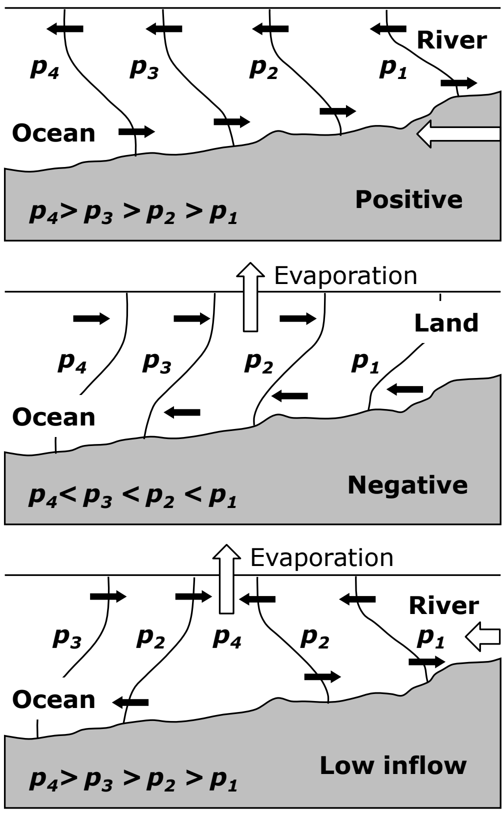

Figure 2.4. Classification of estuaries based on water balance (redrawn from Valle-Levinson, 2010).

The above definitions and classifications confirm the great variety of estuarine types and the challenging task to define and classify estuaries (Table 2.1).

Table 2.1. Summary of estuaries classifications.

CLASSIFICATION CRITERIA	TYPES OF ESTUARIES	
Geomorphological (Cameron and Pritchard, 1963)	*Coastal plains* or *drowned valleys** *Fjords* *Bar built estuaries* *Tectonic original estuaries*	
Water column stratification (Pritchard, 1955; Cameron and Pritchard, 1963)	*Salt wedge** *Strongly stratified* *Weakly stratified* *Vertically mixed*	
Tidal range (Hayes, 1975; McLusky and Elliot, 2004)	*Microtidal*: tidal amplitude less than 2 meters* *Mesotidal*: tidal amplitude between 2 and 4 meters *Macrotidal*: tidal amplitude between 4 and 6 meters *Hypertidal*: tidal amplitude higher than 6 meters	
Estuarine Hydrodynamics: stratification and circulation (Hansen and Rattray, 1966)	*Type 1*: lagoons or bar-built estuaries	Subtype 1a: vertically mixed or slightly-stratified Subtype 1b estuaries show 'appreciable' vertical stratification
	Type 2: reasonable well-developed gravitational and longitudinal circulation, with contribution of adventive and diffusive processes to landwards salt transfer	Subtype 2a: well-mixed or weakly-stratified Subtype 2b: stratified estuaries
	Type 3: fjords (deep basins with strong surface outflow and very small depth-averaged flows)	Subtype 3a: moderately stratified Subtype 3b: highly stratified
	Type 4: salt wedge or highly stratified*	
Water balance (Valle-Levinson, 2010)	*Positive:* freshwater inputs exceed freshwater losses* (well-established longitudinal density gradient) *Inverse* or *negative*: freshwater losses from evaporation exceed freshwater inputs *Low-inflow*: small influence from river discharge.	

* Classification of the Ebro Estuary according to each classification scheme.

2.2 Estuaries: relevance, benefits and anthropogenic pressures concern

Estuaries are complex ecosystems, largely recognized for their high productivity and their interest from social, economic and conservational perspectives (Ysebaert *et al*., 1998; Pierson *et al*., 2002; Russell *et al*., 2006; Bianchi, 2006; Vasconcelos *et al*., 2007). From an ecological point of view, estuaries are source of food, shelter and spawning-nursery areas for many organisms, *e.g*. macroinvertebrates and fish (including commercial species), or waders and waterfowls especially dependent on estuaries for breeding, feeding or sheltering in winter time. Furthermore, estuarine related habitats such as mudflats, coastal lagoons, barrier beaches, deltaic and flood plains, salt marshes or seagrass beds and meadows, are also valuable ecosystems that host unique biological communities.

Historically, the amount of services and resources provided by estuaries, served as an incentive to immigration and settlement for many human populations; since, thousands of years ago important human civilizations thrived around estuaries such as Tigris-Euphrates, Nile, Indus, Usumacinta, and Yellow rivers (Day *et al*., 2012); firstly because of food resources but later also for commerce purposes. Recent studies indicate that 61% of the world population lives along the coastal areas (Bianchi, 2006), and likewise in the past, several World's largest cities are located around estuarine systems, *e.g*. London, New York, Sao Paulo, Buenos Aires or Shanghai. As a result of coastal colonization, there is a plethora of negative effects over the natural environment (Newman *et al*., 2002; McLusky and Elliot, 2004; Amiard-Triquet and Rainbow, 2009). This fact gets especially worse during the last centuries because of industrial revolution and rapid human population growth (demographic revolution) (Kapitza, 2009; Slaus and Jacobs, 2011). Assorted human activities, such as industry, agriculture, livestock farming, fishing or land claim, take place around coastal areas threatening their ecological integrity, their economic value and even affecting public health (Schlacher and Woolbridge, 1996; Edgar *et al*., 2000; McLusky, 1999; McLusky and Elliot, 2004; Dauer *et al*., 2000; Bianchi, 2006; Dauvin, 2007; Elliot and Quintino, 2007; Gray and Elliot, 2009; Day *et al*., 2012).

The main anthropogenic pressures associated to those activities can be grouped depending on the impacts caused in the ecosystem. **Physical or hydrological pressures**, such as channelization, dredging, drainage and harbor construction, produce habitat loss and alteration impacts. **Enrichment pressures**, including industrial wastewater and urban sewage effluents, agriculture and farmland runoffs or fish farming wastes (Justic *et al.*, 1995; McLusky and Elliot, 2004; Zaldívar *et al.*, 2008; Gray and Elliot, 2009), are promoting the accumulation of pollutants *i.e.* heavy metals, toxic compounds, hydrocarbon substances (Cantillo, 1998; Navarro-Ortega *et al.*, 2010) and provoking high nutrient concentrations in water and organic matter excess in rivers, estuaries and bays (Boynton *et al.*, 1995; Day *et al.*, 1997; Nedwell *et al.*, 1999; Navarro-Ortega *et al.*, 2010). High nutrient loads produce direct ecological impacts over biological communities (Karlson *et al.*, 2002), mostly associated with eutrophication process (Bock *et al.*, 1999; Wang *et al.*, 1999; Hänninen *et al.*, 2000). Besides, organic matter enrichment causes episodes of hypoxia and low redox potential values. These facts disturb biological communities' composition, trophic structure and biomass (Pearson and Rosenberg, 1978; Grebmeier *et al.*, 1988; Díaz, 2001). The impacts associated to **enrichment pressures** are magnified in estuaries due to their sheltered nature; they act as traps of sediments and contaminants (McLusky and Elliot, 2004); furthermore, in Mediterranean climate basins where water scarcity occurs during the summer period, the concentration of these toxic compounds increases producing a serious environmental risk (Navarro-Ortega *et al.*, 2010). There is another group of pressures that produce **changes in community composition**, some examples are: overfishing, promoting species replacement; commercial activity, enhance alien species introduction (Day *et al.*, 2012); and, harbors and marinas, that cause habitat loss, change the hydrographic patterns and the sedimentary regime and disrupting consequently the biota (McLusky and Elliot, 2004). Finally, not long ago, a **global effect pressure** is altering aquatic ecosystem environmental balance, the **climate change** produces severe, and in some cases, irreversible impacts at landscape level, such as global warming and sea level rise for coastal areas (Crooks and Turner, 1999;

Cloern, 2001; Bianchi, 2006, Slaus and Jacobs, 2011; Kernan, 2015); thus, the **climate change** is considered the most important pressure impacting coastal ecosystems (Day *et al.*, 2012; Ibáñez *et al.*, 2014). **Climate change** intrinsic temperature changes (mainly warming) favors the expansion and establishment of invasive alien species to the detriment of autochthonous ones (Dukes and Mooney, 1999; Stachowicz *et al.*, 2002; Ricciardi, 2007; Rahel and Olden, 2008, Rahel *et al.*, 2008; Kernan, 2015). Besides, permanent drowned areas by sea level rise allow to invasive species to reach new habitats such as flood plains, salt marshes, gorges and ravines that frequently act as sanctuaries for native species.

During the last decades, the concern about environmental issues has largely increased among scientists, managers and general society. Detecting the environmental health and functioning of ecosystems has become one of the main themes of modern ecology (Karr and Chu, 1999; Bortone, 2004). In order to deal with the anthropogenic pressures-impacts, and to reverse the severe ecological decline of aquatic ecosystems, statements, monitoring programs and environmental laws have been enacted in many countries. Relevant examples are the Clean Water Act (CWA, 1972), the Environmental Monitoring and Assessment Program (EMAP) and the National Estuary Program (NEP) all developed by the United States Environmental Protection Agency (EPA); the Water Framework Directive-2000/60/EC (WFD) (European Parliament, 2000), the Marine Strategy Framework Directive-2008/56/EC (MSFD) (European Parliament, 2008), and in recent times the Division for Oceans Affairs and the Law of the Sea of the United Nations (UN), announced the Oceans Compact. Estuaries are directly taken into account by the EMAP-Estuaries (EMAP-E), the NEP and by the WFD. In the last case, the ecological status (ES) assessment of European water bodies (WB), including estuaries, is based in the status of biological communities.

The WFD, enacted in 2000, provides a basis for the conservation, protection and improvement the ecological integrity of all WB, including groundwater, inland surface, coastal and transitional waters (TW), in which estuaries are included. The objectives defined in the WFD may be summarized into an overall goal: to ensure that all WB achieving the 'good' ES by 2015 through different key actions that have to be undertaken by European Union Member States (EUMS) to support the implementation of the WFD. This process implies the identification of WB typologies, the description of reference condition (RC) for each WB type defined (undisturbed condition; for detailed information see WFD, 2000/60/EC-Annex II and V), and the development of classification schemes based on ecological assessment of biological indicators (biological quality elements (BQEs) in accordance with WFD definitions). These classification schemes must be endorsed by hydromorphological and physic-chemical quality elements. According to the WFD, estuaries are included in the TW category, defined as: *'bodies of surface water in the vicinity of river mouths which are partly saline in character as a result of their proximity to coastal waters but which are substantially influenced by freshwater flows*'. For TW, the BQEs to be considered are phytoplankton, aquatic flora, fish and benthic invertebrates (European Parliament, 2000). Before determining the ES of a monitored WB, it is necessary to develop assessment tools or biotic indices (BIs), and then compare this BI data with type-specific RC BI data; thus, deriving an ecological quality ratio (EQR) expressed as a numerical value ranging between 0 and 1 (result of dividing biological value observed by RC biological value). This range is divided into five categories (*e.g.* using percentile or equidistant partition), each one corresponding to one of the following ES classes: 'High' status corresponds to the values closest to 1 and 'Bad' status is represented by lowest values, the intermediate classes are 'Good', 'Moderate' and 'Poor' status. The boundaries among classes are different within European eco-region, depending on EUMS types and the classification tools developed. 'High/Good' and 'Good/Moderate' boundaries should be established through intercalibration exercises in order to ensure their agreement with WFD normative definitions, and also to validate that the different methodologies used are comparable among EUMS (Borja *et al*., 2009).

2.3 Aquatic macroinvertebrates: definition, ecology and indicator potential

The term 'macroinvertebrates' refers to organisms that are large enough to be seen with a naked eye (usually greater than 500 microns) and lacking a backbone (invertebrate) (McDonald *et al*., 1991). Macroinvertebrates inhabit all types of aquatic ecosystems from high to low latitudes, such as a mountain streams, large rivers, wetlands or lakes and even really harsh environments such as phytotelmata, hot springs, saltpans or the Mediterranean temporary ponds and streams. Examples of aquatic macroinvertebrates include insects at larval (holometabolous), nymph (heterometabolous) or adult forms (this differentiation takes relevance since different stages of the same species can perform different ecological roles), crustaceans (mainly isopods, amphipods and decapods), mollusks (bivalves, gastropods) and annelids (oligochaetes, polychaetes and hirudinids); among others. Their ecological significance relies in the fact that they play important functional roles on ecosystem ecology. For example, they are major components of food web, as primary consumers, as consumers at intermediate trophic levels or being main food source for higher trophic levels. They also mediate in nutrient, carbon and detritus cycling (Wallace and Webster, 1996; Bianchi, 2006); and they act as 'ecosystem engineers', causing physical structuring of ecosystems, mainly by bioturbation, biodeposition, burrowing or substrate accretion (Jones *et al*., 1987; and references therein). Besides, macroinvertebrates are a suitable biological indicator for environmental monitoring and assessment programs because they are permanently in water and therefore constantly affected by its physical, chemical and biological condition. In addition, macroinvertebrates show many relevant characteristics such as sensitiveness to human influences (Pearson and Rosenberg, 1978; Dauer, 1993; Grall and Glemarec, 1997; Dauer *et al*., 2000; Simboura and Zenetos, 2002; Bustos-Baez and Frid, 2003; Rakocinski and Zapfe, 2005; Perus *et al*., 2007; and many others), relative long life cycles (they may show the cumulative impacts of pollution), they are relatively sedentary and have limited dispersal abilities (so they are unable to avoid deteriorating of water and sediment quality), great species richness and abundance (comprising a wide range of tolerances to stress and pollution), well-known taxonomy, ability to colonize a great variety of microhabitats and relatively easy and inexpensive to sample.

For all these reasons, macroinvertebrates are extensively studied in aquatic ecosystems including estuaries, where there is a long tradition in benthic macroinvertebrates research: classical community description studies and ecological response of biota to estuarine gradients (*e.g*. Remane, 1934; Remane and Schlieper, 1958, 1971; Carriker, 1967; Barnes, 1974; Morrisey *et al*., 1992) or research on the effects of pollution or other anthropic pressures (*e.g*. Pearson and Rosenberg, 1978, Warwick, 1986; Dauer, 1993; Grall and Glémarec, 1997). This knowledge established the keystone for current estuarine macroinvertebrate ecology and for the ecological assessment and monitoring programs. In this sense, during the last decade, estuarine ecology publications have increased rapidly (Duarte *et al*., 2015); in the case of Europe, a great number of studies were performed under the WFD implementation representing an important backing.

Regarding the indicator potential of macroinvertebrates, and their use in biological assessment and management programs, it takes special relevance due to the idiosyncrasy of estuaries as transitional environments. In ecological terms, estuaries are interface systems between rivers and sea, characterized by variable hydrological, morphological and chemical conditions. The close connection between riverine and marine habitats implies that a broad range of physicochemical factors are occurring in a relatively small area producing strong environmental gradients, this leads to a patchy distribution of organisms along estuaries (Morrisey *et al*., 1992). As a consequence, estuaries are stressful systems where biological communities must cope with a wide variety of constrains (Morrisey *et al*., 1992; Bortone, 2004; Gray and Elliott, 2009). The interplay between ecological processes (biotic and abiotic) determines biological communities' variation across spatial and temporal scales (Borcard *et al*., 1992; Constable, 1999; Benedetti-Cecchi *et al*., 2000). Identifying how biological communities are structured in response to environmental gradients is a major goal in ecology. However, research on estuaries is mainly focused on the abiotic influence sidestepping biological interactions, such as species competition. This is because the importance of abiotic factors downplays the role of biotic ones; and, probably this is the main reason for considering estuaries as physically controlled environments (Schaffner, 1990; Ysebaert *et al*., 1998, 2003; Josefson and Hansen, 2004; Giberto *et al*., 2007). The species ability to colonize estuarine environments is limited by their physiological tolerance to severe changes in the abiotic factors *e.g*. hydrodynamic processes, depth, water temperature, oxygen, nutrient levels or food availability (Remane and Schlieper, 1971; Brusca and Brusca, 1990; Attrill and Thomas, 1996; Wu and Shin, 1997; Constable, 1999; Ysebaert *et al*., 2003; Dauvin, 2007; Elliot and Quintino, 2007). However, salinity, sediment grain-size and organic matter content are considered the key abiotic factors determining the composition of benthic communities along estuaries (Day *et al*., 1989; Mannino and Montagna, 1997). The restrictive conditions of estuarine environments entail that only a few well-adapted species are able to survive; for this reason, estuaries are areas which have inherent low species richness and high abundances of stress-tolerant (well-adapted) organisms compared with adjacent marine or riverine areas (Biggs and Cronin, 1981; Dauvin, 2007; Day *et al*., 2012).

At the same time, estuaries are ecosystems under the pressure of a great number of human activities causing many kinds of impacts (as shown in the previous section); accordingly, natural and anthropogenic stress co-occurs in estuaries. This confluence, of opposed origin factors, makes difficult the comprehension of macroinvertebrates' distribution along estuaries and also makes challenging to isolate the origin of drivers (natural or anthropic) that cause those variations. The knowledge of an ecosystem, both its ecological functioning and its community dynamics, is essential for bioindicators based assessment. This fact is even more important in estuaries, considering the difficulty of establishing a stressor-response relationship using Biotic Indices (BIs) (Rakocinski and Zapfe, 2005); this is because they are naturally stressed ecosystems, hosting specialized communities, which can be very similar in both impacted and non-disturbed estuarine systems. This difficulty was coined as the term '*Estuarine Quality Paradox*' (Dauvin, 2007; Elliott and Quintino, 2007). Being aware of estuaries' peculiarities, and with the aim of evaluate ecosystem health, several authors developed assessment tools based on benthic invertebrates as BQE, such as AZTI's Marine Biotic Index (AMBI) (Borja *et al*., 2000), Multivariate AMBI (M-AMBI) (Muxika *et al*., 2007), BENTIX (Simboura and Zenetos, 2002), or Benthic Opportunistic Polychaetes Amphipods index (BOPA) (Dauvin and Ruellet, 2007), Mediterranean Occidental index (MEDDOC) (Pinedo *et al*., 2015), among others. These BIs were mainly developed under the guidance of WFD for assessing of transitional and coastal waters. Nevertheless, all of them are widely applied not only in Europe, but also worldwide; this is an indicative of macroinvertebrates indicator relevance.

2.4. Study context: the Ebro Delta-Estuary complex

This thesis was conducted in the Ebro estuary (40º43'10''N, 0º40'30''E) located in the NE of the Iberian Peninsula (Spain) (Fig. 2.5). The Ebro River is 910 km long and has a drainage basin approximately of 85,362 km^2. It is the Spanish river with the highest mean annual flow and one of the most important tributaries to the Mediterranean Sea (*ca.* 12,000 hm^3/year); data obtained at the Ebro Water Authority (CHE) web site (http://www.chebro.es/). Intensive agriculture is the main land-use in the Ebro basin with more than 10,000 km^2 devoted to irrigation, this accounts for the 90% of the water usage in the basin (Ibáñez *et al*., 2008). The entire basin is strongly regulated by *ca.* 190 dams (Batalla *et al*., 2004), managing water for hydropower purposes, irrigation and human consumption. Large reservoirs have altered the annual flow, not only by modifying the natural seasonal flow pattern, but also by preventing flood frequency and intensity (Ibáñez *et al*., 2012a, b; Rovira *et al*., 2012a). Besides, the annual mean flow has decreased since the beginning of the century (Muñoz and Prat, 1989; Muñoz, 1990; Ibáñez *et al*., 1996, 2008). In particular, Mequinenza and Ribarroja reservoirs, located on the main river about 100 km upstream from the river mouth, have a significant regulatory effect over flows in the lower Ebro River (Ibáñez *et al*., 2012a, b; Rovira *et al*., 2012a), and therefore, they are considered as the final responsible of the salt wedge dynamics and macrofaunal trends along Ebro Estuary. Current regulation schedule assures the presence of the salt wedge in the same position for long periods (Ibáñez *et al*., 1995; Sierra *et al*., 2004; Falcó *et al*., 2010; Nebra *et al*., 2014).

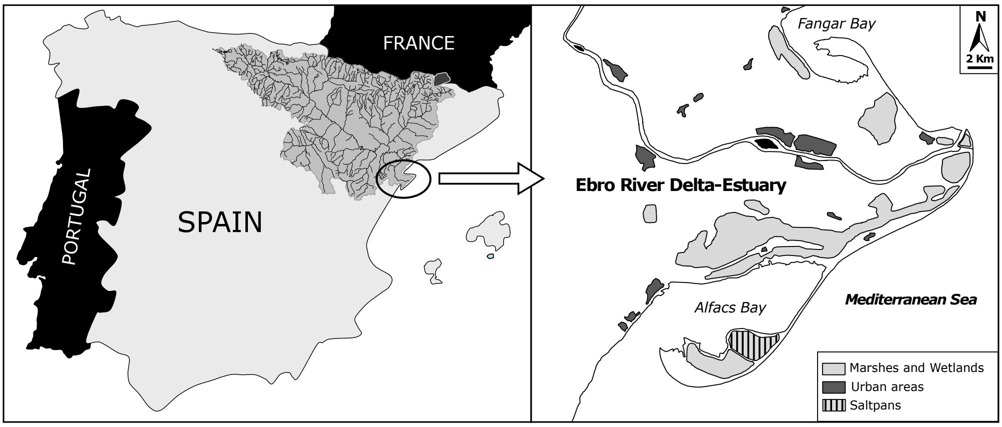

Figure 2.5. Map showing the location of the study area, the Ebro estuary, in the context of the Iberian Peninsula and the Ebro River basin.

The Ebro Delta is one of the largest wetland areas (*ca.* 320 km^2) in the western Mediterranean, and it is considered one of the most important estuarine zones in Europe (Colomé *et al*., 1997; Day *et al.,* 2006). In 2013, the United Nations Educational, Scientific and Cultural Organization (UNESCO) declared the Ebro deltaic plain World Biosphere Reserve. Moreover, a total of 7.736 ha are protected under the Spanish Natural Park figure (including coastal lagoons, freshwater springs, bays and adjacent coastline). The Ebro Delta shows a great diversity of habitats and stands out by its faunal (ornithological and ichthyological) and halophilic floral composition; since many of those species are endemic (Ibáñez *et al.*, 1999). Different human activities occur in this area *e.g.* tourism, shooting, recreational and commercial fishing; but the most important, likewise the whole basin, is agriculture with *ca*. 21.000 ha of Delta dedicated to rice fields. As a consequence the entire Delta-Estuary complex is under permanent anthropogenic pressures. Therefore, its conservation should be a priority task.

The Ebro River flows into the Mediterranean Sea and forms a Type 4-highly stratified or salt wedge estuary (Hansen and Rattray, 1966; Muñoz and Prat, 1989; Muñoz, 1990; Ibáñez, 1993; Ibáñez *et al.*, 1997). The specific characteristics of salt wedge estuaries are: (i) river discharge controls marine intrusion due to the low tidal range (usually tidal amplitude is less than 2 m); (ii) weak mixing forces enhance strong water column stratification and promote the formation of a salt wedge landwards; (iii) vertical profile of density and salinity shows an abrupt change from surface to bottom, friction between fresh and saltwater layers forms a narrow interface called halocline; (iv) isohalines are arranged horizontally and (v) if sediment load is high, a Delta may be formed. The Ebro Estuary is about 32 km long, with a mean width of 240 m and a mean water depth of 7 m. The tidal range in this area is low, *ca*. 20 cm (Cacchione *et al.*, 1990), and the low tidal amplitude promotes the formation of the salt wedge, which is controlled by river discharge (advance, retreat and permanence). Summarizing, salt wedge dynamics in the Ebro Estuary, when river flow exceeds 350-400 $m^3\ s^{-1}$, the salt wedge is pushed seawards and the estuary works as a river, this event was denominated 'fluvial estuarine stretch' by Ibáñez, 1993; on the contrary, the salt wedge reaches its maximum landwards (*ca*. 30-32 km from the river mouth) with flows lower than 100 $m^3\ s^{-1}$ (Ibáñez, 1993; Ibáñez *et al.*, 1997). Regarding anthropogenic activities causing environmental stress on the lower Ebro river and its estuary, the most remarkable impacts are on one hand nutrient enrichment, not only in river water because of input of agricultural and urban sewage effluents on whole basin (Terrado *et al.*, 2006; Falcó *et al.*, 2010), but also in the marine plume (Sierra *et al.*, 2002; Falcó *et al.*, 2010); on the other hand, damming and water regulation cause the worst negative effects on estuarine ecology such as sediment loss (Ibáñez *et al*., 1997), that led to changes in bottom granulometry in addition to habitat loss and Delta regression. Water regulation buffers seasonality of river discharges that are homogenized throughout the year (Muñoz and Prat, 1989). Thus, the only variations occurring in the flow are directly related to hydroelectric power generation or agricultural usage (Muñoz and Prat, 1989; Ibáñez *et al*., 1996, 2008; Sierra *et al*., 2004; Falcó *et al*., 2010). These artificial flows assure the presence of the salt wedge practically in the same position for long periods (Ibáñez *et al*., 1995; Sierra *et al*., 2002; Falcó *et al*., 2010). Additionally, water quality below the halocline gets worse due to different factors: low water renewal rate, the chemical reactions at the sediment surface releasing nutrients, and the deposition of materials; accentuating eutrophication and oxygen depletion through microbial consumption of the DO (Largier 1993; Pierson *et al*., 2002).

2.5. Thesis justification

This PhD thesis is the result of a three years study focused on the ecology of benthic macroinvertebrate community inhabiting the Ebro Estuary, a Mediterranean salt wedge estuary. Research on macroinvertebrate communities is interesting because they integrate information about the functioning of the whole ecosystem as they play essential ecological roles at different ecological scales. Moreover, research focused on estuaries, and more concretely on salt wedge type, needs to be investigated due to the scarce information available.

As already mentioned, regardless of the ecological and socio-economic relevance of estuaries, they have received less attention from limnologist and oceanographers who usually center their research in freshwater or fully marine ecosystems, respectively. Probably, this is because estuaries are transitional ecosystems between rivers and seas and this ‘transitional’ condition implies certain indolence from purist researchers. Regarding estuarine research, the majority of studies focused on macroinvertebrate or other biological communities are conducted on well-mixed temperate estuaries, where longitudinal gradients are well established. Despite the singularities of salt wedge estuaries, research on their biological communities is still neglected, although this type of estuaries is well-represented along microtidal coasts worldwide (as it is shown in the '*2.1 Estuaries: Definition and Classification*' section).

During the whole research period (mainly, scientific articles and thesis manuscript writing) that led to this PhD, bibliographic searches performed on this topic produced only a few results. In fact, most of scientific papers related to salt wedge estuaries have been focused primarily on hydrological research paying no attention to biological communities. In the case of the Ebro Estuary, in spite of constituting the perfect frame for that purpose, due to its heterogeneity and ecological relevance, it showed the same tendency, being extensively studied in relation to its hydrology and salt wedge dynamics (*e.g*. Ibáñez, 1993; Ibáñez *et al*., 1997, 1999; Sierra *et al*., 2002, 2004; Falcó *et al*., 2010, among others). Regarding biological communities, only a few studies performed in the Delta-Estuary complex, provide some 'outdated' information about biological communities (fishes and macroinvertebrates) (*e.g*. De Sostoa, 1983; Muñoz, 1990; Capaccioni-Azzati and Martín, 1992; Muñoz and Prat, 1994; Ibáñez *et al*., 1995; Martín *et al*., 2000). In recent years, the increased interest of studying the ecology of the lower Ebro River and its Delta-Estuary complex increased, together with the WFD impulse, and the increasing environmental concerning, has resulted in several scientific papers and PhD thesis published *e.g*. Rovira *et al*., 2009, 2012b; Cid, 2010; Nebra *et al*., 2011, 2014; Rovira, 2013; Rodríguez-Climent *et al*., 2013; Rodríguez-Climent, 2014.

Finally, recent changes in environmental condition of the Ebro River (mainly, nutrients loads in the whole basin and water regulation) have implied important changes on the estuarine environmental condition, its ecological dynamics and therefore on biological communities. This fact implied that 90's decade studies become obsolete with regards species composition and ecological dynamics. As a consequence, the study of the current macroinvertebrate community of the Ebro Estuary, is an imperative requirement to suggest assessment measures (in combination with other BQEs) to prevent or reduce the environmental decline of the Ebro Estuary.

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3 OBJECTIVES

The main objective of the present PhD thesis was to analyze the ecology of the benthic macroinvertebrate community from the Ebro Estuary in order to evaluate its potential use as biological indicator of highly stratified Mediterranean estuaries. To achieve this goal the study of the macroinvertebrate community was carried out at a high level of taxonomic resolution and its spatiotemporal dynamics in relation with the estuarine environmental gradients was assessed. An exhaustive environmental description of benthic condition of the Ebro Estuary was done, including water physico-chemistry, grain size characterization and total organic estimation in sediments. Furthermore, due to the relevance of river discharge on salt wedge dynamics and therefore on estuarine benthic ecology, a comparison between current salt wedge dynamics and past near natural conditions was done using historical data available at the Ebro basin authority database. Finally, the bioindicator potential of macroinvertebrates to assess the ES according to the WFD criteria was examined throughout the analysis of the response of macrozoobenthos based metrics to the main human pressures in the Ebro estuary, nutrient enrichment and altered flow regime.

This thesis is structured in three chapters, each one corresponding to a scientific paper either published or submitted for its publication in peer reviewed journals. The outcomes of each paper allow the achievement of specific objectives (see below the specific objectives of each chapter), wheras the thre papers altogether allow reaching the main goal. Two chapters are already published and one is currently under review (submitted to *Marine Environmental Research).* Although the second paper is not yet published, the chapter ordination was chosen for a better comprehension and to follow the conclusions presented in this thesis. More concretely, each chapter aimed to respond the following issues:

Chapter I

This chapter covers the detailed description of benthic macroinvertebrate community of the Ebro Estuary at a high level of taxonomic resolution, together with the environmental characterization of the benthic condition of the estuary. Thus, this chapter provides essential information about the Ebro Estuary; more specifically, this chapter pursued the following specific objectives:

- Description and characterization of the benthic macroinvertebrates community from the Ebro Estuary with regard to species composition, abundance and community structure (diversity, equitability and trophic structure).
- Description and characterization of the benthic conditions of the Ebro estuary regarding hydromorphological and physico-chemical parameters.
- Identification of spatial and temporal patterns in the macroinvertebrate community.
- Identify the main environmental drivers explaining the observed patterns.
- Identify the most representative species-complex inhabiting the Ebro Estuary and their comparison with other temperate estuaries.

Chapter II

The second chapter is focused on the analysis of macrofauna response to environmental constraints. Concretely, this chapter analyzes how environmental factors are determining the distribution of the Ebro Estuary macroinvertebrate assemblages identified in the first chapter; more specifically, this chapter pursued the following specific objectives:

- Analyze the degree of association of each species with the environmental parameters and describe the main abiotic factors affecting benthic communities in this type of estuary.
- Study the relationship between the species-environment associations and the observed spatial patterns.
- Describe the distribution pattern, along spatial and temporal scales, that best fits with the Ebro Estuary macroinvertebrate community.
- Based on the observed gradients and patterns of the benthic macroinvertebrates, discuss on the adequacy of consider the Ebro estuary as an ecotone, from an ecological boundary perspective.

Chapter III

Finally, the third chapter purpose is to set a baseline for a suitable ES assessment of Ebro Estuary and, by extension, for Mediterranean salt wedge estuaries, dealing with the main anthropogenic pressures affecting Mediterranean basins such as organic enrichment and water regulation issues. This chapter pursued the following specific objectives:

- Development of synthetic indices describing the most important human pressures in the Ebro estuary influencing the ES, organic and nutrient enrichment and salt wedge dynamics alteration.
- Evaluate the performance of existing BIs developed under WFD criteria throughout the analysis of their responses to the developed pressure indices.
- Evaluate the performance of macroinvertebrates based metrics (individual metrics from the existing BIs and other common metrics used to assess the ES of surface waters) throughout the analysis of their responses to the developed pressure indices.
- Definition of potential macroinvertebrates based metrics to be used in a future BI to assess the ES of Mediterranean highly stratified estuaries.

4 CHAPTERS

CHAPTER I

Community structure of benthic macroinvertebrates inhabiting a highly stratified Mediterranean estuary

Nebra A, Caiola N, Ibáñez C.

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Community structure of benthic macroinvertebrates inhabiting a highly stratified Mediterranean estuary

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ABSTRACT

The community composition and spatial distribution of benthic macroinvertebrates were studied along the Ebro estuary, a highly stratified estuary located in the NE Iberian Peninsula. During the last decade the oligotrophication process occurring in the lower Ebro River and its estuary has allowed a complex benthic macroinvertebrate community to become established; these results contrast with the poor community found there in the early nineties. A total of 214 taxa were identified, and polychaetes dominated the community both in abundance and species richness. The results showed spatial differences in the structure and composition of macroinvertebrates, which suggests that there are two distinct communities along the estuary. Each community was found in a specific stretch (upper and lower estuary) in function of the presence of the salt wedge. The macrobenthos of the upper estuary was dominated by freshwater taxa, but some euryhaline species were also found. The lower estuary showed a marine community typical of shallow Mediterranean environments. The transition between these two communities fits an ecotone model. The highest abundances, richness and diversities were recorded at the lower estuarine stations, especially those closer to the river mouth, whereas the lowest values corresponded to the stations adjacent to the tip of the salt wedge.

***Keywords*:** benthic macroinvertebrates, community structure, distribution patterns, salt wedge, highly stratified estuary, Ebro estuary.

1. INTRODUCTION

The Ebro estuary (NE, Iberian Peninsula) is a salt wedge or highly stratified estuary (Hansen and Rattray, 1966; Ibáñez *et al.*, 1997). The specific characteristics of salt wedge estuaries are: (i) the river discharge controls the marine intrusion mainly due to the low tidal range (usually with an amplitude less than 2 meters); (ii) weak mixing effects cause the water column to be strongly stratified; (iii) the vertical profile of density and salinity shows a marked change with a narrow interface between layers called haloclines; and (iv) the isohalines are arranged horizontally. Although this kind of estuary is well represented along microtidal coasts worldwide (*e.g.* the Mediterranean Sea and the Gulf of Mexico), there is little research on the macroinvertebrate communities that inhabit them. The Ebro estuary has been extensively studied in relation to its hydrology and salt wedge dynamics (*e.g.* Ibáñez *et al.*, 1997, 1999; Sierra *et al.*, 2002, 2004), and some benthic communities of adjacent areas have also been studied (Capaccioni-Azzati and Martín, 1992; Martín *et al.*, 2000). A few studies have focused on the biota of the estuary (*e.g.* Rovira *et al.*, 2009), but only one includes a brief description of its macroinvertebrate community (Ibáñez *et al.*, 1995). Furthermore, this study was performed when the lower Ebro River and its estuary were under severe eutrophic conditions, very different from the present situation. Highly fluctuating estuarine systems produce strong environmental gradients, which leads to a patchy distribution of organisms that must cope with a wide variety of stresses (Morrisey *et al.*, 1992; Gray and Elliott, 2009) due to both natural and anthropogenic factors (McLusky, 1999; Dauer *et al.*, 2000; Dauvin, 2007; Elliott and Quintino, 2007). Therefore, the benthic invertebrate communities, often used as indicators of the health of an ecosystem, can be very similar in both impacted and non-disturbed estuarine systems. This therefore increases the difficulty of distinguishing natural from anthropogenic stresses. The

Estuarine Quality Paradox concept (Dauvin, 2007; Elliott and Quintino, 2007) refers to the challenge of detecting anthropogenic impacts in naturally stressed systems using biological assessment methods. In Mediterranean regions and particularly in the Iberian Peninsula, besides the spatial fluctuation there is strong temporal environmental variability in the aquatic systems due to limited water availability during part of the year (Caiola *et al.*, 2001; Ferreira *et al.*, 2007a). This variability is exacerbated by a long history of human-induced pressures that have led to serious changes in the natural ecological cycles of estuarine systems from this region (Ferreira *et al.*, 2007b). Therefore, identifying the factors that structure the benthic macroinvertebrate community of the Ebro estuary will provide a clearer understanding of the ecological functioning of the system both at the spatial and temporal scales. Moreover, it will help to interpret the recent changes in the estuarine system observed during the last two decades (Ibáñez *et al.*, 2008). Therefore, this study establishes a robust basis so that macroinvertebrates can be used as indicators of the ecological status of the Ebro estuary.

The purpose of this study was to examine the macroinvertebrate community of the Ebro estuary with regard to species composition, community structure and distribution patterns along spatial and temporal scales and to describe the main abiotic factors affecting benthic communities in this type of estuary.

2. MATERIALS AND METHODS

2.1 Study area

The study was conducted in the Ebro estuary (40º43’10’’N, 0º40’30’’E) located in the NE of the Iberian Peninsula (Catalonia, Spain) (Fig.1). The Ebro is 910 km long and has a drainage area of 85,362 km^2; it is the Spanish river with the highest mean annual flow and one of the most important tributaries to the Mediterranean Sea. The main land use in the basin is agriculture with more than 10,000 km^2 of irrigation, corresponding to approximately 90% of the water usage in the basin (Ibáñez *et al.*, 2008). The whole basin is strongly regulated by nearly 190 dams (Batalla *et al.*, 2004). These affect the mean annual flow, which has decreased greatly since the beginning of the century to the present (Ibáñez *et al.*, 1996). The Ebro estuary is highly stratified (30 km long, 240 m mean width and 6-8 m mean depth) and the microtidal amplitude of the Mediterranean Sea, about 20 cm (Cacchione *et al.*, 1990), promotes the formation of a salt wedge. The river discharge controls the salt wedge dynamic (advance, retreat and permanence): when the flow exceeds 350-400 $m^3 s^{-1}$ the salt wedge is pushed from the river channel, and the salt wedge reaches its maximum distance upstream (30-32 km from the river mouth) with flows lower than 100 $m^3 s^{-1}$ (Ibáñez *et al.*, 1997).

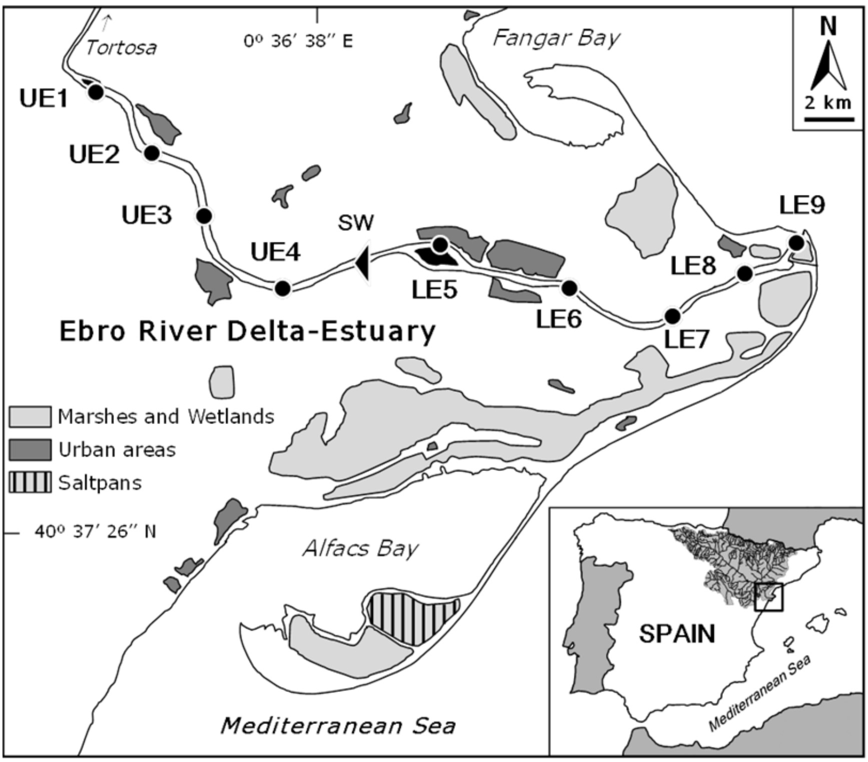

Figure 1. Location of the Ebro estuary and its deltaic plain showing the nine sampling stations. UE, upper estuary stations; LE, lower estuary stations; SW, position of the salt wedge tip.

2.2 Sampling design and laboratory procedures

Nine sampling stations were established in order to cover the whole estuarine stretch of the Ebro River (Fig. 1). Each station was sampled seasonally (summer 2007 to spring 2008). On each sampling occasion, three sediment samples were collected using a Ponar grab (0.046 m^2). The samples were washed in situ through a 0.5-mm mesh sieve to separate macroinvertebrates from sediment, and the organisms retained were immediately fixed with buffered 10% formalin. Later in the laboratory, all macroinvertebrates were sorted, counted and identified under a stereomicroscope to the lowest possible taxonomic level. Two sediment aliquots of 30 g and 200 g were taken from each grab and stored at -20ºC to estimate the total organic matter (TOM) with the loss on ignition method following Kristensen and Andersen (1987), and grain-size characterization according to Holme and McIntyre (1984). Bottom water samples were collected at each station with a water pump, preserved on ice in the absence of light, transported to the laboratory and stored at -20ºC until analysis. Posterior processing included estimating the total chlorophyll and pheophytin concentration using the colorimetric method (Jeffrey and Humphrey, 1975), the dissolved and total nutrient concentration (PO_4, P_T, NH_4, NO_2, NO_3, N_T and SiO_4) following Koroleff (1977) and the suspended solid concentration (Total suspended solids (TSS, mg l^{-1}) and organic suspended solids (OSS, mg l^{-1})) in compliance with the UNE-EN 872 norm (AENOR, 1996). In addition, physicochemical and hydromorphological characteristics were recorded on each sampling occasion. An YSI 556 multi-parameter probe was used to measure water temperature (°C), dissolved oxygen (mg l^{-1}), oxygen saturation (%), pH, salinity and conductivity (mS cm^{-1}). Water depth (m) was measured using a Speedtech SM-5 depth-meter sounder. Water flow velocity (m s^{-1}) was recorded with a Valeport m.001 current-meter, and water transparency was estimated using a Secchi disc. The accumulated permanence time (in days) of the salt wedge was calculated using daily mean flow values measured 40 km upstream from the river mouth (Tortosa) by counting the accumulated days before each sampling occasion with mean flow values lower than

350 m^3 s^{-1}. This data is available at the Ebro Water Authority (CHE) web site (http://www.chebro.es/).

2.3 Data analysis

The following community descriptive parameters were calculated for each station and season (n=36): total abundance (N), density (D, ind m^{-2}), richness (S), Shannon-Wiener's diversity index (H', as $\log_2$), Margalef index (d), Simpson dominance index (1-λ') and Pielou's evenness index (J'). In addition, species were classified with the constancy index (Dajoz, 1971) into five categories according to the number of stations in which any given taxa was found in relation to the total number of stations: constant (>76%), very common (51-75%), common (26-50%), uncommon (13-25%) and rare (<12%). Each species was classified into feeding guilds based on the available literature. The feeding guilds included deposit feeders (DF), grazers (G), omnivores (O), parasites (Pa), predators (Pr) and suspension feeders (SF). Appendix 1 provides a list of the taxa, together with their feeding guild, that are mentioned in the text. Non-parametric multivariate techniques were used as described by Field *et al.* (1982) to identify the possible macroinvertebrate communities. A similarity matrix was computed using the Bray-Curtis coefficient (Legendre and Legendre, 1998) after the four root transformation was applied to the abundance data to downweight the contribution of the most abundant taxa to the similarity (Clarke and Warwick, 2001). All the other statistical analyses were performed using the different routines available in the Multivariate Ecological Research software package PRIMER V6 (Clarke and Gorley, 2006). The stations and taxa were ordered using non-metrical multidimensional scaling (MDS) (Clarke and Warwick, 2001). A similarity percentage analysis (SIMPER) that examines the contribution of each variable to the average resemblances between sample groups was performed. This analysis was also used to identify taxa that contributed to dissimilarity among stations and estuary domains that were pre-determined by ordination analysis. Differences in the community composition were identified using the 1-way analysis of similarities test (ANOSIM) that hypothesizes for differences between groups of samples (defined a priori) through randomization methods on a resemblance matrix. Finally, the relationship between the community structure and environmental variables was investigated with the BIOENV routine, which maximizes a rank correlation (Spearman's coefficient) between resemblance matrices derived from biotic and environmental data, iterating for all possible combinations of environmental variables (Clarke and Warwick, 2001). A Spearman's coefficient value close to 0 indicates a weak relation between the community and environmental variables, whereas a value close to 1 indicates that the environmental variables selected explain the community structure.

3. RESULTS

3.1 Water and sediment features

The Ebro estuary has a sand dominated bottom and a relatively low TOM percentage in both the upper (UE) and lower (LE) parts and throughout the entire year (Table 1). During the study period the salt wedge was only found in the lower estuary stations. At these stations, the accumulated permanence time was different in each season: 55, 143, 257 and 344 days respectively for summer, autumn, winter and spring. The null point (the tip of the salt wedge) was located between UE4 and LE5 in all sampling periods. Nutrient concentrations were higher in the upper estuary stretch (Table 1) except for the ammonia, nitrite, phosphate and silicate concentrations in spring and total phosphorous in summer. The chlorophyll concentrations showed marked differences between the upper and lower estuary; the UE stretch had the highest values during winter and spring, whereas the maximum values in the LE stretch were in summer/autumn. Levels of total pheophytin were lower in the UE stretch except for during the two last seasons. The UE stretch always had seasonal mean water flow velocities higher than the LE stretch. The values of TSS and OSS were higher in the LE stretch in summer, autumn and winter, whereas in spring the UE stretch showed the maximum values.

Table 1. Sediment characteristics and water physicochemical parameters (seasonal mean±standard deviation, n=4) in the two different stretches. TOM, total organic matter in sediment; Transp., transparency; DO, dissolved oxygen; Cond., conductivity; Sal., salinity; TDS, total dissolved salts; TSS, total suspended solids; OSS, organic suspended solids.

	Upper Estuary				Lower Estuary			
	Summer	**Autumn**	**Winter**	**Spring**	**Summer**	**Autumn**	**Winter**	**Spring**
Mud (%)	11.31 ± 14.59	11.31 ± 14.59	21.08 ± 28.43	1.53 ± 2.08	15.89 ± 10.90	15.89 ± 10.90	25.27 ± 20.78	6.51 ± 5.75
Sand (%)	73.35 ± 23.41	73.35 ± 23.41	57.93 ± 33.30	88.78 ± 16.81	79.84 ± 13.01	79.84 ± 13.01	74.32 ± 21.06	85.36 ± 18.10
Gravel (%)	15.34 ± 27.22	15.34 ± 27.22	20.99 ± 36.79	9.69 ± 17.68	4.27 ± 6.26	4.27 ± 6.26	0.41 ± 0.29	8.13 ± 12.52
TOM (%)	2.67 ± 1.03	2.67 ± 1.03	2.89 ± 1.11	2.45 ± 1.88	4.03 ± 1.22	4.03 ± 1.22	4.36 ± 1.59	3.70 ± 2.21
Depth (m)	3.50 ± 1.73	4.25 ± 1.89	3.75 ± 2.22	4.25 ± 2.06	6.00 ± 1.41	6.80 ± 1.48	6.00 ± 1.58	6.00 ± 1.58
Velocity (m s^{-1})	0.13 ± 0.06	0.17 ± 0.05	0.14 ± 0.05	0.42 ± 0.10	0.06 ± 0.04	0.10 ± 0.09	0.05 ± 0.05	0.25 ± 0.21
Transp. (m)	2.40 ± 0.71	2.68 ± 0.78	1.98 ± 0.73	2.21 ± 0.22	2.37 ± 0.27	1.89 ± 0.23	1.88 ± 0.13	1.60 ± 0.22
T (ºC)	24.26 ± 0.40	22.80 ± 0.07	11.12 ± 0.36	16.32 ± 0.14	22.07 ± 0.19	22.30 ± 0.83	13.27 ± 0.04	15.30 ± 0.51
DO (mg l^{-1})	7.85 ± 0.47	7.89 ± 1.22	13.82 ± 0.69	7.94 ± 0.69	5.25 ± 1.22	6.00 ± 2.4	10.32 ± 0.83	6.72 ± 2.11
DO (%)	94.00 ± 6.26	92.00 ± 14.25	126.28 ± 7.02	81.30 ± 7.24	74.10 ± 17.64	84.78 ± 33.61	123.90 ± 10.11	74.36 ± 16.52
Cond.(mS cm^{-1})	0.95 ± 0.01	1.37 ± 0.00	1.12 ± 0.03	1.04 ± 0.00	51.27 ± 0.53	51.51 ± 0.71	43.21 ± 0.50	25.00 ± 19.78
Sal.	0.47 ± 0.01	0.72 ± 0.00	0.77 ± 0.02	0.62 ± 0.00	35.97 ± 0.31	35.98 ± 0.40	36.89 ± 0.45	20.02 ± 16.27
TDS (g l^{-1})	0.62 ± 0.01	0.93 ± 0.00	0.99 ± 0.02	0.81 ± 0.00	35.30 ± 0.27	35.30 ± 0.34	36.19 ± 0.39	20.09 ± 15.95
Chlorophyll (µg l^{-1})	0.09 ± 0.06	1.16 ± 0.24	1.07 ± 0.91	2.83 ± 2.82	1.01 ± 0.80	2.83 ± 1.06	0.79 ± 0.23	0.69 ± 0.41
Pheophytin (µg l^{-1})	0.05 ± 0.02	1.06 ± 0.10	1.00 ± 0.55	3.43 ± 2.28	0.31 ± 0.18	1.38 ± 0.32	0.66 ± 0.26	0.87 ± 0.50
pH	8.20 ± 0.06	8.25 ± 0.05	7.89 ± 0.09	8.00 ± 0.02	7.98 ± 0.06	8.28 ± 0.15	7.94 ± 0.05	7.83 ± 0.11
PO4 (mg l^{-1})	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.01 ± 0.01	0.03 ± 0.01
P_T (mg l^{-1})	0.08 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.11 ± 0.02	0.05 ± 0.02	0.02 ± 0.02	0.04 ± 0.02
NH_4 (mg l^{-1})	0.02 ± 0.02	0.04 ± 0.03	0.02 ± 0.02	0.19 ± 0.14	0.05 ± 0.02	0.09 ± 0.12	0.05 ± 0.02	0.20 ± 0.30
NO_2 (mg l^{-1})	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.01 ± 0.00	0.04 ± 0.01
NO_3 (mg l^{-1})	2.08 ± 0.16	1.85 ± 0.34	3.52 ± 0.05	4.45 ± 0.15	0.04 ± 0.03	0.04 ± 0.02	0.10 ± 0.02	3.26 ± 0.89
N_T (mg l^{-1})	2.42 ± 0.08	2.43 ± 0.04	3.52 ± 0.05	5.37 ± 0.08	0.28 ± 0.07	0.20 ± 0.12	0.10 ± 0.02	4.39 ± 1.13
SiO_4 (mg l^{-1})	1.89 ± 0.06	0.85 ± 0.17	1.01 ± 0.15	1.21 ± 0.13	0.42 ± 0.48	0.47 ± 0.33	0.17 ± 0.16	1.28 ± 0.31
TSS (mg l^{-1})	3.05 ± 0.98	3.56 ± 2.68	2.91 ± 1.66	14.69 ± 11.25	20.60 ± 2.17	24.99 ± 3.78	16.91 ± 32.53	5.84 ± 3.51
OSS (mg l^{-1})	1.94 ± 0.52	1.47 ± 0.81	0.99 ± 0.21	3.20 ± 2.31	4.75 ± 1.11	4.71 ± 1.02	1.76 ± 2.27	1.52 ± 0.75
OSS (%)	66.49 ± 18.14	46.07 ± 8.76	43.07 ± 21.78	23.19 ± 2.59	22.92 ± 3.70	18.72 ± 1.53	27.34 ± 12.39	27.04 ± 2.95

3.2 Macroinvertebrate abundance, taxa richness and diversity

During one year of seasonal sampling in the Ebro estuary a total of 21,805 individuals were collected belonging to 214 different taxa that comprised 151 species, 115 families, 57 orders, 20 classes and 9 phyla (Supplementary material Appendix 1). Annelida was the dominant phylum and accounted for 71.07% of the total abundance. Polychaeta and Oligochaeta contributed with 49.64% and 21.42% respectively. Spionidae was the most abundant family (28.56%) due to the contribution of the most dominant species *Streblospio benedicti* (24.10% of the total abundance). Another dominant phylum was Arthropoda, which contributed 15.56% of the total abundance, with Malacostraca accounting for 10.37% of the total abundance. Mollusca was the third most abundant phylum with 12.09% of the total abundance, and Bivalvia contributed 10.61% of the total abundance. In terms of species richness, Polychaeta contributed with 49 different taxa (40 species) and Bivalvia with 37 taxa (32 sp), followed by Gastropoda with 29 taxa (18 sp) and Insecta with 24 taxa (14 sp). Applying Dajoz's constancy index (considering the 9 stations), 1% of the taxa were found constant, 8% very common, 27% common, 20% uncommon and 44% were rare. Applying the constancy index to UE stations revealed that 9% of the taxa were constant, 14% very common, 19% common, 58% were uncommon and no taxa were rare; whereas in the LE stretch 22% of the taxa were constant, 16% very common, 20% common and 42% were uncommon.

Total density values throughout seasons ranged from 216 to 20,022 ind m^{-2} (Table 2). The highest densities were found at the mouth (station LE9) due to the high abundance of the polychaete *S. benedicti*. Intermediate densities were found in the uppermost stations UE1 and UE2 with a large contribution of Tubificidae and the introduced bivalve *Corbicula fluminea*. The lowest densities corresponded to stations UE3, UE4 and LE5 in the middle part of the estuary. Station LE9 had the highest richness values with a maximum of 69 taxa and an annual mean value of 48 taxa; other stations located near the river mouth (LE8 and LE7) also reached high values of richness, whereas stations UE3, UE4 and LE5 showed the lowest richness values

(Table 2). Diversity indices showed the same tendency as density and richness, with low values at stations located near the limit of the salt wedge (Table 2). In terms of the trophic structure, the deposit feeders (32%), suspension feeders (29%) and predators (17%) were the dominant feeding guilds in the entire estuary. The contribution of the different feeding guilds in the UE stretch was: deposit feeders (38%), predators (22%), grazers (19%), suspension feeders (14%), omnivores (5%) and parasites (3%). The trophic structure of the LE stretch was dominated by suspension feeders (35%) and deposit feeders (30%).

3.3 Analysis of benthic macroinvertebrate communities

Two different communities were determined according to the ordination of stations and taxa of the MDS analysis based on macroinvertebrate abundance. The ordination showed two definite groups of sampling stations: those corresponding to the upper estuary (UE) and lower estuary (LE) respectively (Fig. 2). The UE group (UE1-UE4) included stations located in the upper estuary stretch and corresponded to a freshwater community, whereas the second group comprised the lower estuary stations (LE5-LE9) and had a community with a large marine influence. In addition, we also applied the MDS analysis considering lower taxonomic categories *e.g.* genus and family; the results obtained showed the same grouping of stations regardless of the taxonomic level employed in the ordinations. Significant differences in community composition were found between these two groups (ANOSIM r: 0.891, $p<0.001$). Significant differences were also found among stations (ANOSIM global r: 0.694, $p<0.001$) except for the following pairs: UE1-UE3, UE3-UE4, UE4-LE5, LE5-LE6, LE6-LE7, LE6-LE8, LE7-LE8, LE7-LE9 and LE8-LE9, $p>0.05$ (Table 3).

Table 2. Community descriptive parameters for each sampling station and season. N, total abundance per 0.14 m^2; D, density (ind m^{-2}); S, richness; H'(log_2), Shannon-Wiener diversity index; d, Margalef index; 1-λ', Simpson's index; J', Pielou's evenness; DF (%), deposit feeders; G (%), grazers; O (%), omnivores; Pa (%), parasites; Pr (%), predators; SF (%), suspension feeders. See Figure 1 for sampling station codes.

			Community indices					**Trophic structure**					
Station	**Season**	**Density**	**S**	**H'(log_2)**	**d**	**1-λ'**	**J'**	**DF**	**G**	**O**	**Pa**	**Pr**	**SF**
UE1	Summer	2792	11	1.96	1.68	0.67	0.57	54.55	0.00	9.09	9.09	9.09	18.18
UE2	Summer	4820	25	2.16	3.69	0.59	0.47	44.00	16.00	4.00	8.00	12.00	16.00
UE3	Summer	830	6	2.02	1.05	0.72	0.78	33.33	0.00	16.67	16.67	0.00	33.33
UE4	Summer	491	3	1.45	0.47	0.61	0.91	0.00	0.00	33.33	0.00	0.00	66.67
LE5	Summer	216	4	0.63	0.88	0.19	0.31	50.00	25.00	25.00	0.00	0.00	0.00
LE6	Summer	1457	7	1.03	1.13	0.32	0.37	57.14	0.00	14.29	0.00	0.00	28.57
LE7	Summer	2670	23	2.52	3.72	0.67	0.56	47.83	4.35	8.70	0.00	4.35	34.78
LE8	Summer	2583	23	3.18	3.74	0.82	0.70	56.52	0.00	8.70	0.00	8.70	26.09
LE9	Summer	11212	32	0.48	4.22	0.09	0.10	25.00	3.13	15.63	0.00	18.75	37.50
UE1	Autumn	491	13	2.79	2.84	0.80	0.75	30.77	23.08	7.69	0.00	23.08	15.38
UE2	Autumn	2403	23	3.15	3.79	0.80	0.70	34.78	13.04	4.35	0.00	34.78	13.04
UE3	Autumn	1335	8	1.29	1.34	0.42	0.43	62.50	0.00	0.00	0.00	25.00	12.50
UE4	Autumn	505	12	2.57	2.59	0.78	0.72	58.33	0.00	16.67	0.00	16.67	8.33
LE5	Autumn	2020	11	2.00	1.77	0.60	0.58	54.55	0.00	0.00	0.00	0.00	45.45
LE6	Autumn	599	21	3.94	4.53	0.93	0.90	52.38	0.00	4.76	4.76	23.81	14.29
LE7	Autumn	2316	31	3.19	5.20	0.75	0.64	35.48	0.00	6.45	3.23	16.13	38.71
LE8	Autumn	2648	36	2.84	5.93	0.66	0.55	30.56	5.56	5.56	2.78	8.33	47.22
LE9	Autumn	13485	69	2.84	9.03	0.68	0.47	31.88	1.45	7.25	7.25	17.39	34.78
UE1	Winter	9632	21	0.79	2.78	0.18	0.18	33.33	19.05	0.00	4.76	23.81	19.05
UE2	Winter	4906	17	1.57	2.45	0.41	0.38	47.06	17.65	11.76	5.88	5.88	11.76
UE3	Winter	981	4	1.05	0.61	0.48	0.53	50.00	0.00	0.00	0.00	25.00	25.00
UE4	Winter	1522	6	0.99	0.93	0.34	0.38	66.67	0.00	0.00	0.00	16.67	16.67
LE5	Winter	2756	19	2.64	3.03	0.78	0.62	47.37	5.26	0.00	5.26	5.26	36.84
LE6	Winter	4278	27	3.30	4.07	0.86	0.69	51.85	3.70	7.41	3.70	14.81	18.52
LE7	Winter	6934	62	3.81	8.88	0.83	0.64	46.77	3.23	4.84	3.23	16.13	25.81
LE8	Winter	3413	48	4.04	7.63	0.87	0.72	50.00	0.00	8.33	2.08	14.58	25.00
LE9	Winter	20022	58	1.66	7.19	0.35	0.28	37.93	1.72	6.90	5.17	18.97	29.31
UE1	Spring	18319	21	0.98	2.55	0.27	0.22	44.44	22.22	0.00	5.56	11.11	16.67
UE2	Spring	5368	24	1.74	3.48	0.46	0.38	41.67	20.83	8.33	0.00	16.67	12.50
UE3	Spring	1198	9	2.42	1.56	0.78	0.76	55.56	0.00	0.00	0.00	22.22	22.22
UE4	Spring	3802	8	0.34	1.12	0.08	0.11	50.00	12.50	12.50	0.00	12.50	12.50
LE5	Spring	3629	7	0.91	0.96	0.33	0.32	42.86	0.00	14.29	14.29	0.00	28.57
LE6	Spring	1941	28	3.14	4.83	0.80	0.65	46.43	0.00	10.71	0.00	21.43	21.43
LE7	Spring	6486	59	4.15	8.53	0.88	0.71	47.46	1.69	11.86	3.39	15.25	20.34
LE8	Spring	7417	63	4.62	8.94	0.93	0.77	41.27	1.59	12.70	3.17	17.46	23.81
LE9	Spring	1876	33	3.30	5.75	0.81	0.65	48.48	0.00	6.06	3.03	21.21	21.21

Table 3. One-way ANOSIM test to compare the macroinvertebrate communities at different sampling stations. The test results are shown in the lower diagonal of the table. Significant differences between stations (P<0.05) are indicated (*). The R values are shown in bold letters in the upper diagonal of the table. See Figure 1 for sampling station codes.

	UE1	**UE2**	**UE3**	**UE4**	**LE5**	**LE6**	**LE7**	**LE8**	**LE9**
UE1		**0.708**	**0.458**	**0.552**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
UE2	0.029*		**0.719**	**0.635**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
UE3	0.057	0.029*		**0.219**	**0.917**	**0.990**	**1.000**	**1.000**	**1.000**
UE4	0.029*	0.029*	0.143		**0.302**	**0.688**	**0.849**	**0.885**	**0.880**
LE5	0.029*	0.029*	0.029*	0.114		**0.219**	**0.542**	**0.667**	**0.604**
LE6	0.029*	0.029*	0.029*	0.029*	0.143		**0.167**	**0.115**	**0.448**
LE7	0.029*	0.029*	0.029*	0.029*	0.029*	0.171		**0.000**	**0.240**
LE8	0.029*	0.029*	0.029*	0.029*	0.029*	0.200	1.000		**0.083**
LE9	0.029*	0.029*	0.029*	0.029*	0.029*	0.029*	0.086	0.229	

The BIOENV analysis showed that the combination of salinity, dissolved phosphate, total phosphorous, ammonia and the distance from the mouth have a large influence on the structure of the macroinvertebrate communities (r=0.741). The combination of salinity, dissolved phosphate, ammonia and nitrate explained the differences in taxa abundance in the upper estuary (r=0.308). However, within the community of the lower estuary, the combination of ammonia, total chlorophyll, sand percentage, the highest correlation and explained the main differences in the macroinvertebrate abundance data (r=0.681).

4. DISCUSSION

The whole Ebro estuary is dominated by sand; however, the percentage of fine deposits such as clay or mud was higher in the lower stretch due to flocculation and settling processes and low velocities recorded at the salt wedge (Sierra *et. al.*, 2002). During the study period the bottom water layer of the estuary showed important differences in physicochemical features between the lower and upper estuary stretches. We found freshwater stations (UE1-UE4) that were not exposed to marine intrusions, and saltwater stations (LE5-LE9) that were permanently exposed to marine intrusions and had a well stratified water column. At LE stations, salinity in the salt wedge decreased upstream with small fluctuations but with values always higher than 30, which evidences the weak mixing between water layers. In highly stratified estuaries the salt wedge dynamics are complex and can be explained by a combination of hydromorphological factors, such as the tide amplitude, river channel cross section and flow, and the freshwater runoff is one of the main factors determining the salt wedge regime (Ibáñez *et al.*, 1997). Nevertheless, in the lower estuary the salt wedge was present on all sampling occasions and the permanence time almost reached a complete year. Although other long periods of marine intrusion in the Ebro estuary have been recorded before (Ibáñez *et al.*, 1995), under natural conditions this period should be approximately 6 months per year (Ibáñez *et al.*, 1997). These conditions of the quasi permanent presence of the salt wedge in the lower estuary stretch are exacerbated by the strong flow regulation and the almost total absence of peak flows, which leads to reduced turbulence and therefore to highly stable density-thermal stratification (Ibáñez *et al.*, 1995, 1996).

The present conditions of nutrient loading of the Ebro estuary are quite different from the past situation of eutrophication (Ibáñez *et al.*, 1995). Under eutrophic conditions, and with long periods of permanence of the salt wedge in the lower estuary at the same time, the water quality was worse below the wedge than above it due to organic matter deposition and low water renewal. This organic enrichment caused oxygen depletion through microbial consumption (Ibáñez *et al.*, 1995; Casamayor *et al.*, 2001). Recent changes in the nutrient content of the river, especially the reduction of phosphates, have reduced the primary production in the upper layer, whereas in the lower layer it has increased due to higher light penetration (Falco *et al.*, 2010); thus, the hypoxic conditions in the lower layer have decreased (Casamayor *et al.*, 2001; Ibáñez *et al.*, 2008).

Under the present oligotrophication process, the long periods of salt wedge permanence ensure the stability of the water column, which allows the complexity of the benthic communities to increase, as suggested by Sousa *et al.* (2006a). The present situation is very different to that of the early nineties, when a survey conducted in October 1992 showed an impoverished macroinvertebrate community (only seven different taxa were found) due to eutrophication, which caused severe anoxic episodes below the halocline (Ibáñez *et al.*, 1995).

The benthic macroinvertebrate community in the Ebro estuary shows considerable spatial and temporal differences, with a complex structure and composition. The multivariate analysis defined two different communities: one from the lower and one from the upper estuary stretch. In contrast, the pattern described in more mixed estuaries (Rundle *et al.*, 1998; Ysebaert *et al.*, 1998; Sousa *et al.*, 2008) supports the idea that these systems work as a continuum of overlapping communities along the salinity gradient, which fits with the ecocline boundary model suggested by Attrill and Rundle (2002). However, the weak longitudinal salinity gradient and the narrow transition zone between fresh and marine water suggest that the Ebro estuary fits much better into an ecotone model, when ecotone is defined as an area of relatively rapid change that produces a narrow ecological zone between two different and homogeneous community types (Van der Maarel, 1990).

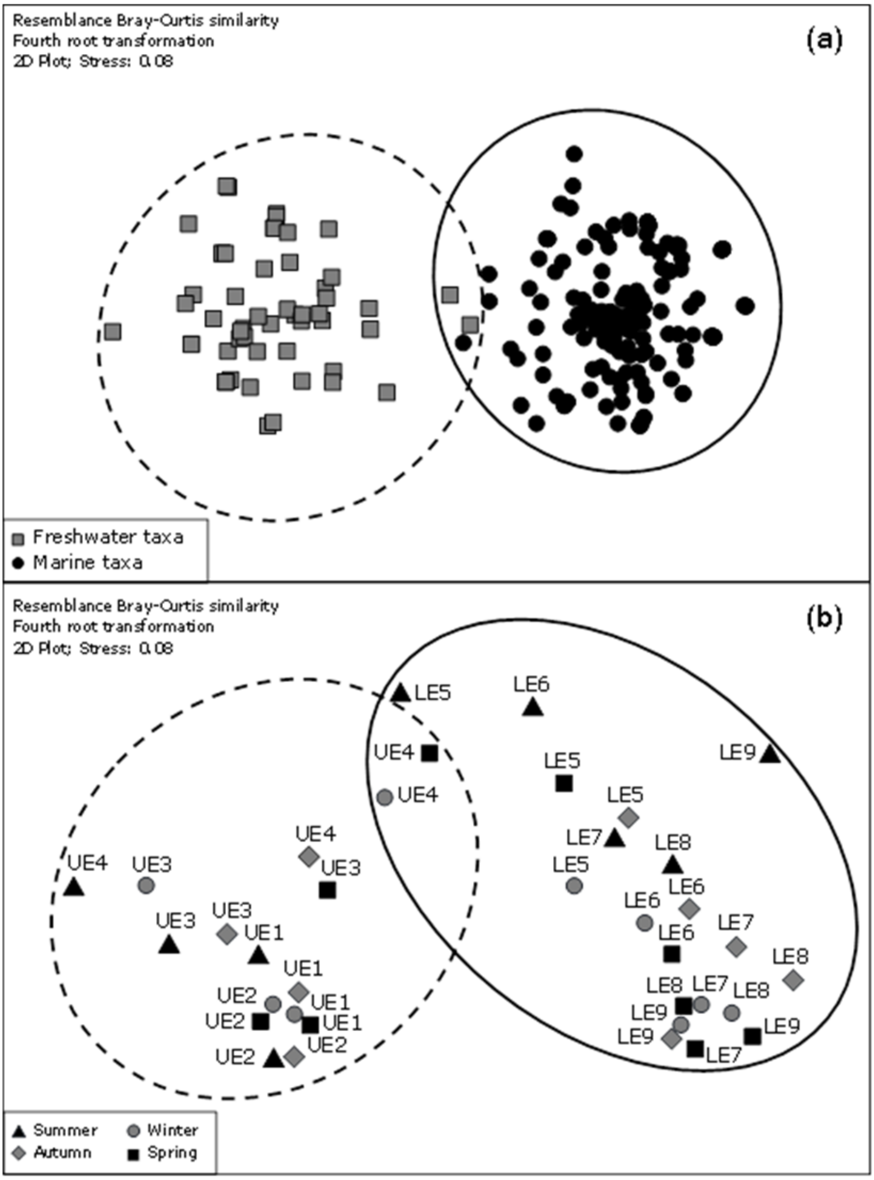

Figure 2. Two dimensional MDS plots based on Bray-Curtis similarities of fourth-root transformed macroinvertebrate abundance data: (a) ordination using inter-species resemblance matrix of nine stations; (b) ordination of the nine stations sampled in the Ebro estuary. The dashed line and the solid line encircle the freshwater and marine communities respectively. See Figure 1 for sampling station codes.

The upper stretch of the Ebro estuary was characterized by an impoverished macroinvertebrate community dominated by the non-indigenous bivalve *C. fluminea,* which tends to acquire an invasive pattern (Sousa *et al.*, 2006b), together with tolerant taxa such as Tubificidae, Naididae (Oligochaeta) and abundant Chiromidae. The amphipod *C. orientale* was well-represented in number of individuals but its presence was restricted to stations UE3 and UE4 located close to the salt wedge tip due to its euryhaline nature. The salt wedge community was dominated in terms of abundance by the Polychaeta and Malacostraca classes, followed by the phylum Mollusca. Nevertheless, in terms of richness it was dominated by molluscs, polychaetes and crustaceans in this order. This pattern was slightly different from those found in other temperate intertidal areas, where polychaetes are the most diverse group, followed by molluscs and crustaceans (Ysebaert *et al.*, 1998; Rodrigues *et al.*, 2006). Comparing our results with those from other European estuarine ecosystems we found that the Ebro estuary was colonized in its mouth area by typical marine species associated with the *Abra alba-Lagis koreni* community (colonizing fine sediments rich in organic matter) and with the *Nephtys* spp. community (colonizing sandy sediments). These two communities are widely distributed throughout European estuarine and coastal areas (Dauvin, 2000, 2007; Martín *et al.*, 2000; Van Hoey *et al.*, 2004; Puente *et al.*, 2008). In addition to these communities, we also found tolerant groups dominated by Capitellidae and Spionidae (Polychaeta), together with *Corbula gibba*, which usually colonizes disturbed areas; whereas in the upper stations close to the null point the community was dominated by eurybiontic taxa like *Hediste diversicolor*, *Perinereis cultrifera*, *Heteromastus filiformis*, *C. orientale* and *Cyathura carinata*. These species are also very common in other European estuaries and coastal areas (Marques *et al.*, 1993; Ysebaert *et al.*, 1998, 2003; Martín *et al.*, 2000; Chainho *et al.*, 2006; Rodrigues *et al.*, 2006; Sousa *et al.*, 2006a, 2008).

Currently, the Ebro estuary shows high levels of richness compared with other European estuaries (Rodrigues *et al.*, 2006). The trophic structure is well represented with six different trophic guilds. Deposit feeders, suspension feeders and predators are dominant, which suggests that different resources are available (Brown *et al.*, 2000). In the upper stretch the diversity and richness decreased seawards, with minimum values found close to the null point because the salinity fluctuation is a physiological barrier for stenohaline freshwater and marine taxa (Mannino and Montagna, 1997). However, diversity and richness at the salt wedge stations declined with increasing distance from the sea, which is a recurring tendency in mixed estuaries (Remane and Schleiper, 1971; Schlacher and Woolbridge, 1996). In the Ebro estuary this impoverishment tendency could be explained by the increase in organic matter, ammonia and total phosphorous towards the tip of the salt wedge in combination with the salinity fluctuations in the same area.

The present study provides baseline data that can be used in future ecological studies on this important Mediterranean estuarine ecosystem, as well as in comparisons with other highly stratified estuaries. Complementary studies are necessary to improve our understanding of the spatial and temporal variability of the macrozoobenthic estuarine community. This knowledge could be an important tool for conserving the biodiversity in the Ebro estuary and could be used to develop biological indices for assessing its ecological status according to the Water Framework Directive of the European Union.

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SUPPLEMENTARY MATERIAL

The following Appendix is available through the web page

http://www.icm.csic.es/scimar/supplm/sm75n3577sm.pdf

APPENDIX 1. List of the identified taxa that were found at all the stations over the entire study period. The stations where each taxon was found are also listed. See Figure 1 for sampling station codes; FG, feeding guild (see Table 2 for feeding guild codes); CI, Constancy index; Ct, constant; VC, very common; C, common; UC, uncommon; R, rare.

Taxa	**Summer**	**Autumn**	**Winter**	**Spring**	**FG**	**CI**	**CI UE**	**CI LE**
PHYLUM CNIDARIA								
Class Anthozoa								
Diadumene sp.	LE9				Pr	R		UC
PHYLUM PLATYHELMINTHES								
Class Turbellaria								
Dugesia sp.	UE2	UE2		UE2	Pr	R	UC	
Turbellaria indet.		UE2	UE1		Pr	UC	C	
PHYLUM NEMERTINA								
Class Enopla								
Nemertina indet.		LE6,7,9	LE5,6,7,8,9	LE6,7,8,9	Pr	VC		Ct
Prostoma graecense (Böhmig, 1892)		UE2	UE1,2,4	UE2	Pr	C	VC	
PHYLUM NEMATODA								
Nematoda indet.	UE1,2,3	LE9	UE1,2; LE9	UE1	Pa	C	VC	UC
PHYLUM MOLLUSCA								
Class Gastropoda								
Aplysiidae indet.	LE9	LE8			G	UC		C
Bittium reticulatum (da Costa, 1778)		LE8,9	LE7,8,9	LE7,8,9	DF	C		VC
Buccinum sp.	LE9				O	R		UC
Chrysallida sp.		LE6,7,8,9	LE5,6,7,8,9	LE5,7,8,9	Pa	VC		Ct
Eulimella polita (Verrill, 1872)		LE9			Pa	R		UC
Ferrissia clessiniana (Jickeli, 1882)	UE2	UE2		UE2	G	R	UC	
Gyraulus albus (Müller, 1774)				UE1,2,4	G	C	VC	
Haminoea navicula (da Costa, 1778)			LE6,7		G	UC		C
Hinia limata (Chemnitz, 1795)	LE9	LE9		LE9	Pr	R		UC
Hydrobia sp.	LE7		LE5	LE7	G	UC		C
Hydrobia ulvae (Pennant, 1777)			LE7		G	R		UC
Mangelia sp.		LE9			O	R		UC

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Melanella polita (Linnaeus, 1758)			LE9		Pa	R		UC
Nassarius mutabilis (Linnaeus, 1758)		LE9	LE9		O	R		UC
Nassarius pygmaeus (Lamarck, 1822)	LE7,8,9	LE9			O	C		VC
Nassarius sp.		LE7,9			O	UC		C
Neverita josephinia Risso, 1826	LE9	LE9			Pr	R		UC
Odostomia conoidea (Brocchi, 1814)				LE8	Pa	R		UC
Odostomia sp.		LE9			Pa	R		UC
Physella acuta (Draparnaud, 1805)	UE2	UE1,2	UE1,2	UE1,2	G	UC	C	
Radix auricularia (Linnaeus, 1758)			UE2		G	R	UC	
Radix peregra (Müller, 1774)	UE2		UE1	UE2	G	UC	C	
Retusa truncatula (Bruguière, 1792)	LE8	LE9	LE7	LE6,7,8	Pr	C		Ct
Rissoa sp.		LE9	LE9		G	R		UC
Rissoa ventricosa Desmarest, 1814				LE8	G	R		UC
Tricolia sp.		LE8			G	R		UC
Turbonilla lactea (Linnaeus, 1758)		LE9	LE7	LE7	Pa	UC		C
Turritella sp.		LE9			SF	R		UC
Class Bivalvia								
Abra alba (Wood, 1802)	LE9	LE5,7,8,9	LE7,8,9	LE7,8,9	SF	C		Ct
Abra nitida (Müller, 1776)		LE9	LE9	LE8	SF	UC		C
Acanthocardia echinata (Linnaeus, 1758)		LE7,8,9			SF	C		VC
Acanthocardia paucicostata (Sowerby, 1841)	LE8	LE6,7	LE7,8,9	LE7,8	SF	C		Ct
Acanthocardia tuberculata (Linnaeus, 1758)				LE9	SF	R		UC
Cerastoderma edule (Linnaeus, 1758)				LE8	SF	R		UC
Cerastoderma glaucum (Poiret, 1789)		LE8	LE5,7		SF	C		VC
Circomphalus casina (Linnaeus, 1758)	LE7	LE8	LE8		SF	UC		C
Corbicula fluminea (Müller, 1774)	UE1,2,3,4	UE1,2,3,4	UE1,2,3,4	UE1,2,3	SF	C	Ct	
Corbula gibba (Olivi, 1792)	LE7,9	LE5,6,7,8,9	LE5,6,7,8,9	LE5,6,7,8	SF	VC		Ct
Donax semistriatus Poli, 1795	LE9	LE9			SF	R		UC
Donax sp.		LE9			SF	R		UC
Donax trunculus Linnaeus, 1758	LE7				SF	R		UC
Donax venustus Poli, 1795		LE8			SF	R		UC
Dosinia lupinus (Linnaeus, 1758)		LE8,9	LE7,8,9	LE8,9	SF	C		VC

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Gari fervensis (Gmelin, 1791)		LE9			SF	R		UC
Gastrana fragilis (Linnaeus, 1758)			LE9		SF	R		UC
Glycymeris glycymeris (Linnaeus, 1758)		LE9			SF	R		UC
Laevicardium crassum (Gmelin, 1791)		LE8			SF	R		UC
Lutraria lutraria (Linnaeus, 1758)	LE9				SF	R		UC
Mactra corallina (Linnaeus, 1758)	LE7,9	LE7,8			SF	C		VC
Mactra sp.		LE9	LE9		SF	R		UC
Musculus discors (Linnaeus, 1767)	LE7,8	LE7,9	LE5,7	LE6,7,8	SF	VC		Ct
Mytilus galloprovincialis Lamarck, 1819				LE8	SF	R		UC
Pandora inaequivalvis (Linnaeus, 1758)	LE7,8	LE7,8,9	LE8,9	LE7	SF	C		VC
Pharus legumen (Linnaeus, 1758)		LE9		LE9	SF	R		UC
Pitar rudis (Poli, 1795)		LE8	LE8		SF	R		UC
Scrobicularia plana (da Costa, 1778)	LE9			LE8	SF	UC		C
Solemya togata (Poli, 1795)			LE9		SF	R		UC
Solen sp.		LE9	LE5		SF	UC		C
Spisula subtruncata (da Costa, 1778)	LE8,9	LE9	LE7	LE7,8,9	SF	C		VC
Tapes philippinarum (Adams and Reeve, 1850)			LE7	LE7	SF	R		UC
Tapes pullastra (Unspecified)	LE7	LE7,8			SF	UC		C
Tapes sp.		LE8,9	LE6,7,8,9	LE7,8,9	SF	C		Ct
Tellina albicans (Gmelin, 1791)		LE8,9	LE7,9	LE7	SF	C		VC
Tellina sp.		LE8,9	LE7,9	LE6,9	SF	C		Ct
Tellina tenuis da Costa, 1778		LE9	LE5,8,9	LE7	SF	C		Ct
Class Scaphopoda								
Antalis novemcostata (Lamarck, 1818)				LE8	Pr	R		UC
Antalis sp.		LE8,9	LE9	LE7,8	Pr	C		VC
PHYLUM ANNELIDA								
Class Hirudinea								
Helobdella stagnalis (Linnaeus, 1758)	UE2				Pr	R	UC	
Piscicola geometra (Linnaeus, 1758)	UE2				Pa	R	UC	
Class Oligochaeta								
Haplotaxidae indet.			UE3		DF	R	UC	
Lumbricidae indet.	UE2			UE1	DF	UC	C	

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Naididae indet.	UE1,2	UE1,2,3	UE1,2,4; LE5	UE1,2,3,4	DF	VC	Ct	UC
Tubificidae indet.	UE1,2,3; LE5	UE1,2,3,4	UE1,2,3	UE1,2,3	DF	VC	Ct	UC
Class Polychaeta								
Ampharete grubei Malmgren, 1865	LE6,7,8	LE6,7,8,9	LE6,7,8,9	LE6,7,8	DF	C		Ct
Aricidea sp.	LE8,9	LE6,7,8,9	LE7,8,9	LE6,7,8,9	DF	VC		Ct
Armandia cirrhosa Filippi, 1861	LE6,7,8	LE5,6,7,9	LE5,6,7,9	LE7,8	DF	VC		Ct
Capitella capitata (Fabricius, 1780)	LE6		LE5,6,7,8,9	LE6,8,9	DF	VC		Ct
Capitellidae indet.	LE8	LE7			DF	UC		C
Caulleriella zetlandica (McIntosh, 1911)	LE8	LE6,7,8,9	LE6,7,8,9	LE6,7,8,9	DF	C		Ct
Cirratulus cirratus (Müller, 1776)			LE7,8,9	LE7,9	DF	C		VC
Clymenura clypeata (Saint-Joseph, 1894)			LE7	LE7,8	DF	UC		C
Diopatra neapolitana Delle Chiaje, 1841			LE8,9	LE7,9	DF	C		VC
Eteone picta Quatrefagues, 1865		LE6,7,9	LE7,9	LE7	Pr	C		VC
Euclymene oerstedi (Claparède, 1863)			LE7,8,9	LE6,7,8	DF	C		Ct
Eunice harassii Audouin and Edwards, 1834		LE8,9	LE7		DF	C		VC
Ficopomatus enigmaticus (Fauvel, 1923)	LE6				SF	R		UC
Glycera sp.		LE6,9	LE6,7,8,9	LE6,7,8,9	Pr	C		Ct
Glycera tesselata Grube, 1840				LE9	Pr	R		UC
Glycera tridactyla Schmarda, 1861	LE8,9	LE6,7			Pr	C		Ct
Harmothoe sp.		LE6,9	LE7,8,9		Pr	C		Ct
Hediste diversicolor (Müller, 1776)	LE7,8	LE6,7,8	LE7,8	LE5,6,7,8	O	C		Ct
Heteromastus filiformis (Claparède, 1864)		LE5,6	LE7,8,9	LE6,7,8,9	DF	VC		Ct
Hydroides norvegicus Gunnerus, 1768		LE7			SF	R		UC
Lagis koreni Malmgren, 1866	LE9		LE6,9		DF	UC		C
Laonice cirrata (Sars, 1851)			LE8,9		DF	UC		C
Lepidonotus squamatus (Linnaeus, 1758)			LE7,9	LE8	Pr	C		VC
Lumbrineris sp.		LE7,8,9	LE6,7,8	LE7,8	Pr	C		Ct
Magelona papillicornis Müller, 1858		LE9		LE7,9	DF	UC		C
Melinna palmata Grube, 1870		LE6,7,8,9	LE5,7,8,9	LE6,7,8	DF	VC		Ct
Micronephthys maryae San Martín, 1982	LE7	LE7,8,9	LE7,8,9	LE6,7,8,9	Pr	C		Ct
Neosabellides oceanica (Fauvel, 1909)			LE6,7,8	LE7	DF	C		VC
Nephtys assimilis Örsted, 1843				LE9	Pr	R		UC

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Nephtys cirrosa (Ehlers, 1868)	LE9				Pr	R		UC
Nephtys hombergii Lamarck, 1818			LE9		Pr	R		UC
Nephtys sp.				LE6,8	Pr	UC		C
Nereididae indet.			UE4; LE6,7	UE4	DF	C	UC	C
Notomastus sp.		LE9	LE7,8,9		DF	C		VC
Oriopsis armandi (Claparède, 1864)	LE7,8,9	LE5,7,9	LE5,6,9	UE4;LE5,8	SF	VC	UC	Ct
Paradoneis lyra (Southern, 1914)		LE7,9	LE6,7,8,9	LE7,8,9	DF	C		Ct
Perinereis cultrifera (Grube, 1840)	LE5,6	UE4	LE6	UE4; LE8	O	VC	UC	VC
Phyllodoce mucosa Örsted, 1843	LE9	LE9	LE9		Pr	R		UC
Phylo foetida (Claparède, 1869)			LE8	LE7	DF	UC		C
Pista cristata (Müller, 1776)	LE7,8	LE6,7,8,9	LE7,8,9	LE7,8	DF	C		Ct
Prionospio malmgreni Claparède, 1869		LE7,8,9	LE7,8,9	LE7,8,9	DF	C		VC
Pseudopolydora antennata (Claparède, 1869)	LE7,8	LE5,6,9	LE5,6,7,8,9	LE6,7,8,9	DF	VC		Ct
Sabella pavonina Savigny, 1822			LE8	LE7,8	SF	UC		C
Sabellidae indet.	LE6		LE8,9	LE6	SF	C		VC
Serpula vermicularis Linnaeus, 1767		LE5,6,7,8	LE7		SF	C		Ct
Sigambra parva (Day, 1963)		LE9	LE7,8,9	LE7,8	Pr	C		VC
Spio filicornis (Müller, 1776)	LE8	LE5,6,7,9	LE5,6,7,8,9	LE6,7,8,9	DF	VC		Ct
Streblospio benedicti Webster, 1879	LE7,8,9	LE5,6,7,8,9	LE5,6,7,8,9	LE5,6,8,9	DF	VC		Ct
Syllidia armata Quatrefages, 1866			LE6,7,8,9	LE6,7,8,9	Pr	C		Ct
PHYLUM ARTHROPODA								
Class Arachnida								
Acaridida indet.			UE1		Pr	R	UC	
Halacaridae indet.				UE3	Pr	R	UC	
Hydrozetes sp.		UE2			Pr	R	UC	
Lebertia sp.	UE2	UE2,4			Pr	UC	C	
Sperchon sp.		UE2		UE2	Pr	R	UC	
Torrenticola sp.		UE2,3	UE3		Pr	UC	C	
Class Pantopoda								
Nymphon gracile Leach, 1814				LE7	O	R		UC
Class Branchiopoda								
Daphnia longispina (Müller, 1776)	LE5				G	R		UC

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Eurycercus lamellatus (Müller, 1776)				UE1	G	R	UC	
Ilyocryptus sordidus (Liévin, 1848)			UE1		G	R	UC	
Simocephalus exspinosus (Koch, 1841)		UE1			G	R	UC	
Simocephalus vetulus (Müller, 1776)				UE1	G	R	UC	
Class Ostracoda								
Cyprideis torosa (Jones, 1850)			LE7	LE8	DF	UC		C
Fabaeformiscandona fabaeformis (Fischer, 1851)		UE2	UE1,2	UE1,4	DF	C	VC	
Herpetocypris brevicaudata (Kaufmann, 1900)		UE3			DF	R	UC	
Herpetocypris sp.		UE4			DF	R	UC	
Class Copepoda								
Acanthocyclops latipes (Lowndes, 1927)			UE1		SF	R	UC	
Canuella furcigera Sars, 1903			LE7		SF	R		UC
Centropages chierchae Giesbrecht, 1889			LE6		SF	R		UC
Cyclops sp.				UE1,3	SF	UC	C	
Eucyclops serrulatus (Fischer, 1851)			UE1		SF	R	UC	
Macrocyclops albidus (Jurine, 1820)		UE1,2	UE1	UE1	SF	UC	C	
Class Malacostraca								
Ampelisca brevicornis (Costa, 1853)			LE9		SF	R		UC
Ampelisca sp.			LE7		SF	R		UC
Ampelisca typica (Bate, 1856)	LE8,9	LE9	LE9		SF	UC		C
Apseudes latreillii (Milne-Edwards, 1828)	LE9	LE8,9			DF	UC		C
Bathyporeia sp.				LE9	DF	R		UC
Bodotria arenosa Goodsir, 1843				LE9	DF	R		UC
Corophium orientale Schellenberg, 1928	LE5,6,7,8,9	UE4;LE5,6,9	UE4; LE5,6	UE3,4;LE5,6	DF	Ct	C	Ct
Corophium rotundirostre Stephensen, 1915			LE7,8,9	LE7,8,9	DF	C		VC
Cumopsis goodsir (Van Beneden, 1861)		LE9			DF	R		UC
Cyathura carinata (Krøyer, 1847)	LE7		LE7,8	LE7,8	DF	UC		C
Decapoda indet.	LE9			LE7,9	O	UC		C
Diastylis sp.		LE8	LE8	LE6,7,8	DF	UC		C
Echinogammarus longisetosus Pinkster, 1973	UE1,3,4	UE4	UE2	UE2	O	C	Ct	
Gammarus aequicauda (Martyinov, 1931)				LE8	O	R		UC
Iphinoe sp.	LE9	LE9		LE7,8	DF	UC		UC

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI	CI
							UE	LE
Lembos sp.			LE7		DF	R		UC
Lembos spiniventris (Stebbing, 1895)				LE7	DF	R		UC
Leptocheirus pilosus Zaddach, 1844	LE9		LE7	LE8	SF	C		VC
Leucothoe incisa (Robertson, 1892)	LE9		LE7,8	LE7,8	O	C		VC
Liocarcinus corrugatus (Pennant, 1777)			LE9		O	R		UC
Medorippe lanata (Linnaeus, 1767)			LE9		O	R		UC
Microprotopus sp.		LE9			O	R		UC
Monoculodes acutipes Ledoyer, 1983	LE9		LE8	LE6,7,8,9	O	C		Ct
Pariambus typicus (Kroyer, 1844)		LE8	LE8,9	LE6,7,8	O	C		Ct
Perioculodes longimanus (Bate and Westwood, 1868)				LE7,8	DF	UC		C
Phtisica marina Slabber, 1769			LE6,7	LE7,8	O	C		VC
Praunus flexuosus (Müller, 1776)	LE9				SF	R		UC
Pseudocuma longicorne (Bate, 1858)	LE8	LE9			DF	UC		C
Sphaeroma serratum (Fabricius, 1787)				LE8	O	R		UC
Synchelidium haplocheles (Grube, 1864)	LE7	UE4			DF	UC	UC	UC
Synchelidium sp.	LE8				DF	R		UC
Upogebia pusilla (Petagna, 1792)	LE9				SF	R		UC
Upogebia sp.		LE9			SF	R		UC
Class Insecta								
Baetis fuscatus (Linnaeus, 1761)	UE2	UE2,3		UE2	DF	UC	C	
Baetis pavidus Grandi, 1949	LE7		LE6,7		DF	UC		C
Caenis luctuosa (Burmeister, 1839)	UE1,2,3	UE1,2	UE1,2	UE1,2	DF	C	VC	
Ceraclea dissimilis (Stephens, 1836)				UE2	DF	R	UC	
Ceraclea sobradieli (Navás, 1917)	UE2			UE2	DF	R	UC	
Chironomus sp.	LE7,8	UE4	UE1	UE1,3; LE6	DF	VC	VC	VC
Choroterpes picteti (Eaton, 1871)	UE2				DF	R	UC	
Coenagrion pulchellum (Van der Linden, 1825)				UE1	Pr	R	UC	
Coenagrionidae indet.		UE1	UE1		Pr	R	UC	
Drypos sp.		UE1	UE1		G	R	UC	
Ecnomus tenellus (Rambur, 1842)	UE2	UE1,2	UE2	UE2	O	UC	C	
Ephoron virgo (Olivier, 1791)	UE1,2,3,4			UE2	SF	C	Ct	
Hydropsyche exocellata Dufőur, 1841	UE2	UE2	LE5	UE2	SF	UC	UC	UC

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Hydroptila sp.	UE2	UE2		UE2	G	R	UC	
Mystacides azurea (Linnaeus, 1761)	UE2				DF	R	UC	
Orthotrichia angustella (McLachlan, 1865)			UE2		G	R	UC	
Pseudocloeon atrebatinus Eaton, 1870	UE2	UE2	UE2		DF	R	UC	
Psychomyia pusilla (Fabricius, 1781)				UE2	DF	R	UC	
Sf. Orthocladiinae indet.	UE1,2; LE7	UE1,2,4	UE1,2,4; LE5,6,7	UE1,2; LE5,7,8	DF	Ct	VC	Ct
Sf. Tanypodinae indet.	UE1	UE1,2,3,4	UE1	UE1,2,3,4	Pr	C	Ct	
Simulium erithrocephalum (De Geer, 1776)	UE2		UE2		SF	R	UC	
Tr. Chironomini indet.	UE1	UE4	UE2	UE2	DF	C	VC	
Tr. Tanytarsini indet.	UE1,2	UE2,3	UE1,2	UE1,2,3	DF	C	VC	
Trithemis annulata (Palisot de Beauvois, 1807)		UE1			Pr	R	UC	
PHYLUM PHORONIDA								
Class Phoronida								
Phoronis ovalis Wright, 1856				LE6	SF	R		UC
Phoronis psammophila Cori, 1889		LE5,7,8,9	LE6,7,8	LE6,7,8	SF	VC		Ct
PHYLUM ECHINODERMATA								
Class Holothuroidea								
Thyone sp.			LE7	LE7,8	DF	UC		C
Class Ophiuroidea								
Amphipholis squamata (Delle Chiaje, 1828)	LE9	LE9	LE7,8	LE8	DF	C		VC
Amphiura chiajei Forbes, 1843			LE9	LE7	DF	UC		C
Class Echinoidea								
Fibulariidae indet.	LE9				DF	R		UC
Echinocardium sp.		LE9			DF	R		UC

CHAPTER II

Benthic macrofaunal dynamics and environmental stress, across a riverine-marine boundary, in a salt wedge Mediterranean estuary.

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ABSTRACT

The spatial distribution of benthic macroinvertebrate community in relation to environmental factors was studied along the Ebro Estuary (NE Iberian Peninsula), a salt wedge Mediterranean estuary. Both ordination methods and generalized additive models were performed to identify the different benthic assemblages and their relationship to abiotic factors. Our results showed a strong relationship between macrofaunal assemblages and the predominant environmental gradients (*e.g.* salinity); thus revealing spatial differences in their structure and composition. Two different stretches were also identified, namely the upper (UE) and the lower Ebro Estuary (LE). UE showed riverine characteristics and hence a freshwater community; whereas LE was influenced by marine intrusion and sustained a complex marine-origin community. However, within each stretch, water and sediment characteristics played an important role in explaining species composition differences among sampling stations. Moreover, outcomes suggested a total species replacement pattern, instead of the nestedness pattern usually associated with well-mixed temperate

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estuaries. Both sharp species turnover together with the estuarine stratification point out that the Ebro Estuary is working, in terms of ecological boundaries, under an ecotone model. Finally, despite obvious differences with well mixed estuaries (*i.e.* lack of tide influence, stratification and species turnover), the Ebro Estuary shares important ecological attributes with well-mixed temperate estuaries.

Keywords: Macroinvertebrates; highly stratified estuary; salinity; ecotone; multivariate analysis; GAMs; large river.

INTRODUCTION

Estuaries are critical transition zones linking freshwater and marine systems, where a broad range of physicochemical factors co-occur in a small geographical area (Levin *et al*., 2001; Elliot and Whitfield, 2011). The close connection between riverine and marine habitats assures a rapid and constant flux-exchange of energy, materials and organisms; for this reason, estuaries are commonly described as stressful (*e.g*. Elliot and Whitfield, 2011; Day *et al*., 2012) and biologically dynamic ecosystems (*e.g*. Ahel *et al*., 1996; Nebra *et al*., 2011). Estuarine environmental gradients impose physiological constraints on biota; only a few specialized species are capable of withstanding them, resulting in extremely poor communities compared with those from adjacent riverine and marine areas (McLusky and Elliott, 2004; Dauvin, 2007; Elliot and Whitfield, 2011; Day *et al*., 2012). Among the different abiotic factors that are known to affect the macrofaunal distribution and diversity within estuaries, of both natural and anthropogenic origin, the salinity variation has been identified as the main driver in structuring estuarine communities. Although the relationship between salinity gradient and macrofaunal patterns has been extensively studied (*e.g*. Remane 1934; Attrill, 2002; Attrill and Rundle, 2002; Giberto *et al*., 2007; Whitfield *et al*., 2012); nevertheless, the definition of estuary in relation with tidal influence and the salinity gradient formation is still controversial (*e.g*. McLusky, 1999; Elliott and McLusky, 2002; Tagliapietra *et al*., 2009; Potter *et al*., 2010; Telesh and Khlebovich, 2010). Recent studies still associate its definition with tidal influence as the main mixing agent forming the salinity gradient (Elliott and McLusky, 2002; Tagliapietra *et al*., 2009). Since the development of the Remane diagram (Remane, 1934), several modifications and new models have been developed to explain the richness pattern of biota along the estuarine salinity gradient (Whitfield *et al*., 2012); one of them included ecological boundary concepts for estuaries like ecotone and ecocline (Attrill and Rundle, 2002). Considering that the riverine-marine interface is the most obvious landscape boundary in aquatic ecology and that ecological boundaries are matter of contemporary ecology, it is clear that estuarine boundaries require more clarification and will probably influence future studies (Rundle, 1998; Strayer *et al*., 2003).

All of the aforementioned is applicable to well-mixed estuaries; but what occurs in rivers emptying into non-tidal seas that normally form stratified type estuaries? Should these river-mouths be considered estuaries according to currently accepted definitions of estuary? Are salt wedge estuaries colonized by different species than those inhabiting well-mixed estuaries? Which would be the best model for describing biota distribution patterns? Can this kind of riverine-marine boundary actually be considered an ecotone or ecocline?

When compared to well-mixed estuaries, in salt wedge estuaries: (i) river discharge controls marine intrusion due to the low tidal range (usually tidal amplitude is less than 2 m); (ii) weak mixing drivers enhance water column stratification promoting the formation of a salt wedge landwards; (iii) vertical profile of density and salinity shows an abrupt change from surface to bottom, friction between fresh and saltwater layers forms a narrow interface called halocline; (iv) isohalines are arranged horizontally and (v) if sediment load is high, a Delta may be formed. As consequence, in salt wedge estuaries environmental fluctuations are not gradual due to the lack of mixing events; there is not salinity gradient along the estuary (Ibáñez, 1993); but in contrast with mixed estuaries the biota must be adapted to abrupt changes rather than to gradual ones, not only in salinity terms but also in other environmental features like temperature, E_h (redox potential) or pH. Moreover, salt wedge estuaries become rivers if flows are high enough (mean annual river discharge is close to the critical value determining the formation and breakup of the salt wedge) to expel marine intrusion (Ibáñez, 1997); thus, environmental stress for biota is more pronounced (Elliot and Whitfield, 2011). The material exchange is mainly the result of entrainment process between layers together with the turbulence occurring at the salt wedge tip or null point; the halocline only allows scarce transfer of materials between layers, mainly coarse suspension particles and died organisms coming from the upper layer and salt and nutrients from the lower layer (Dyer, 1997; Lewis, 1997).

In this study we investigate how environmental factors influence macrofaunal community distribution in a distinctive riverine-marine boundary; therefore, the aims of this paper are: first, to relate the distribution and abundance of the different macroinvertebrate species to environmental variables and river disturbances by means of ordination methods, and second, to analyze the response of the macrofaunal populations at the community level to the variation in limnological features along the whole estuarine stretch.

MATERIALS AND METHODS

2.1 Study site

The study was conducted in the Ebro Estuary (Fig. 1) located in the NE of the Iberian Peninsula (40º43'10"N, 0º40'30"E). The Ebro River flows into the Mediterranean Sea and forms a Type 4 (salt wedge or highly stratified) estuary (Hansen and Rattray, 1966; Ibáñez, 1993; Ibáñez *et al*., 1997) of about 32 km long, with a mean width of 240 m and a mean water depth of 7 m. The tidal range is low, *ca*. 20 cm (Cacchione *et al*., 1990), and its low influence promotes the formation of a salt wedge, which is controlled by river discharge (advance, retreat and permanence). Briefly, when river flow exceeds 350-400 m^3 s^{-1} the salt wedge is pushed seawards and the estuary works as a river or 'fluvial estuarine stretch' (Ibáñez 1993), conversely the salt wedge reaches its maximum landwards (*ca*. 30-32 km from the river mouth) at flows lower than 100 m^3 s^{-1} (Ibáñez, 1993; Ibáñez *et al*., 1997).

The basin is highly regulated by approximately 190 dams, managing water for hydropower production, irrigation, and human consumption. Large reservoirs have altered the annual flow not only by modifying the natural seasonal flow pattern but also by preventing flood frequency and intensity (Ibáñez *et al*., 2012; Rovira *et al*., 2012a). The annual mean flow decreased during the last decades (Ibáñez *et al*., 1996, 2008). In particular, Mequinenza and Ribarroja reservoirs (located on the main river about 100 km upstream from the river mouth) have a significant regulatory effect over flows in the lower Ebro River (Ibáñez *et al*., 2012; Rovira *et al*., 2012a), and therefore are considered to be the final responsible of the salt wedge dynamics and macrofaunal trends along Ebro Estuary. Water abstraction and regulation virtually assures the presence of the salt wedge in the same position for long periods (Ibáñez *et al*., 1995; Sierra *et al*., 2004; Falcó *et al*., 2010; Nebra *et al*., 2014).

2.2 Sampling design and laboratory procedures

In order to cover the whole study area, both the estuary and the potential stretch accessible by salt wedge during low flow periods, nine sampling stations were established from the river mouth to 37 km upstream (Fig. 1). Each station was sampled seasonally (from summer 2007 to spring 2008) for benthic macroinvertebrates, sediment traits, dissolved oxygen (DO), nutrient loadings (PO_4, P_T, NH_4, NO_2, NO_3, N_T and SiO_4) and hydromorphological characteristics (depth, flow velocity and water transparency) (see Appendix 1). Data available at the Ebro Basin Authority web site (http://www.chsegura.es; station 9027: Tortosa), were used to calculate the permanence time of the salt wedge in each sampling station and season as a function of the daily average river flows according to Ibáñez (1993). For detailed analysis procedures and units see Nebra *et al*., 2014.

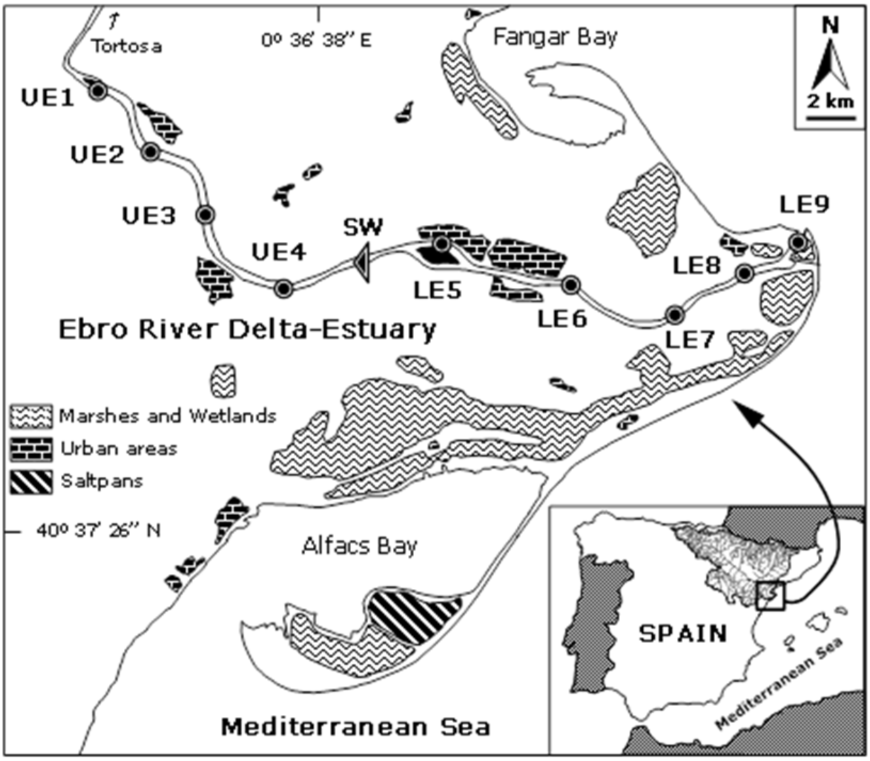

Figure 1. Map showing the Ebro Estuary, its deltaic plain and the location of the nine sampling stations. UE, upper estuary stations; LE, lower estuary stations; SW, salt wedge tip's position.

2.3 Data analysis

The macroinvertebrate abundance and environmental variables data sets were analyzed by means of multivariate ordination techniques, including both indirect (Principal Component Analysis and Detrended Correspondence Analysis; PCA and DCA, respectively) and direct (Canonical Correspondence Analysis; CCA) gradient techniques. A PCA was carried out to explore the relationships and association patterns among environmental variables in the sample sets. Kaiser-Meyer-Olkin's (KMO) measure of sampling adequacy was used to assess the usefulness of a PCA; KMO ranges from 0 to 1 and should be > 0.5 if variables are sufficiently interdependent for PCA to be useful. On the other hand, the structure of the macroinvertebrate community was investigated by means of DCA (*i.e.* indirect gradient technique) and CCA (*i.e.* direct analysis method). Indirect gradient analysis only uses the species × sample matrix in the ordination whereas in direct techniques the ordination results are constrained to optimize their linear relationship to the environmental variables. Indirect and direct gradient analyses are complementary because although direct gradient analysis provide an ordination using the two matrices in a single analysis, indirect techniques are often more robust (McCune, 1997) and can show species gradients because of unmeasured environmental variables. CCA analysis was appropriate as direct technique because a DCA showed that the first axis length exceeded more than four standard deviation units and then a unimodal response model technique was preferable to assess the relationship between macroinvertebrate community and environmental variables (Lepš and Šmilauer 2003).

To further describe salinity and taxa variation (from Class to Family level) along the Ebro Estuary (from mouth to upper estuary limit) Generalized Additive Models (GAMs) (Lepš and Šmilauer, 2003) were also fitted. GAMs are an extension of the generalized linear models that, unlike more conventional regression methods, do not require the assumption of a particular shape for the response variable distribution along the environmental gradient, being a flexible and powerful analytical tool when assessing nonlinear relationships (Lepš and Šmilauer, 2003; Carol *et al.* 2006; Alcaraz *et al.*, 2011). Model complexity was selected by the stepwise selection procedure using the Akaike information criterion (AIC). The AIC not only considers the goodness of fit but also parsimony; penalizing very complex models (Burnham and Anderson, 1998), thus variables without an adequate candidate model are automatically deleted, for instance rare taxa for which there is no evidence of response to the gradient.

Multivariate analyses were performed with Canoco 4.5 (Lepš and Šmilauer, 2003), downweighting rare species and log-transforming species abundances and most environmental variables, because normality and homoscedasticity were clearly improved (Levene's test for equality of variances). The environmental variables in CCA were selected using the forward selection procedure of CANOCO 4.5 (Lepš and Šmilauer 2003), which tests the significance of the variables with Monte Carlo permutation test (499 permutations). GAMs were conducted using R software 3.2.2 through the gam 1.12 package.

RESULTS

3.1 Environmental characterization of the Ebro Estuary

The daily mean river discharges in all four sampling seasons were below 350 m^3 s^{-1}, thus allowing the marine intrusion. However, the permanence time (expressed as accumulated days) the marine intrusion varied among seasons, being 52, 140, 254 and 341 days in summer, autumn, winter and spring, respectively. The presence of the salt wedge produced a sharp change in physico-chemical characteristics along the estuary bottom, clearly differentiating two contrasting stretches, the upper and lower estuary (UE and LE) (Figs. 1, 2, 3 and 4). The salt wedge tip or null point was located between the stations UE4 and LE5 (Fig. 1) in all sampling occasions. The lower estuary (LE5-LE9 stations) was stratified during the whole studied period and had marine water, whereas the upper estuary (UE1-UE4 stations) had fresh water (see salinity values in Appendix 1). Sediment characterization showed an estuarine bottom predominantly sandy and sandy-mud (Appendix 1). Samples were primarily composed of sand (> 65% at 8 stations), mud was abundant in LE stations; except for LE5 station, and gravel was mainly restricted to UE2 station, which had the highest current velocity (Appendix 1). Total organic matter, total suspended solids and organic suspended solids were higher in the LE stations than in the UE, showing a maximum of suspended solids in LE9 stations. With regards to nutrients, in general terms their concentrations were higher in the riverine stretch of the estuary than in the marine zone; with the exception of LE5 station for ammonium and phosphorous compounds (Appendix 1). Dissolved oxygen was never limiting, although LE5 station showed some level of hypoxia in autumn (Fig. 2). Although the temporal and spatial variability in chlorophyll and pheophytin, both variables showed concentration differences between upper and lower estuary stretches. Finally, mean water flow velocities (close to the bottom) were *ca*. 3 times higher in UE stations when compared to LE stations (Appendix 1), except in the LE9 station, where annual mean velocity was 0.25 $m^3 s^{-1}$, probably due to the advection effect.

3.2 Macroinvertebrate community description

A total of 21,805 individuals were collected, comprising 216 different taxa (155 species), and belonging to 9 different phyla. The most abundant taxa were Annelida (71.07% of the total abundance), Arthropoda (15.56%) and Mollusca (12.09%) contributing to richness with 81, 67 and 55 different taxa, respectively. Density values varied seasonally and among sampling stations, ranging from 216 to 20,022 ind m^{-2} (Fig. 3). The highest densities were found close to river mouth (*i.e*. LE8-LE9), mainly because of to abundance of the polychaete *Streblospio benedicti*. The uppermost stations, UE1 and UE2, showed intermediate densities, with a large contribution of Tubificidae and the invasive bivalve *Corbicula fluminea*. Interestingly, lowest densities were recorded at UE3, UE4 and LE5 stations located close to the null point. A similar pattern was observed for richness; stations close to the river mouth had the highest richness values with a maximum of 69 different taxa in LE9, whereas UE3, UE4 and LE5 stations showed the lowest values (see also Fig. 3). For a further description of benthic macrofauna community see Nebra *et al*., 2011.

3.3 Benthic environmental condition and macrofauna communities' response

Many of the environmental variables were interdependent and significantly correlated among them, with the KMO's measure of sampling adequacy (0.56) indicating the usefulness of the PCA. The first two axes of the PCA explained the 52.75% of the total variation, 32.96 and 19.79%, for the first and second axis respectively (Fig. 2). The strongest correlations were observed between velocity and SiO_4, PO_4, NO_2, NO_3 and N_T (r > 0.687 in all cases); these variables were all positively correlated and opposed to conductivity, TOM and mud content. The first PCA axis summarized these correlations displaying an apparent upstream-downstream gradient, and revealed the sharp change in salinity and nutrient loads from the marine influenced LE stations, to nutrient-enriched riverine UE sites. The exceptions were the spring samples of LE6, LE7 and LE9 stations probably as the result of the high level of dissolved N_T recorded in this season. The second PCA axis distinguished a flow gradient, from the low-flows in summer and winter to high flows in autumn and spring (with highest TSS, OSS, chlorophyll and pheophytin, but opposed to organic suspended solids).

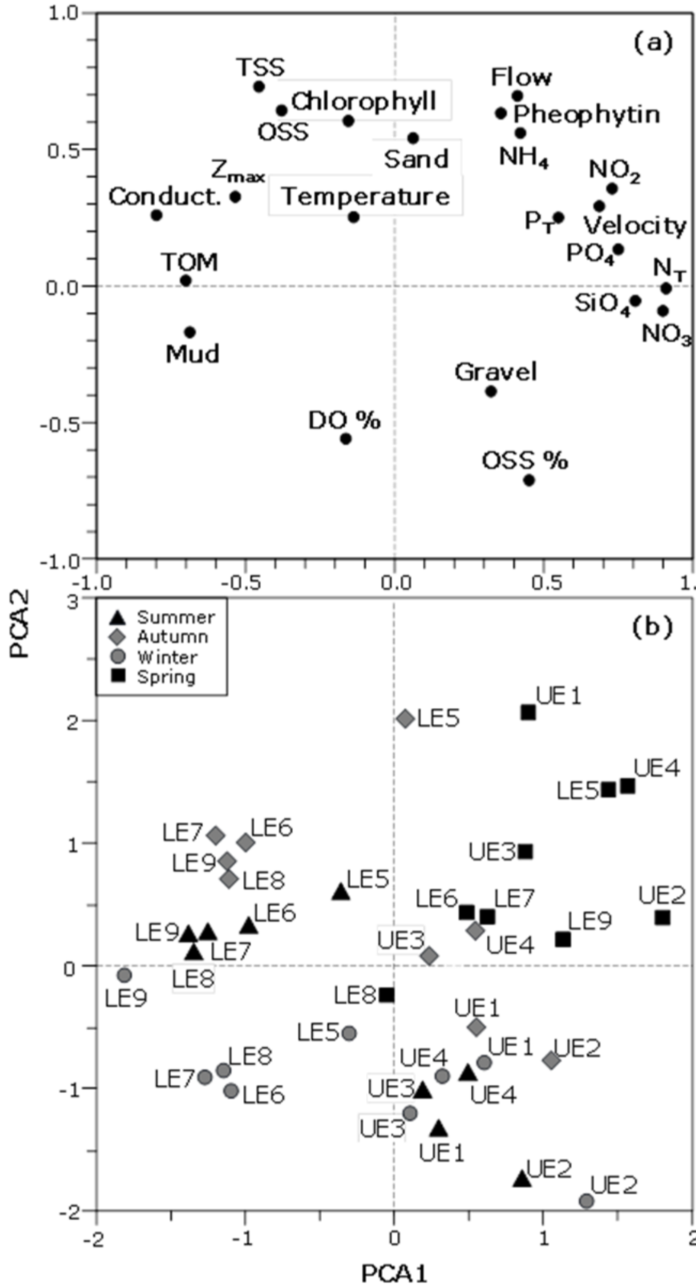

Figure 2. Principal component analysis of environmental variables for the nine sampling stations studied. Factor loadings of the variables (a), and sampling stations scores (b) for the first two principal components are shown.

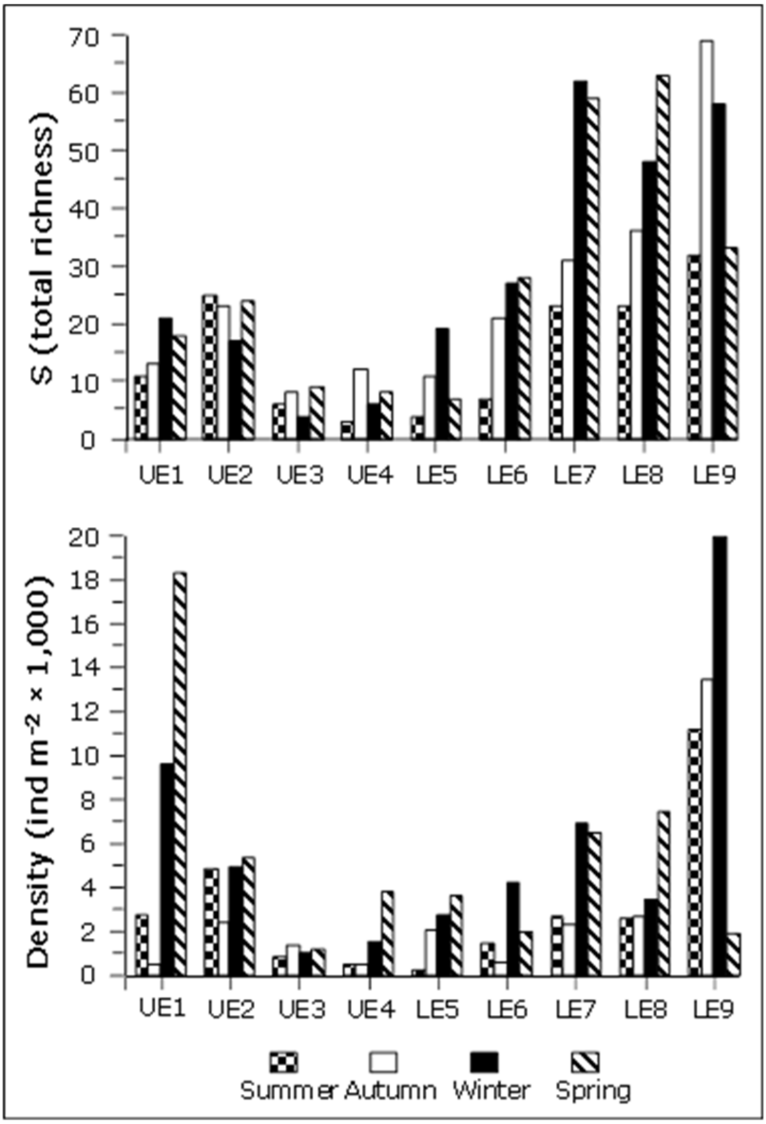

Figure 3. Richness and Density values for UE and LE stations recorded at each sampling occasion.

The first two axes of the DCA of the macroinvertebrate abundance, respectively, explained 20.8% and 6.0% of the total variation, and suggested a similar ordination of the estuarine stations (Fig. 4). The first DCA axis clearly distinguished freshwater (*i.e.* UE1-UE4 stations) from marine taxa (*i.e.* LE5-LE9 stations). The first axis also differentiated stations dominated by euryhaline species; *e.g. Corophium orientale, Perinereis cultrifera, Ficopomatus enigmaticus, Hediste diversicolor* and *Cyathura carinata*, from those stations with strictly freshwater or marine community (Fig. 4). The second DCA axis discriminated mostly in the UE stations group, in this case separating UE1 station dominated by euryoecious taxa as the invasive bivalve *C. fluminea* or the tolerant *Chironomus* sp., *Echinogammarus longisetosus* and Tubificidae oligochaetes, from those stations (UE2-UE3) with more balanced community.

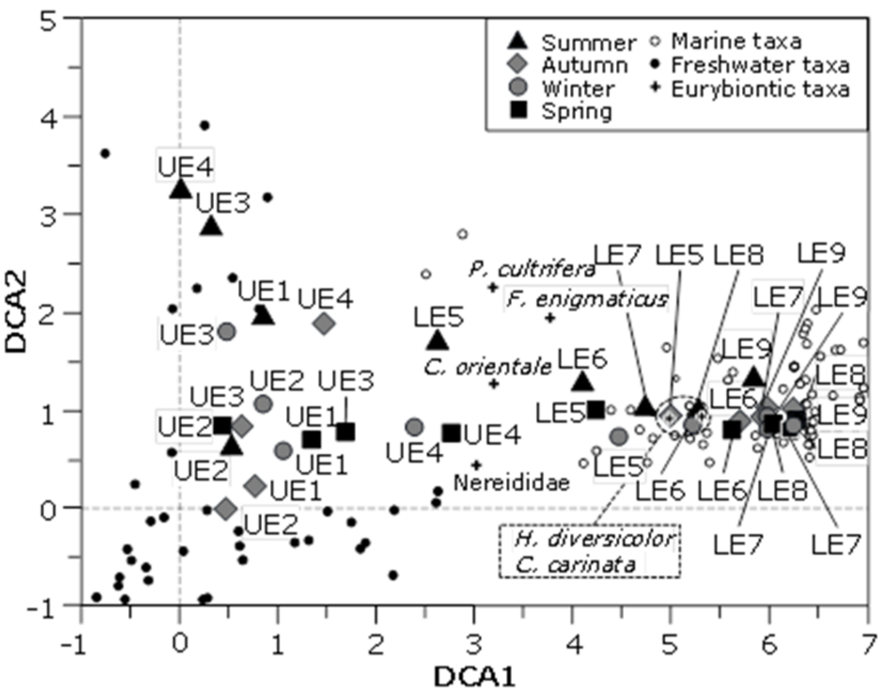

Figure 4. Detrended correspondence analysis of the macrofauna abundance data for the nine stations studied.

3.4 Relationship of environmental factors with the macroinvertebrate community

The CCA analysis of macrofauna abundance confirmed the previously described pattern with two distinct station groups corroborating PCA and DCA stations grouping (Fig. 5). The total taxa-environment variance explained by the first two canonical axes was 52.4% (37.1 and 15.3, respectively), accounting for the 20.6% of the total variance in taxa abundance data. Only eight variables were retained by the forward selection procedure (Fig. 5). The first CCA axis was mainly associated with salinity and nitrate concentration; distinguishing the saltwater LE stations with marine community, from the UE stations characterized by elevated nitrogen compounds concentration and a freshwater community. The second canonical axis separated the stations with high contents of organic suspended solids but low gravel content, for instance UE4 and LE5 stations, characterized by the presence of stress tolerant taxa such as the amphipod *Corophium orientale,* Tubificids oligochaetes and Nereid polychaetes.

Salinity was the most important factor in structuring benthic communities; therefore, riverine and marine stations (*i.e.* UE and LE or pre-salt wedge and salt wedge stations) were analyzed separately. The CCA conducted on UE (*i.e.* pre-salt wedge) macroinvertebrate abundance retained only five variables of the environmental variable initially included (Fig. 6). The first two canonical axes explained the 28.6% of total variation in macroinvertebrate taxa abundance, accounting for the 35.7 and 23.9% of the variance in taxa-environment relation. The first axis was mainly related to depth, separating deeper stations from shallower ones. UE4 station in spring, coinciding with the maximum flow, was characterized by the presence of euryhaline and stress tolerant species such as *P. cultrifera*, *C. orientale* and Nereid polychaetes immature stages. Both UE3 and UE4 stations (displayed across the axis) were dominated by several tolerant taxa, for instance *E. longisetosus*, Tubificidae, Lumbricidae Chironomidae, Nematoda and the invasive *C. fluminea*. The second CCA axis distinguished stations with highest organic matter, chlorophyll and nitrate values and lowest gravel content. This gradient was also translated into a pattern of taxa composition, from UE1 station, characterized by the presence of standing water taxa like *Macrocyclops albidus*, *Cyclops* sp., *Ilyocryptus sordidus* and *Fabaeformiscandona fabaeformis* to UE2 station dominated by the presence of several species of caddisflies and mayflies (Fig. 6b).

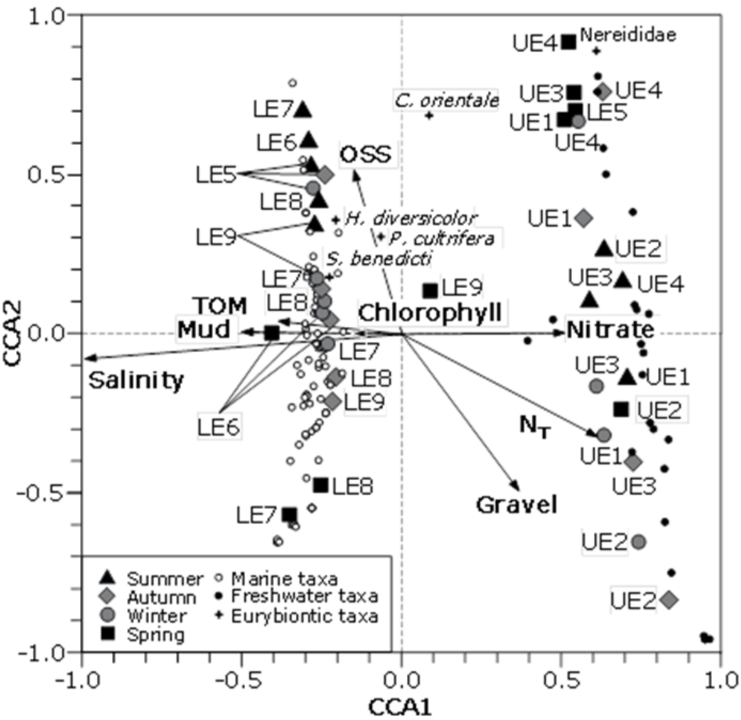

Figure 5. Canonical correspondence analysis triplot of macrofauna abundance data and environmental variables assessed in the nine sampling stations studied. Environmental variables are represented by arrows, which length is proportional to variable importance and orientation represents their correlation with the axes.

Regarding LE stretch (*i.e.* salt wedge stretch); of the environmental variables initially included in the CCA, only 5 were retained by the forward selection procedure (Fig. 7). The first two canonical axes respectively explained 32.6 and 19.8% of the total variation in taxa-environment relationship, accounting for the 22.6% of the total variation in taxa abundance. The first axis separated stations influenced by the salt wedge tip, *i.e.* with instable conditions and higher organic suspended solids concentrations, from those with more stable conditions because of the proximity to river mouth characterized by higher total organic matter and total nitrogen values. Salt wedge influenced stations, with a poor community, were dominated by eurybiontic and tolerant taxa such as *H. diversicolor*, *P. cultrifera*, *C. orientale* and *C. carinata* (Fig. 7). Opposed stations, LE8 and LE9, were colonized by typical marine species associated with the *Abra alba-Lagis koreni* community (colonizing fine sediments, rich in organic matter). Finally, LE7 and LE8 were characterized by a rich community mixed with stress-tolerant taxa, *e.g. Capitella capitata, Heteromastus filiformis* and Spionidae (Polychaeta) together with *Corbula gibba*, which usually colonizes disturbed areas.

Overall, in both groups (pre-salt wedge and salt wedge) warmer samplings were separated by the second CCA axis, showing the greatest productivity and characterized by high organic suspended solids content, benefited by the upper layer transparency (Fig. 6 and Fig. 7).

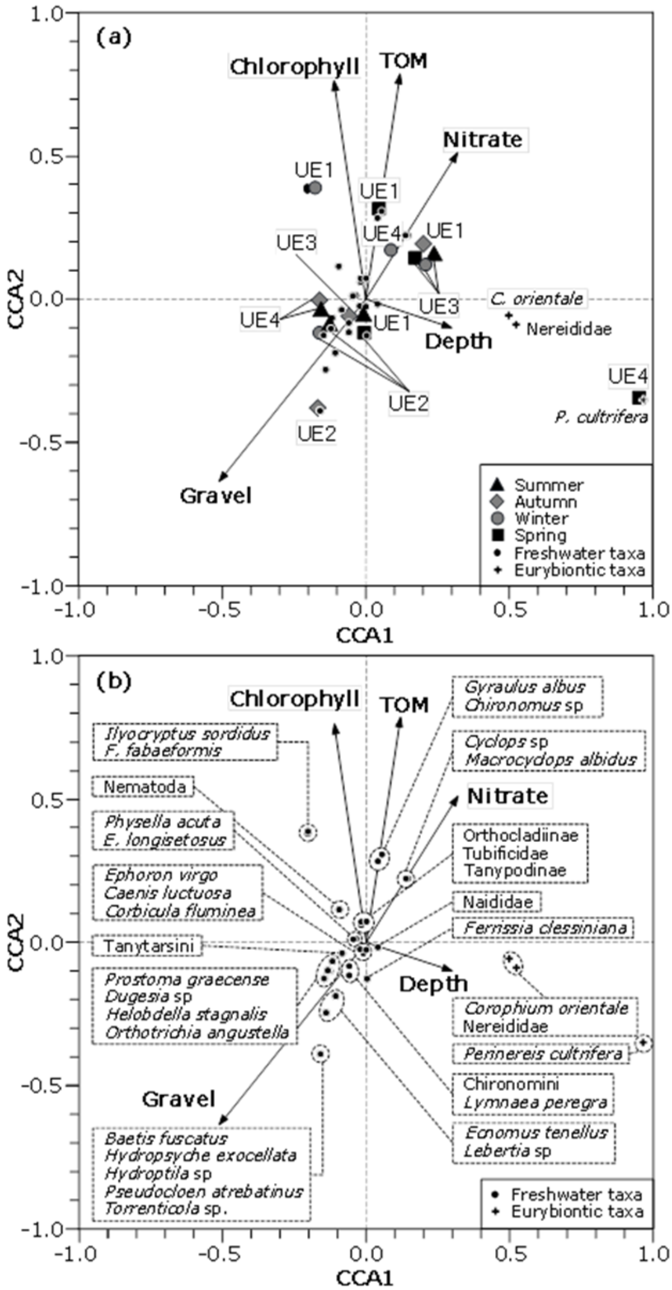

Figure 6. Canonical correspondence analysis of macroinvertebrate abundance data and environmental variables assessed for the riverine stations. a, CCA triplot; b, CCA biplot of environmental variables and macrofauna taxa data.

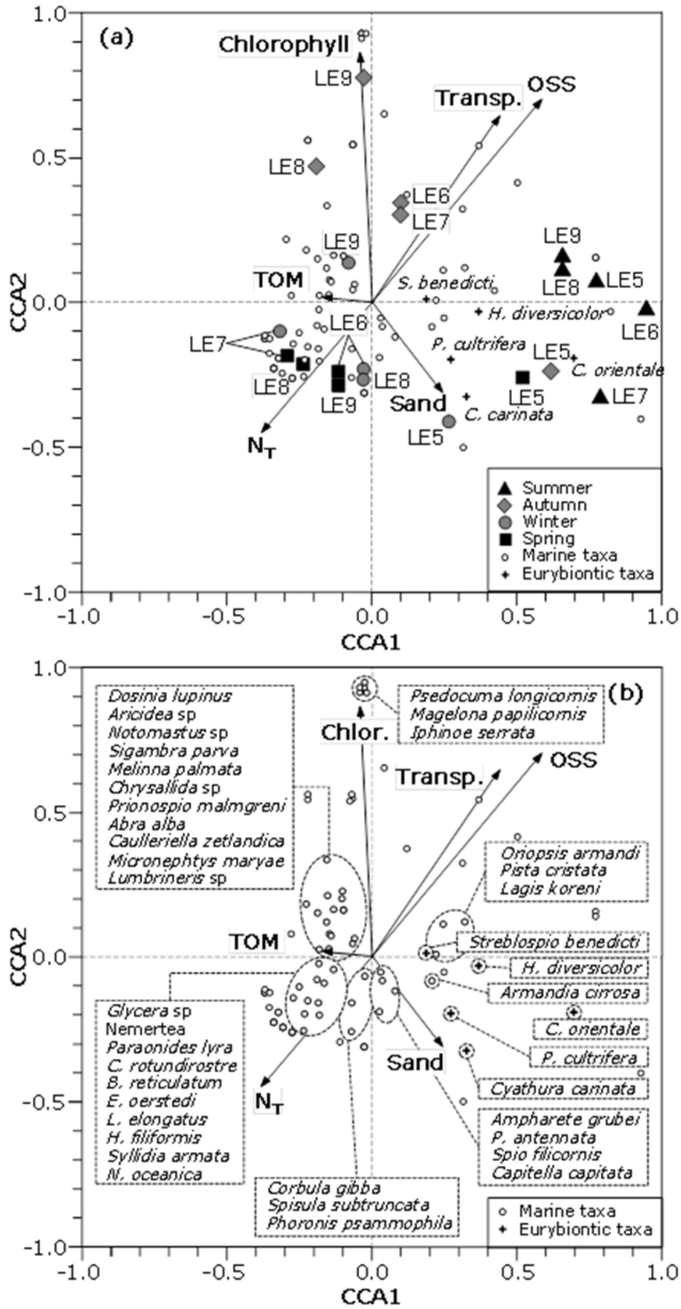

Figure 7. Canonical correspondence analysis of macroinvertebrate abundance data and environmental variables assessed for the marine stations. a, CCA triplot; b, CCA biplot of environmental variables and macrofauna taxa data**.**

The salinity response curve (GAM) to distance to the river mouth (Fig. 8) confirmed the two-stretch pattern observed in ordination methods (*i.e*. PCA and CCA) results. AIC selected a polynomial response for salinity variation along the lower Ebro River (Fig. 8), thus, differentiating the upper and the lower river sections. The response curves (GAMs) also revealed differences in taxa abundance variation, thus confirming and clarifying previous results (Fig. 9 & Fig.10). The response curves for Caenidae, Chironomidae, Physidae, Ephemeroptera, Insecta, Oligochaeta, Basommatophora and Corbiculidae showed a clear increase landwards; downstream once passed null point (shaded area in plots) these taxa almost disappeared (Fig. 9). Opposite, the response curves for Cirratulida, Echinodermata, Opheliida, Eunicida, Spionida, Phyllodocida, Myoida and Veneridae (representatives of brackish and marine waters) showed the inverse tendency with a clear decrease with distance to river mouth; upstream of null point these taxa disappeared (the only exception was the Phyllodocida because of the contribution of euryhaline Nereididae). In both figures, it is noteworthy that salinity change has a clear effect over the distribution of taxa.

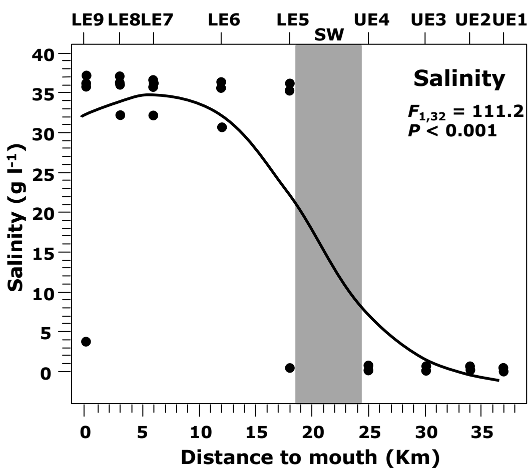

Figure 8. Response curve of salinity with distance to river mouth. The curve is the generalized additive models (GAM) selected by the Akaike information criterion (AIC). SW, salt wedge tip.

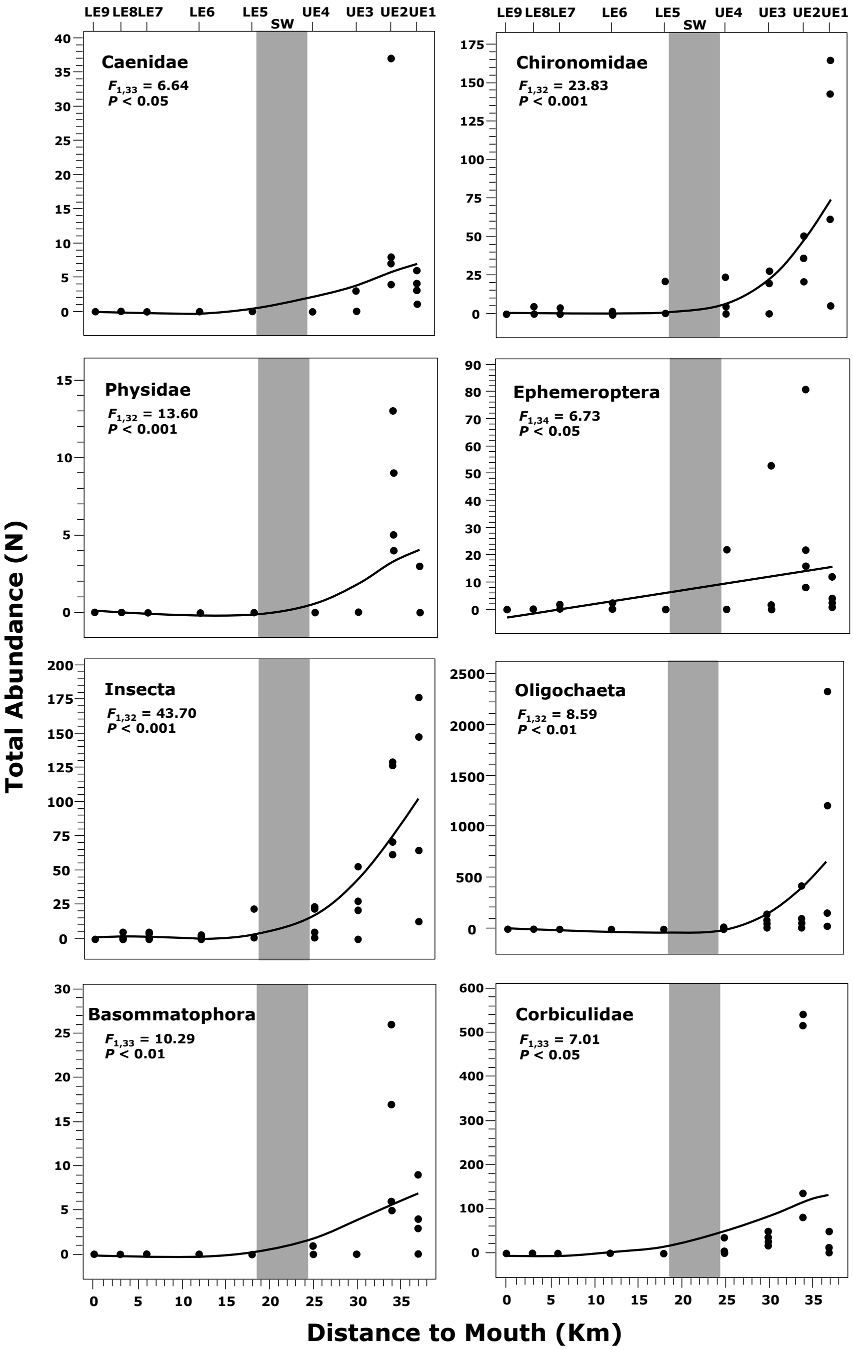

Figure 9. Response curves of UE taxa with distance to river mouth (the eight most representative taxa are shown). The curves are the generalized additive models selected by the Akaike information criterion (AIC). SW, salt wedge tip.

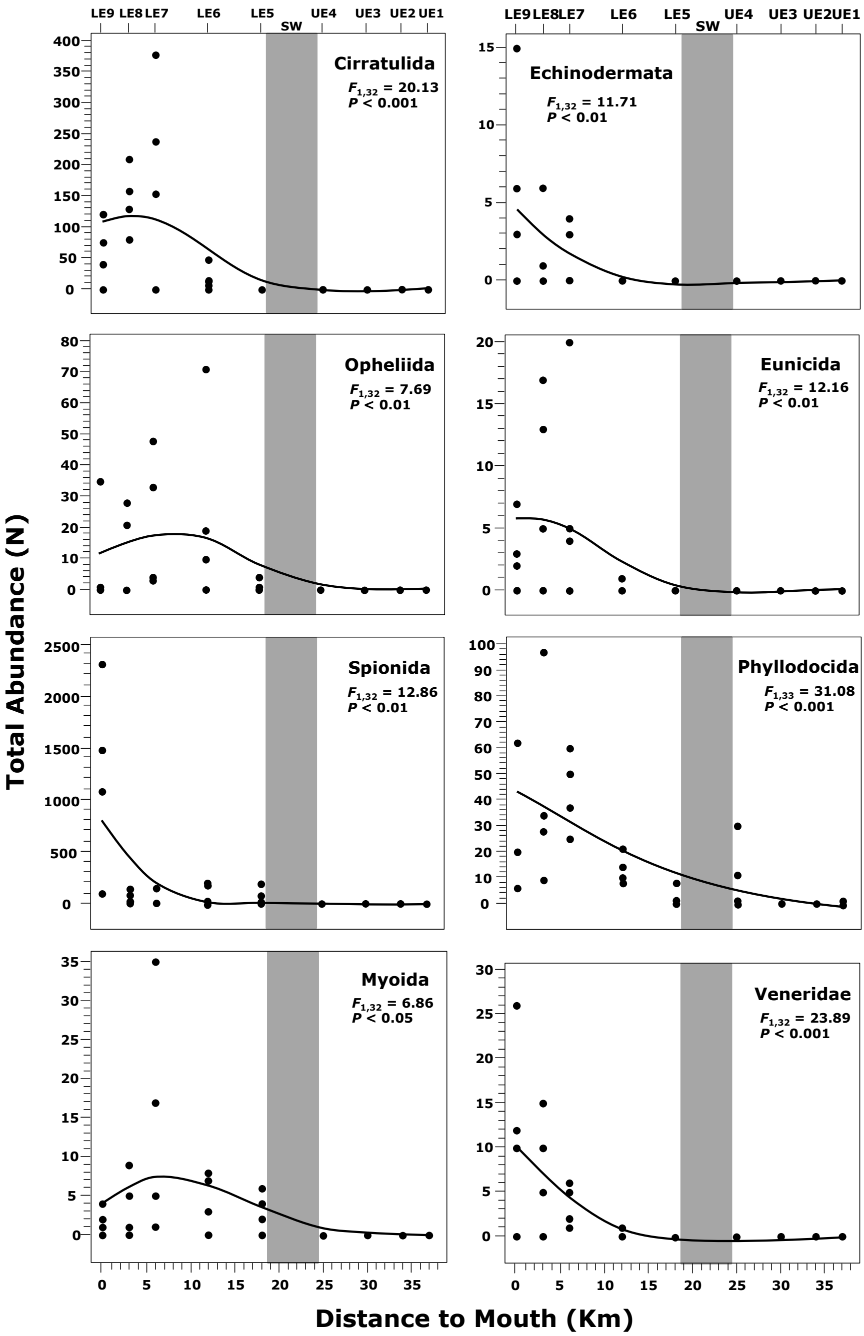

Figure 10. Response curves of LE taxa with distance to river mouth (the eight most representative taxa are shown). The curves are the generalized additive models selected by the Akaike information criterion (AIC). SW, salt wedge tip**.**

DISCUSSION

4.1 Macrofaunal trends at Ebro's riverine-marine boundary

Community ecology research try to respond two central questions: does variability in species composition along environmental gradients and across spatial scales follow non-random patterns? and, consequently, 2) what mechanisms produce these patterns? (Ulrich and Almeida-Neto, 2012). Community trends in relation with salinity gradient are well documented for mixed estuaries (*e.g*. Remane, 1934; Wolff, 1973; Attrill and Thomas, 1996; Giberto *et al*., 2007; Day *et al*., 2012). Mixed estuaries present a relatively extensive gradient zone where a heterogeneous range of transitional assemblages thrive. A well-established salinity gradient acts as an 'ecophysiological selector' for species; each one, with fresh or marine origin, has a salinity optimum and a tolerance range, and when overtaken, the species disappears. The overlapping of populations, with different salinity tolerance range, led the formation of an assemblage-continuum along the estuary with a nested distribution pattern (Ulrich and Almeida-Neto, 2012). This pattern can be traduced, in terms of ecological boundaries, as an ecocline, a gradient zone containing relatively heterogeneous assemblages and environmentally more stable than an ecotone. Ecoclines therefore represent boundaries of progressive change (both spatial and ecological) between two ecosystems, in response to the gradual difference in at least one major environmental factor (Attrill and Rundle, 2002), such as salinity in estuaries. In salt wedge estuaries, such as the Ebro, the river flow mediates the marine intrusion into the river channel, and therefore the community dynamics (Ibáñez, 1993); salinity does not change gradually and the gradient zone is restricted to a narrow and dynamic area around the null point. Because of the stress imposed on biological communities by salinity (tolerance ranges of species are suddenly overtaken); there is a drastic change in species composition, or in our case, a total replacement, upstream and downstream of the null point; thus causing a turnover in the diversity pattern (Ulrich and Almeida, 2012; Barros *et al*., 2014). Our results are consistent with this pattern, two macroinvertebrate communities were clearly differentiated along the Ebro Estuary and with respect to the estuarine null point; without a gradual transition change, a characteristic freshwater community was found restricted to UE stations, and a complex and rich estuarine community downstream the null point (see also Nebra *et al.*, 2011 for detailed communities' description). GAMs outcomes reflected the scarce exchange of species between community types, since only a few euryoecious marine-derived taxa were reported inhabiting both environments (*e.g.* Nereid polychaeta). This narrow riverine-marine interface is interpreted as an ecotonal boundary; a zone characterized by the rapid change between two different homogeneous communities, where fluctuations create a time-series of strongly different, but individually, homogeneous environments (Gosz, 1993; Attrill and Rundle, 2002; Acha *et al.*, 2015).

Ecosystem boundaries has been largely studied on terrestrial ecosystems (Attrill and Rundle, 2002; Strayer *et al.*, 2003; Acha *et al.*, 2015), but have received less attention in aquatic ecosystems. Only a few recent estuarine studies have been focused (although from different approaches) partially or totally in ecological boundaries (*e.g.* Attrill and Rundle, 2002; Elliot and Whitfield, 2011; Whitfield *et al.*, 2012; Basset *et al.*, 2013; Conde *et al.*, 2013). In spite of considering estuarine boundaries as ecotones or ecoclines, all these works deal with the redefinition of estuarine boundaries; since, different boundary types have different structural and functional characteristics; thus it is highlighted the necessity of accounting for the correct interpretation of ecological boundaries and the application of proper biodiversity models, and in this way prevent misleading designs, tests of theories, and study comparisons as suggested by Strayer *et al.* (2003).

4.2 Environmental stress along Ebro Estuary

Salinity was the most important stressor explaining the macroinvertebrate community turnover along the Ebro Estuary. Our results also showed small-scale variation in macroinvertebrate community within both upper and lower estuary. Both richness and diversity decreased from the lower and upper estuary towards to the null point where the environmental stress is maximum (Nebra *et al*., 2011); seasonal variations were evident both in richness and abundance terms but maintaining this pattern. Our results are consistent with estuarine models predicting richness increase seawards (*e.g*. Remane, 1934; Rundle *et al*., 1998; Attrill, 2002; Attrill and Rundle, 2002; Whitfield *et al*., 2012; Barros *et al*., 2014); nevertheless, all these models suggest that richness and diversity decrease landwards achieving minimum values in the 4-8 salinity zone because this salinity range represent a ecophysiological constraint for biota (Elliot and Whitfield, 2011). However, the Ebro Estuary is a stratified type estuary (Ibáñez *et al*., 1997) consequently, both marine and freshwater do not mix; and hence, community variation within each stretch is due to secondary factors that generate a similar impoverishment pattern. Within each estuarine stretch, the secondary factors affecting richness and diversity were grain size, total organic matter, and despite of seasonal variations chlorophyll concentration, which is mediated by nutrient concentrations (see Ibáñez *et al*., 2012). In accordance with those results, several species found close to the null point were typical of the oligohaline area of temperate estuaries (Ysebaert *et al*., 1998; Nebra *et al*., 2011; Whitfield *et al*., 2012); demonstrating in part their euryoecious character more than their euryhaline nature.

Additionally to the natural environmental stress affecting estuarine macroinvertebrate community, we identified one artificial factor directly affecting community ecology of Ebro estuary; river flow regulation creates an artificial environmental stability in the Ebro Estuary by maintaining stratification for long periods, and thus enhancing functional and structural features of the salt wedge. Environmental stability increases the ecotone perception, because in natural regime conditions the salt wedge can be flushed out from the river channel breaking the estuarine stratification in several occasions all over the year, especially during wet periods (Ibáñez, 1993). Under natural instability conditions, riverine-marine boundary (gradient zone) could be consequently wider in both time and space, probably making difficult the establishment of stenobiontic taxa, and thus resulting in a community dominated by better adapted euryoecious species (Elliot *et al.*, 2007). In these conditions, euryhaline taxa could extend their distribution ranges along the river channel following the marine intrusion (natural diel advance or retreat). In this situation, species nestedness increase and differentiate between the strictly fresh from the marine community is also more difficult; moreover, ecotone characteristics (abrupt salinity change and species turnover) may be obscured or even misidentified as an ecocline (see Remane diagram redrawn by Atrill and Rundle, 2002). Regardless the natural origin of Ebro riverine-marine interface, in the current context our results are consistent with those previously reported by Attrill and Rundle (2002) and Acha *et al.* (2015); they found that sharpest ecotones are because of anthropogenic activity, and their ecological effects are intensified with the persistence of conditions over time, not only over the physic environment but also over communities. Macroinvertebrate community complexity in the Ebro Estuary increased in both richness and diversity with the permanence of the salt wedge (*i.e.* stratification). Although these findings are supported by those of Elliot and Whitfield (2011), since as they suggested in an ecotone is expected a biodiversity increase due to the transition area between two adjacent systems. Nevertheless, the same authors pointed out that this pattern is not found in estuarine ecotones; therefore, we should consider the Ebro Estuary as the first documented case of an estuarine ecotone with really complex benthic communities *e.g.* macroinvertebrates and diatoms since, recently performed studies in the Ebro Estuary reported 160 different species of diatoms (Rovira *et al.*, 2012b).

CONCLUSIONS

Trying to respond to introduction questions: what occurs in rivers emptying into non-tidal seas? Should these river-mouths be considered special estuaries? Are stratified estuaries colonized by different species? Which is the most suitable distribution model for biota? Can this kind of riverine-marine boundary be considered an ecotone or ecocline? Despite of the lack of tide influence and a gradual salinity gradient, the

Ebro river-mouth must be considered functional and ecologically as an estuary at least in marine intrusion condition (Ibáñez, 1993). Both structural and functional differences with well-mixed estuaries are obvious (*e.g.* stratification, ecotone, community turnover); but the Ebro Estuary founds are consistent with the estuarine paradigms proposed by Perillo, 1995 and Elliot and Whitfield (2011). For instance, the Ebro Estuary shares important characteristics with north temperate mixed estuaries; it is fundamentally colonized by typical taxa from temperate estuaries such as the *Abra alba-Lagis koreni* species complex (Puente *et al*., 2008; Nebra *et al*., 2011; Reizopoulou *et al*., 2013). Furthermore, Nereid polychaetes and representatives of the genus *Corophium*, which are usually found inhabiting the oligohaline area (the most osmotic stressful zone) of mixed estuaries, were recorded just in the most stressful zone and less productive zone of the estuary, around the null point or ecotonal region. According to the most common biodiversity models (*e.g.* Remane, 1934; Attrill and Rundle, 2002; Telesh and Khlebovich, 2010) the Ebro Estuary presented an impoverishment bias from mouth to landwards, identifying its '*Artenminimum*' (*i.e.* the critical zone characterized by minimal species richness) in the null point surrounding area. The relationship of *Artenminimum* and salinity is still matter of controversy (see Conde *et al*., 2013 for detailed discussion). But according to our results, an ecotonal region is compatible with the concept of *Artenminimum*, supporting the assertion that organisms' distribution within estuaries (*i.e.* diversity patterns) is highly influenced more by variation than by absolute salinity regimes (Wolff, 1973; Reizopoulou *et al*., 2013). Therefore, we stand up for a re-definition of estuary concept with the aim of including all estuaries flowing into non-tidal seas, similarly as done by Potter *et al*., 2010, to include hypersaline estuaries.

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APPENDIX 1. Water physic-chemical parameters (annual mean and standard deviation) and sediment characteristics of the 9 sampling stations. Cond., conductivity; TDS, total dissolved salts; DO, dissolved oxygen; TSS, total suspended solids; OSS, organic suspended solids TOM, total organic matter in sediment.

	Upper Estuary stretch				Lower Estuary stretch				
	UE1	**UE2**	**UE3**	**UE4**	**LE5**	**LE6**	**LE7**	**LE8**	**LE9**
Mouth distance (km)	37.00	34.00	30.00	25.00	18.00	12.00	6.00	3.00	0.00
Salinity (g l^{-1})	0.65 ± 0.13	0.64 ± 0.13	0.64 ± 0.13	0.65 ± 0.13	26.98 ± 17.58	34.79 ± 2.66	35.34 ± 2.00	35.57 ± 2.20	28.40 ± 16.35
Cond.(mS cm^{-1})	1.11 ± 0.19	1.11 ± 0.19	1.11 ± 0.18	1.13 ± 0.18	36.62 ± 24.1	45.69 ± 6.09	46.49 ± 5.97	46.79 ± 6	38.14 ± 21.85
TDS (g l^{-1})	0.84 ± 0.16	0.84 ± 0.16	0.83 ± 0.16	0.85 ± 0.17	26.56 ± 17.18	34.28 ± 2.36	34.77 ± 1.77	34.97 ± 1.95	28.03 ± 15.64
DO (mg l^{-1})	9.43 ± 2.92	8.90 ± 2.92	9.18 ± 3.06	9.99 ± 3.23	6.21 ± 3.59	6.36 ± 3.36	6.82 ± 2.42	7.55 ± 2.20	8.41 ± 1.56
DO (%)	98.68 ± 21.50	93.18 ± 19.77	96.63 ± 21.50	105.10 ± 20.85	73.50 ± 34.82	80.90 ± 37.53	88.10 ± 26.72	98.48 ± 25.85	105.45 ± 17.76
Temperature (ºC)	18.33 ± 6.10	18.55 ± 6.12	18.78 ± 6.03	18.84 ± 6.06	18.68 ± 4.97	17.94 ± 4.39	18.10 ± 4.76	18.14 ± 4.74	18.33 ± 4.41
pH	8.03 ± 0.20	8.07 ± 0.16	8.11 ± 0.15	8.14 ± 0.18	7.91 ± 0.08	7.99 ± 0.24	8.01 ± 0.24	8.05 ± 0.21	8.10 ± 0.22
PO_4 (mg l^{-1})	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.01 ± 0.02
P_T (mg l^{-1})	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.02
NH_4 (mg l^{-1})	0.04 ± 0.05	0.05 ± 0.06	0.06 ± 0.03	0.13 ± 0.18	0.30 ± 0.30	0.06 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.07
NO_2 (mg l^{-1})	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.03	0.01 ± 0.02	0.01 ± 0.01	0.01 ± 0.02
NO_3 (mg l^{-1})	3.07 ± 1.19	2.94 ± 1.26	3.01 ± 1.32	2.89 ± 1.20	1.08 ± 1.98	0.87 ± 1.63	0.85 ± 1.6	0.49 ± 0.88	1.01 ± 1.93
N_T (mg l^{-1})	3.40 ± 1.36	3.43 ± 1.41	3.45 ± 1.39	3.47 ± 1.41	1.53 ± 2.57	1.38 ± 2.37	1.17 ± 1.93	0.78 ± 1.20	1.36 ± 2.44
SiO_4 (mg l^{-1})	1.26 ± 0.39	1.15 ± 0.52	1.20 ± 0.55	1.35 ± 0.40	1.03 ± 0.48	0.64 ± 0.64	0.39 ± 0.55	0.40 ± 0.38	0.46 ± 0.55
Chlorophyll (µg l^{-1})	2.65 ± 3.08	0.75 ± 0.47	0.87 ± 0.70	0.88 ± 0.72	0.91 ± 0.52	0.87 ± 0.95	1.56 ± 1.51	1.94 ± 1.45	1.37 ± 1.04
Pheophytin (µg l^{-1})	2.43 ± 3.02	0.99 ± 0.86	1.01 ± 0.85	1.12 ± 1.13	1.18 ± 0.73	0.78 ± 0.47	0.77 ± 0.56	0.61 ± 0.43	0.69 ± 0.32
Velocity (m s^{-1})	0.17 ± 0.14	0.30 ± 0.17	0.18 ± 0.09	0.22 ± 0.16	0.11 ± 0.19	0.07 ± 0.11	0.06 ± 0.01	0.08 ± 0.05	0.25 ± 0.19
Transparency (m)	2.79 ± 0.89	2.50 ± 0.42	2.21 ± 0.55	1.78 ± 0.21	1.93 ± 0.33	2.04 ± 0.28	1.8 ± 0.26	1.76 ± 0.35	2.15 ± 0.49
TSS (mg l^{-1})	9.21 ± 14.52	2.83 ± 2.25	4.85 ± 2.62	7.32 ± 4.07	14.43 ± 9.34	13.06 ± 10.69	12.52 ± 10.61	13.35 ± 12.87	32.06 ± 30.53
OSS (mg l^{-1})	2.41 ± 2.75	1.40 ± 0.85	1.59 ± 0.33	2.20 ± 0.75	3.43 ± 2.30	2.92 ± 2.18	2.61 ± 1.91	2.76 ± 2.35	4.21 ± 2.29
OSS (%)	48.05 ± 22.93	59.66 ± 26.71	37.27 ± 13.08	33.83 ± 13.72	25.87 ± 6.56	27.2 ± 9.76	23.94 ± 5.1	23.39 ± 4.13	19.62 ± 9.70
Mud (%)	12.36 ± 6.46	0.18 ± 0.11	31.56 ± 24.48	1.13 ± 0.88	1.91 ± 1.20	13.22 ± 6.04	16.69 ± 10.34	15.27 ± 0.54	32.36 ± 21.25
Sand (%)	86.69 ± 6.56	43.66 ± 16.41	66.53 ± 24.69	96.55 ± 2.70	97.86 ± 1.11	84.33 ± 4.24	82.47 ± 9.99	69.41 ± 12.75	65.13 ± 19.93
Gravel (%)	0.95 ± 0.11	56.16 ± 16.31	1.91 ± 0.21	2.33 ± 1.82	0.23 ± 0.08	2.45 ± 1.80	0.85 ± 0.35	15.33 ± 12.21	2.51 ± 1.91
TOM (%)	3.37 ± 0.76	1.30 ± 0.28	3.55 ± 0.24	2.47 ± 1.43	2.09 ± 0.24	4.86 ± 0.54	3.64 ± 1.30	5.07 ± 1.79	4.47 ± 1.04

CHAPTER III

Towards a suitable ecological status assessment of highly stratified Mediterranean estuaries: A comparison of benthic invertebrate fauna indices

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Towards a suitable ecological status assessment of highly stratified Mediterranean estuaries: A comparison of benthic invertebrate fauna indices

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ABSTRACT

Biotic indices developed to assess the ecological status of coastal waters according to the European Water Framework Directive (WFD) often show discrepancies when they are applied in transitional environments. Although several indices have been widely used in transitional waters throughout Europe, there is still a lack of knowledge about their suitability assessing ecological status. We evaluated the performance of most common used biotic indices and community parameters (*e.g.* Multivariate AZTI's Marine Biotic Index (M-AMBI), BENTIX, Benthic Opportunistic Polychaetes Amphipods index (BOPA), diversity indices, species richness, abundance) that have been proposed in the scope of WFD, using data of macroinvertebrate community coming from a special case of transitional water body, the highly stratified Ebro estuary. Additionally, we tested their ability to respond to the main pressures affecting the Ebro estuary, the hydrological alteration due to regulation and the pollution pressure due to nutrient enrichment. Estimation of

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hydrological alteration was based on flow historical data (period from 1913 to 1963), that we assumed as 'hydrological reference conditions' for Ebro estuary. Pollution pressure was estimated by means of PCA analysis including organic and nutrient enrichment related variables, expressed as a synthetic index by PCA factor scores extraction. All the community parameters were able to detect changes in macrofauna composition along the estuarine gradients and were able to differentiate between the impoverished stations and the healthier ones. Regarding indices, the ratings were contradictory and only M-AMBI classified the stations in the correct way. Strong significant correlations were found between indices and metrics and the calculated pressures; nevertheless, these correlations showed a paradoxical result, since increasing hydrological alteration benefited the macrofauna, achieving great complexity. Other identified limitations of biotic indices were the opposite classifications, overestimation of ecological status and low resolution ability. We conclude that for transitional water ecosystems, where each water body has particular characteristics, is difficult the use of 'common biological' assessment tools as the results of this study, among others (more details in discussion section), have demonstrated. Nevertheless, M-AMBI seemed to work in the correct way, so further investigation about its use for transitional waters is necessary. The development of new strategies such as the use of historical data, the use of metrics as a complement for the assessment could be a reliable alternative.

Keywords: Ecological status, stratified estuary, hydrological alteration, transitional waters, biotic indices, benthic indicators, Water Framework Directive.

INTRODUCTION

Estuaries are interface systems between rivers and sea, characterized by unstable hydrological, morphological and chemical conditions, resulting in stressful habitats where biological communities are structured along strong environmental gradients

(Day *et al*., 1989; Dauvin, 2007). Nevertheless, these complex ecosystems are largely recognized by their high productivity and their importance, from both economic and conservation perspectives (Ysebaert *et al*., 1998; Edgar *et al*., 1999; McLusky, 1999; Pierson *et al*., 2002; Russell *et al*., 2006).

The rapid human population growth during the last century has increased the pressures over these areas threatening their ecological integrity, their economic value and even affecting public health (Schlacher and Wooldridge, 1996; Edgar *et al*., 1999; McLusky, 1999; Dauer *et al*., 2000; Dauvin, 2007; Elliott and Quintino, 2007; Gray and Elliott, 2009). The main anthropogenic pressures affecting estuaries are industrial wastewater, urban sewage effluents, agriculture and farmland runoff, fish farming and harbors (Justic *et al*., 1995; McLusky and Elliott, 2004; Zaldívar *et al*., 2008; Gray and Elliott, 2009). These activities cause an excess of nutrients in Water Bodies (WBs), increase the organic matter loads and even promote the accumulation of dangerous pollutants in the sediment such as heavy metals, toxic compounds and hydrocarbon substances (Boynton *et al*., 1995; Day *et al*., 1997; Cantillo, 1998; Nedwell *et al*., 1999; Navarro-Ortega *et al*., 2010). High nutrient loads produce direct ecological impacts over biological communities (Karlson *et al*., 2002), mostly associated with eutrophication processes (Bock *et al*., 1999; Wang *et al*., 1999; Hänninen *et al*., 2000). Besides, organic enrichment causes episodes of hypoxia and low redox potential values. These facts disturb composition, trophic structure and biomass of the biological communities (Pearson and Rosenberg, 1978; Grebmeier *et al*., 1988; Díaz, 2001).

In Mediterranean aquatic ecosystems, the impacts produced by these pressures are magnified by the strong seasonal and interannual hydrological variability (Caiola *et al*., 2001a; Ferreira *et al*., 2007a, b). Moreover, human responses to this hydrological fluctuation involve flow regulation measures, such as reservoirs, that frequently disrupt aquatic ecosystems, producing accentuated environmental changes (Caiola *et al*., 2001b).

The European Union reacted to the severe ecological decline of aquatic ecosystems by proclaiming Water Framework Directive (WFD) in 2000 (European Parliament, 2000). The WFD provides a basis for the conservation, protection and improvement the ecological integrity of all WBs, including groundwater, inland surface water, coastal and transitional waters. According to the WFD the estuaries are classified as Transitional Waters (TWs); defining them as: bodies of surface water in the vicinity of river mouths which are partly saline in character as a result of their proximity to coastal waters but which are substantially influenced by freshwater flows.

Ecological quality assessment of a water body must be based on the status of different biological quality elements (*e.g*. benthic invertebrate fauna or aquatic flora) and endorsed by hydro- morphological and physicochemical quality elements. The status of these elements is determined by the deviation they exhibit from the type-specific reference conditions, at undisturbed or nearly undisturbed situations (WFD, 2000/60/EC -Annex V). Benthic invertebrates have been identified as key biological element for Ecological Status (ES) assessment of TWs; they play important roles in the ecology of aquatic ecosystems and respond to anthropogenic stress (Pearson and Rosenberg, 1978; Dauer, 1993; Grall and Glemarec, 1997 Dauer *et al*., 2000; Simboura and Zenetos, 2002; Bustos-Baez and Frid, 2003; Perus *et al*., 2007). Nevertheless, within estuarine ecosystems it is difficult to establish a stressor-response relationship using Biotic Indices (BIs) since they are naturally stressed ecosystems; this difficulty was coined as the term 'Estuarine Quality Paradox' (Dauvin, 2007; Elliott and Quintino, 2007).

Moreover, in highly stratified estuaries, like the study case, obtaining such a response is even more difficult because both natural and anthropic hydrological variations (spatial and temporal) produce rapid and abrupt changes in biological communities (Nebra *et al*., 2011). Therefore, establishing reference conditions for these systems (the basis for the development of BI according to the WFD criteria) is a challenging task.

Since the apparition of the WFD in the year 2000, some BIs based on soft-bottom benthic invertebrate communities such as the AZTI's Marine Biotic Index (AMBI) (Borja *et al*., 2000), the multivariate AMBI (M-AMBI) (Borja *et al*., 2004; Muxika *et al*., 2007), BENTIX (Simboura and Zenetos, 2002) and Benthic Opportunistic Polychaetes Amphipods index (BOPA) (Dauvin and Ruellet, 2007) have proved to be very useful tools in assessing the ES of coastal and TWs, especially regarding nutrient and organic enrichment. However, the estuarine systems where these indices were developed correspond to 'well-mixed' type, which are systems with different ecological dynamics compared with 'highly stratified' estuaries like the Ebro estuary.

The present study analyzes the performance of M-AMBI, BENTIX and BOPA indices developed under the scope of the WFD, to the main anthropic pressures on the Ebro estuary, a highly stratified Mediterranean estuary. It is expected that results obtained could assist on the development of a suitable ES assessment approach for salt wedge estuaries.

METHODS

2.1 Study site

The Ebro estuary (Fig. 1) is a highly stratified or salt-wedge estuary located at the NE of the Iberian Peninsula (40º43'10''N, 0º40'30''E). The microtidal regime of the Mediterranean Sea about 20 cm (Cacchione *et al*., 1990), promotes the formation of a salt wedge whose dynamics (advance, retreat and permanence) is controlled by the river discharge. Continuous river flow values exceeding 350-400 m^3 s^{-1} pushes the salt wedge from the river channel and the estuary becomes a river. Conversely, when the river discharge is lower than 100 m^3 s^{-1}, the salt wedge reaches its maximum distance upstream 30-32 km from the river mouth (Ibáñez *et al*., 1997); intermediate flows together with the bathymetry of river-bed placed the salt wedge in different positions (Ibáñez *et al*., 1996). The main land use in the basin (85,362 km^2) is agriculture with more than 10,000 km^2 of irrigation, corresponding to approximately

90% of the water usage in the basin (Ibáñez *et al*., 2008). The main human impacts in the lower Ebro river and therefore its estuary are the strong flow regulation in the whole basin by nearly 190 dams (Batalla *et al*., 2004) and the nutrient enrichment of river water due to the input of agricultural and urban sewage effluents (Sierra *et al*., 2002; Terrado *et al*., 2006; Falcó *et al*., 2010). Nevertheless, during the last 15 years, an improvement of urban sewage treatment together with the restriction in the use of phosphate-based compounds dimmed the eutrophication process (Ibáñez *et al*., 2008, 2012a, b).

2.2 Sampling design and laboratory procedures

In order to cover the whole study area nine sampling stations were established from the river mouth to 37 km upstream. This stretch included the estuarine freshwater reach potentially accessible by salt wedge (Fig. 1). Each station was sampled seasonally (July and October 2007; January and April 2008) for benthic macroinvertebrates, in each sampling occasion three sediment samples were collected using a Ponar grab (0.046 m^2), sediment grain size and total organic matter (TOM), dissolved oxygen (DO), chlorophyll a, pheophytin, total suspended solids (TSS), organic suspended solids (OSS), nutrient loadings: phosphate (PO_4), total phosphorous (P_T), ammonia (NH_4), nitrite (NO_2), nitrate (NO_3), total nitrogen (N_T) and hydromorphological char- acteristics (depth, flow velocity and water transparency) (see Nebra *et al*., 2011 for detailed sampling and analysis procedures).

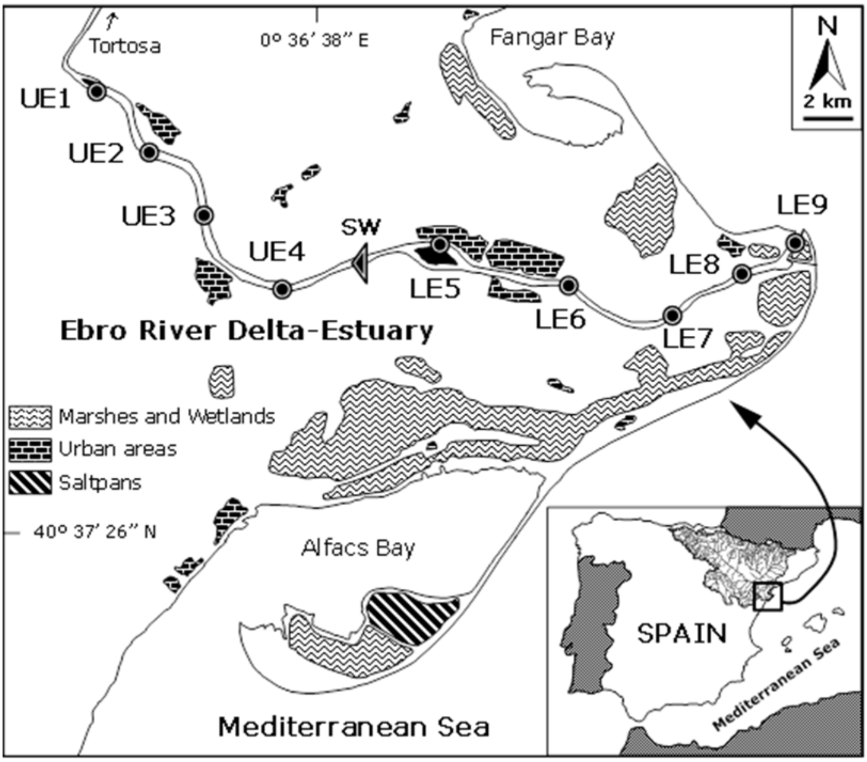

Figure 1. Map of the Ebro River basin and its delta showing the studied estuary with the position of the nine sampling stations. UE, upper estuary stations; LE, lower estuary stations; SW, null point position.

2.3 Biotic indices computation

The benthic macroinvertebrates of the Ebro estuary were structured in two contrasting communities associated with the upper estuary (UE) and lower estuary (LE) stretches, fresh and saltwater respectively; regardless of the sampling season due to maintained flows throughout the year (Nebra *et al.*, 2011). Therefore, the sensitivity of the BIs and metrics to human disturbance was analyzed separately, using specific BIs and metrics for these two stretches. For the UE, the applied BI was the Iberian Biological Monitoring Working Party (IBMWP) (Alba- Tercedor *et al.*, 2002) adapted for WFD requirements by Catalan Water Agency (ACA, 2006) see

Table 1 for ES Boundaries and further information. Some commonly used freshwater macro- invertebrate metrics were also computed; these were: the percentages of functional feeding groups (grazers, deposit feeders, parasites, predators and suspension feeders) and the number and ratios (total and relative) of invertebrate orders comprising sensitive taxa (Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, Bivalvia and Odonata). Regarding LE stations M-AMBI, BENTIX and BOPA were applied (Table 1); the computation of the three marine indices (M-AMBI, BENTIX and BOPA) is based on the frequencies of functional or ecological groups that are considered as metrics. In these cases, besides the BI score, the metrics' individual scores were also analyzed. The descriptions, codes and predictable response to human pressures of the computed indices and metrics are summarized in Tables 3, 4 and 5. Moreover, the number of taxonomic ranks (families and genera), Shannon-Wiener diversity, Margalef diversity, Simpson dominance and Pielou's evenness indices as well as community structure and abundance descriptors (total abundance, density and taxa richness) were calculated.

2.4 Hydrological alteration

Prior to river regulation in the early sixties, big floods were common in the lower Ebro (Ibáñez *et al*., 1996); the suppression of such floods together with minimum flow conditions in summer and autumn led to an altered salt wedge dynamics in the Ebro estuary. For this reason an estimation of hydrological alteration was made with the aim of quantifying the effect over the biological communities. The Hydrological Pressure for the Ebro estuary was expressed as the deviation of the salt wedge dynamics from the 'expected natural dynamics' that it would correspond to each sampling occasion. For this, the dynamics of the salt wedge (position, probability of occurrence and permanence time in days) was calculated for each sampling station and occasion as a function of the daily average river flows. The position was estimated following Ibáñez *et al*. (1996), permanence time in days was estimated by counting the accumulated days before each sampling occasion with mean flow values lower than 350 $m^3\ s^{-1}$ according to Ibáñez (1993), data available at Ebro Basin Authority web site (station 9027: Tortosa). Then, the dynamics of salt wedge (position, presence probability and permanence in days) was computed for each month and each sampling station during the period from 1913 to 1963 using Ebro Water Authority daily average flow values at Tortosa station. This time series represents the natural flow period, *i.e.* period before the construction of the two dams responsible for the lower Ebro regulation. The Hydrological Pressure was finally expressed as the absolute value of the deviation of the salt wedge presence (expressed as probability and time) from the monthly average probability and number of days of the salt wedge during natural flows period.

Table 1. Biotic indices value ranges and ES boundaries used in this paper to estimate the sampling stations ES categories.

Biotic index	Index value	ES	Index requirements
IBMWP	$100 < \text{IBMWP}$	High	**PRIP**: Decrease **TR**: Family **MSP**: D-frame net 500 µm, qualitative. **FA** : Freshwater
	$61 \leq \text{IBMWP} < 100$	Good	
	$35 \leq \text{IBMWP} < 61$	Moderate	
	$15 \leq \text{IBMWP} < 35$	Poor	
	$0 < \text{IBMWP} < 15$	Bad	
M-AMBI	$0.77 < \text{M-AMBI} \leq 1.00$	High	**PRIP**: Decrease **TR**: Usually genus or species level **MSP**: Grab, replicates, > 1mm, quantitative. **FA** : Coastal and transitional waters
	$0.53 < \text{M-AMBI} \leq 0.77$	Good	
	$0.39 < \text{M-AMBI} \leq 0.53$	Moderate	
	$0.20 < \text{M-AMBI} \leq 0.39$	Poor	
	$0.00 < \text{M-AMBI} \leq 0.20$	Bad	
BENTIX	$4.5 \leq \text{BENTIX} < 6.0$	High	**PRIP**: Decrease **TR**: Usually genus or species level **MSP**: Grab, replicates, > 1mm, quantitative **FA** : Coastal and transitional waters
	$3.5 \leq \text{BENTIX} < 4.5$	Good	
	$2.5 \leq \text{BENTIX} < 3.5$	Moderate	
	$2.0 \leq \text{BENTIX} < 2.5$	Poor	
	$0.0 \leq \text{BENTIX} < 2.0$	Bad	
BOPA	$0.00000 \leq \text{BOPA} \leq 0.04576$	High	**PRIP**: Increase **TR**: Genus and species level. **MSP**: Grab, replicates, > 1mm, quantitative **FA** : Coastal and transitional waters
	$0.04576 < \text{BOPA} \leq 0.13966$	Good	
	$0.13966 < \text{BOPA} \leq 0.19382$	Moderate	
	$0.19382 < \text{BOPA} \leq 0.26761$	Poor	
	$0.26761 < \text{BOPA} \leq 0.30103$	Bad	

2.5 Data analysis

Principal Component Analysis (PCA) was performed separately for each estuarine stretch (UE and LE) with the organic pollution related variables (DO, nutrients, chlorophyll a, pheophytin and organic matter in sediment and in suspension). Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was used to assess the usefulness of a PCA. KMO ranges from 0 to 1 and should be > 0.5 if variables are sufficiently interdependent for PCA to be useful (Tabachnick and Fidell, 2001). Once obtained the final PCAs, the two first factors of each PCA were merged by summing (inverting the values if their trends were opposed) as a combined index of Pollution Pressure. One way ANOVA followed by Post-hoc (LSD) test were carried out among stations for testing environmental parameters differences. To test the response of the BIs and metrics to the anthropogenic disturbance gradient, a correlation analysis was carried out with the Pollution Pressure index and with Hydrological Pressure index (probability and time). The measured variables were log or square root transformed (for absolute values and percentages, respectively) because homoscedasticity and linearity were clearly improved. Statistical analyses were performed using STATISTICA 8 software.

RESULTS

3.1 Benthic environmental condition

During the study period daily mean river discharge was always below 350 m^3 s^{-1} allowing the penetration of the salt wedge. This fact divided the estuary in two different stretches 'upper and lower estuary' (UE and LE); the UE (stations UE1-UE4) had freshwater (0.65 g l^{-1} ± 0.005) (Table 2); whereas the LE five stations (LE5- LE9) had marine water ranging from (26.98 g l^{-1} ± 17.58) to (35.57 g l^{-1} ± 2.20), ANOVA revealed no significant differences for salinity within each stretch. LE stations permanence time of salt wedge was different for each sampling occasion 52, 140, 254 and 341 days for summer, autumn, winter and spring, respectively. The null point or salt wedge's tip was always located at the same position, between UE4-LE5 stations (25-18 Km from river mouth). Regarding nutrient concentrations, in general terms were higher in river water than in sea water (Table 2); especially for phosphates, nitrates, total nitrogen and silicate. Regarding LE stretch, LE5 showed the highest nutrient values such as PO_4 (0.03 mg l^{-1} ± 0.01), P_T (0.06 mg l^{-1} ± 0.03) and NH_4 whose concentration value was five times greater than values reported for other stations (0.30 mg l^{-1} ± 0.30). Regarding LE stations, ANOVA ($P < 0.05$) revealed significant differences among LE5 and the rest of stations for PO_4 (post-hoc test, $P < 0.0307$ to 0.0438), P_T (post-hoc test, $P < 0.0297$ to 0.0448) and NH_4 (post-hoc test, $P < 0.0099$ to 0.0229). The other nutrients showed an increase tendency from LE9 to LE5, from river mouth to null point. DO values were higher in UE stretch ranging from (8.90 ± 2.92) to (9.99 ± 3.23); whereas in LE stations oxygen concentrations decreased upstream, the lowest values were recorded at stations close to the null point (Table 2). Chlorophyll a concentrations were slightly higher in LE stations but without significant differences between UE and LE stations

(0.25 µg l^{-1} ± 0.10 to 0.47 µg l^{-1} ± 0.07, respectively). Pheophytin levels in the UE stations were close to 1.00 µg l^{-1}; except for the station UE1 in which the pheophytin largely exceeded the 2.00 µg l^{-1}. The LE stations presented pheophytin levels ranging from 0.61 µg l^{-1} ± 0.43 to 1.18µg l^{-1} ± 0.73. The percentage of organic matter in sediment was slightly higher in LE stations ranging from (2.09 ± 0.24) to (4.86 ± 0.54); ANOVA ($P < 0.05$) revealed significant differences in TOM content, among stations UE1-UE2 (post-hoc test, $P < 0.0041$), UE2-UE3 (post-hoc test, $P < 0.0024$) and LE5 and the rest of stations (except LE7) (post-hoc test, $P < 0.0019$ to 0.0089).

The first two axes of the PCA for the UE explained 87.17% (62.00% and 25.17%, respectively) of the total variation. KMO (0.561) indicated the usefulness of the PCA; NO_2, NO_3 and NH_4 were positively correlated with the first PCA axis. In contrast, TOM, OSS were positively correlated with second axis. The first and second axis of PCA for the LE explained 53.4% and 23.89% respectively of the total variation. KMO (0.692) indicated the usefulness of the PCA; NO_2, NO_3 and PO_4

were positively correlated with the first PCA axis; the second axis was mainly related with NH_4 and OSS.

3.2 Macroinvertebrate abundance, taxa richness and diversity

A total of 21,805 individuals were collected belonging to 214 different taxa; for more detailed community results see Nebra *et al*. (2011). Higher densities were found at LE stretch (Fig. 2), especially at those stations near river mouth. LE9 station showed an annual mean of 11,650 ind m^{-2}. Intermediate density values were found in upper estuary stations due to the contribution of Tubificidae and the non-indigenous taxon *Corbicula fluminea*. The lowest densities corresponded to stations UE3, UE4, LE5 and LE6 located around null point. This pattern was similar in taxa richness terms (Fig. 2); station LE9 showed the highest richness values around 50 different taxa of annual mean. Stations located near river mouth LE8 and LE7 reached high values of richness too; whereas stations UE3, UE4 and LE5 showed the lowest values. Regarding diversity indices, all of them showed higher values in LE stations (Fig. 2) with a decreasing tendency towards null point.

Table 2. Water physic-chemical parameters (annual mean and standard deviation) and sediment characteristics of the 9 sampling stations.

	Upper Estuary stretch				Lower Estuary stretch				
	UE1	**UE2**	**UE3**	**UE4**	**LE5**	**LE6**	**LE7**	**LE8**	**LE9**
Mouth distance (km)	37.00	34.00	30.00	25.00	18.00	12.00	6.00	3.00	0.00
Salinity	0.65 ± 0.13	0.64 ± 0.13	0.64 ± 0.13	0.65 ± 0.13	26.98 ± 17.58	34.79 ± 2.66	35.34 ± 2.00	35.57 ± 2.20	28.40 ± 16.35
DO (mg l^{-1})	9.43 ± 2.92	8.90 ± 2.92	9.18 ± 3.06	9.99 ± 3.23	6.21 ± 3.59	6.36 ± 3.36	6.82 ± 2.42	7.55 ± 2.20	8.41 ± 1.56
DO (%)	98.68 ± 21.50	93.18 ± 19.77	96.63 ± 21.50	105.10 ± 20.85	73.50 ± 34.82	80.90 ± 37.53	88.10 ± 26.72	98.48 ± 25.85	105.45 ± 17.76
PO_4 (mg l^{-1})	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.01 ± 0.02
P_T (mg l^{-1})	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.02
NH_4 (mg l^{-1})	0.04 ± 0.05	0.05 ± 0.06	0.06 ± 0.03	0.13 ± 0.18	0.30 ± 0.30	0.06 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.07
NO_2 (mg l^{-1})	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.03	0.01 ± 0.02	0.01 ± 0.01	0.01 ± 0.02
NO_3 (mg l^{-1})	3.07 ± 1.19	2.94 ± 1.26	3.01 ± 1.32	2.89 ± 1.20	1.08 ± 1.98	0.87 ± 1.63	0.85 ± 1.6	0.49 ± 0.88	1.01 ± 1.93
N_T (mg l^{-1})	3.40 ± 1.36	3.43 ± 1.41	3.45 ± 1.39	3.47 ± 1.41	1.53 ± 2.57	1.38 ± 2.37	1.17 ± 1.93	0.78 ± 1.20	1.36 ± 2.44
SiO_4 (mg l^{-1})	1.26 ± 0.39	1.15 ± 0.52	1.20 ± 0.55	1.35 ± 0.40	1.03 ± 0.48	0.64 ± 0.64	0.39 ± 0.55	0.40 ± 0.38	0.46 ± 0.55
Chlorophyll (µg l^{-1})	2.65 ± 3.08	0.75 ± 0.47	0.87 ± 0.70	0.88 ± 0.72	0.91 ± 0.52	0.87 ± 0.95	1.56 ± 1.51	1.94 ± 1.45	1.37 ± 1.04
Pheophytin (µg l^{-1})	2.43 ± 3.02	0.99 ± 0.86	1.01 ± 0.85	1.12 ± 1.13	1.18 ± 0.73	0.78 ± 0.47	0.77 ± 0.56	0.61 ± 0.43	0.69 ± 0.32
TSS (mg l^{-1})	9.21 ± 14.52	2.83 ± 2.25	4.85 ± 2.62	7.32 ± 4.07	14.43 ± 9.34	13.06 ± 10.69	12.52 ± 10.61	13.35 ± 12.87	32.06 ± 30.53
OSS (mg l^{-1})	2.41 ± 2.75	1.40 ± 0.85	1.59 ± 0.33	2.20 ± 0.75	3.43 ± 2.30	2.92 ± 2.18	2.61 ± 1.91	2.76 ± 2.35	4.21 ± 2.29
OSS (%)	48.05 ± 22.93	59.66 ± 26.71	37.27 ± 13.08	33.83 ± 13.72	25.87 ± 6.56	27.2 ± 9.76	23.94 ± 5.1	23.39 ± 4.13	19.62 ± 9.70
Mud (%)	12.36 ± 6.46	0.18 ± 0.11	31.56 ± 24.48	1.13 ± 0.88	1.91 ± 1.20	13.22 ± 6.04	16.69 ± 10.34	15.27 ± 0.54	32.36 ± 21.25
Sand (%)	86.69 ± 6.56	43.66 ± 16.41	66.53 ± 24.69	96.55 ± 2.70	97.86 ± 1.11	84.33 ± 4.24	82.47 ± 9.99	69.41 ± 12.75	65.13 ± 19.93
Gravel (%)	0.95 ± 0.11	56.16 ± 16.31	1.91 ± 0.21	2.33 ± 1.82	0.23 ± 0.08	2.45 ± 1.80	0.85 ± 0.35	15.33 ± 12.21	2.51 ± 1.91
TOM (%)	3.37 ± 0.76	1.30 ± 0.28	3.55 ± 0.24	2.47 ± 1.43	2.09 ± 0.24	4.86 ± 0.54	3.64 ± 1.30	5.07 ± 1.79	4.47 ± 1.04

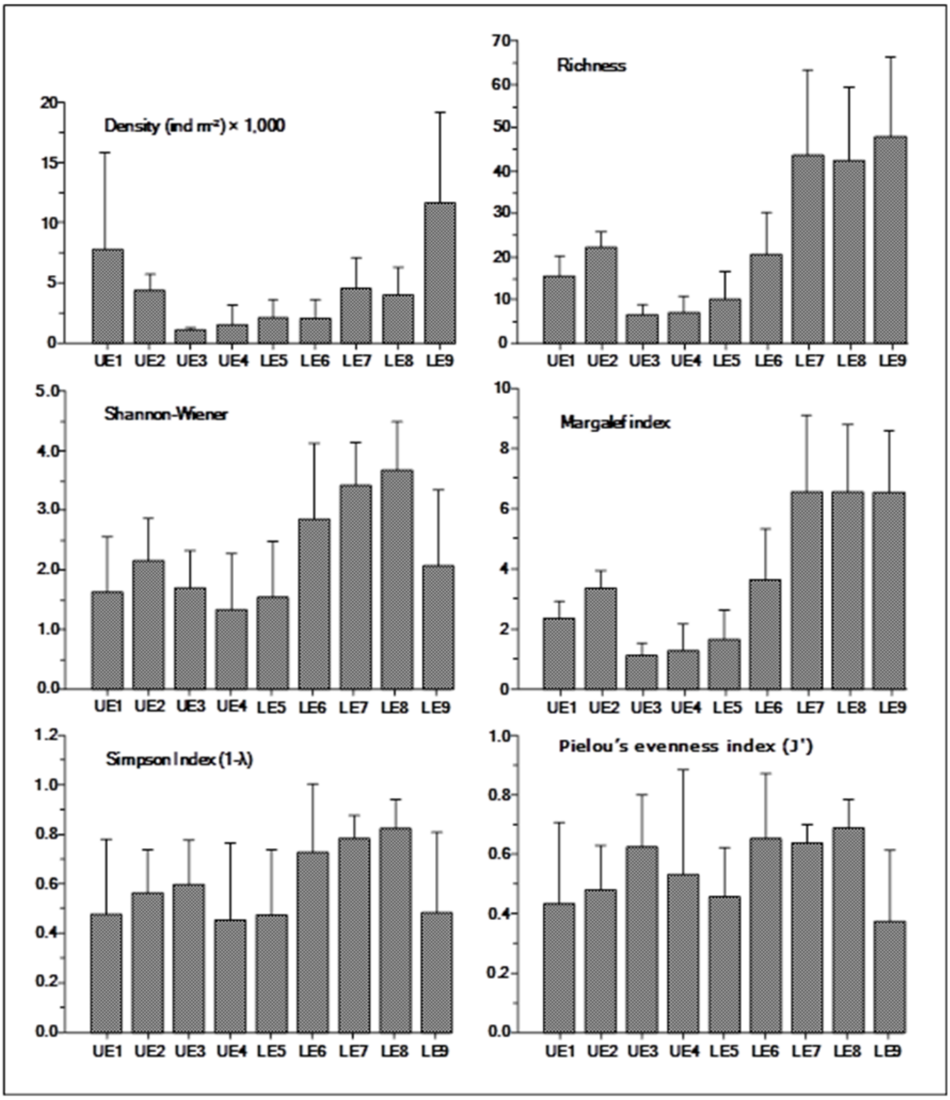

Figure. 2. Community descriptive parameters (annual mean ± standard deviation bars, *n* = 4) recorded at each station. See Fig. 1 for sampling stations' codification.

3.3 Benthic biological indices and individual metrics

Due to the dynamic of the Ebro estuary specific BIs and metrics were computed for each stretch (UE and LE).

IBMWP

All the families found in UE stations computed for IBMWP calculation. Concerning UE stations, 12.4% were classified as 'Good', 18.8% as 'Moderate', 31.3% as 'Poor' and 37.5% as 'Bad'. There wasn't any station achieving 'High' ES. The worst ES ratings corresponded to stations UE3 and UE4 which ranged between 'Bad' and 'Poor' (Fig. 3); UE2 ranged between 'Moderate' and 'Good' achieving this category in summer and spring. Station UE1ratings ranged from 'Bad' to 'Moderate'. Spearman correlation coefficient reported significant correlations among IBMWP and 10 analyzed variables (Table 3). The Pollution Pressure showed strong negative correlation with IBMWP. Regarding community parameters richness measures, density and Margalef index showed significant and strong positive correlation with IBMWP. Total organic matter in sediment showed significant negative correlation with IBMWP.

M-AMBI

The percentage of non-scoring taxa in LE stations was very low (0.14% ± 0.30). results showed that 25.00% of LE stations were classified as 'High', 45.00% as 'Good', 15.00% as 'Moderate' and 15.00% as 'Poor'; there were no 'Bad' ES rating (Fig. 3). Worst ES ratings corresponded to LE5 which ranged between 'Poor' and 'Moderate' (Fig. 3); LE6 ranged between 'Poor' and 'Good' reaching this category in the last three sampling occasions. LE7-LE8 showed 'Good' and 'High' ratings in the two first and two last sampling occasions respectively. The LE9 ES ranged between 'Moderate' and 'High'.

Table 3. Significant Spearman correlation coefficients among the BI scores, hydrological pressure expressed as the deviation of wedge occurrence probability and as deviation of permanence time from natural flow regime conditions, pollution pressure, environmental parameters and the community descriptive parameters (UE stretch n = 16; LE stretch n = 20).

Calculated Pressures	**IBMWP**	**M_AMBI**	**BENTIX**	**BOPA**
H. Pressure (p)	–	0.624[b]	-0.570[b]	0.530[a]
H. Pressure (days)	–	0.660[b]	-0.599[b]	0.554[a]
P. Pressure	-0.505[a]	-0.484[a]	0.520[a]	-0.474[a]
Environ. parameters				
DO (mg l^{-1})	–	–	-0.489[a]	–
NH_4 (mg l^{-1})	–	-0.490[a]	0.464[a]	–
P_T (mg l^{-1})	0.580[a]	–	–	–
TSS (mg l^{-1})	–	–	–	-0.476[a]
OSS (mg l^{-1})	–	-0.458[a]	–	-0.512[a]
TOM (%)	-0.648[b]	0.529[a]	-0.552[a]	0.502[a]
Community parameters				
Richness (S)	0.940[b]	0.902[b]	-0.838[b]	0.516[a]
Number of Families	0.957[b]	–	–	–
Number of Genera	0.933[b]	–	–	–
Density (ind m^{-2})	0.523[a]	0.501[a]	-0.588[b]	–
Margalef index (d)	0.942[b]	0.933[b]	-0.825[b]	0.582[b]
Pielou's evenness (J')	–	0.629[b]	–	0.663[b]
Shannon-Wiener index (H')	–	0.863[b]	–	0.742[b]
Simpson index (1-λ')	–	0.758[b]	–	0.707[b]
Deposit feeders (%)	–	–	0.570[b]	–
Grazers (%)	0.816[b]	–	–	–
Predators (%)	–	0.748[b]	-0.887[b]	0.589[b]
Suspension Feeders (%)	-0.573[a]	–	–	–

DO, dissolved oxygen; P_T, total phosphorous; TSS, total suspended solids; OSS, organic suspended solids; TOM, total organic matter in sediment.

[a] $p < 0.05$.

[b] $p < 0.01$.

Spearman correlation reported significant correlations among M-AMBI and 13 analyzed factors (Table 3). The Hydrological Pressure as probability and time reported strong positive correlation with this index; conversely the Pollution Pressure showed negative correlation. Regarding community parameters, all of them showed significant and strong positive correlation with M-AMBI. Ammonium was negatively correlated with M-AMBI.

BENTIX

Similarly to M-AMBI, BENTIX index showed similar percentages of non-scoring taxa 0.18% ± 0.30. Within LE stretch, the 25.00% of stations were classified as 'High', 5.00% as 'Good', 55.00% as 'Moderate' and 15.00% as 'Poor'; there were no 'Bad' ES ratings. Contrary to M-AMBI, best ES ratings corresponded to LE5 which ranged between 'Moderate' and 'High'; this rating was achieved in three of the sampling occasions (Fig. 3). LE6 ranged between 'Poor' and 'High' achieving 'Moderate' rating in two sampling occasions. LE7-LE8 showed similar pattern with 'Moderate' category in the three last sampling occasions; station LE9 ratings ranged between 'Poor' and 'Moderate'. Spearman correlation coefficient reported significant correlations among BENTIX and 11 analyzed variables (Table 3). Both measures of Hydrological Pressure showed strong negative correlation with BENTIX, nevertheless the Pollution pressure was positively correlated. Richness and Margalef index showed significant and strong negative correlation with BENTIX. Regarding environmental parameters, DO showed negative correlation with BENTIX.

BOPA

According to this index, the benthic estuarine condition ranged between 'High' and 'Poor' ES categories; there were no 'Bad' ES rating. A 45.00% of LE stations were classified as 'High', 25.00% as 'Good', 20.00% as 'Moderate' and 10.00% as 'Poor'. Best ES ratings corresponded to station LE5 which reached 'High' ES in all sampling occasions; following LE9 with 'High' category in three of the four sampling occasions (Fig. 3). LE6 ranged between 'High' and 'Poor' ES and together with LE8 were the only ones reaching 'Poor' rating with BOPA. LE7 ratings ranged between 'Moderate' and 'High' and showed 'Moderate' category in two sampling occasions; LE8 was the only station that did not reach 'High' ES rating with BOPA.

Spearman correlation analysis indicated significant correlations among BOPA and 12 analyzed variables (Table 3). BOPA was positively correlated with Hydrological Pressure and negatively with Pollution Pressure. With regard to community descriptive parameters, richness, Margalef index, Pielou's evenness, Shannon-Wiener's diversity index and Simpson index showed significant strong positive correlations with BOPA; TSS and OSS showed negative correlation with BOPA.

Individual metrics

Spearman correlation coefficients indicated strong significant negative correlations among Pollution Pressure for UE stretch and metrics such as Shannon-Wiener index and EPT (see Table 4 for data and abbreviations). In contrast, Hydrological pressure for UE reach was strong and positively correlated with the percentage of EPT (%) and EP/Total (%), despite of the predicted response to increasing perturbation (PRIP) for these two metrics. Spearman correlation coefficients indicated strong significant correlations among Pollution Pressure for LE stretch and six metrics in the expected way of their PRIP. Nevertheless, four metrics *e.g*. BENTIX- GI (%) a sensitive species group, showed a correlation not according with their PRIP. Hydrological pressure for LE stretch showed strong correlations with the great part of the metrics; nevertheless these correlations were not according with their PRIP (Table 5).

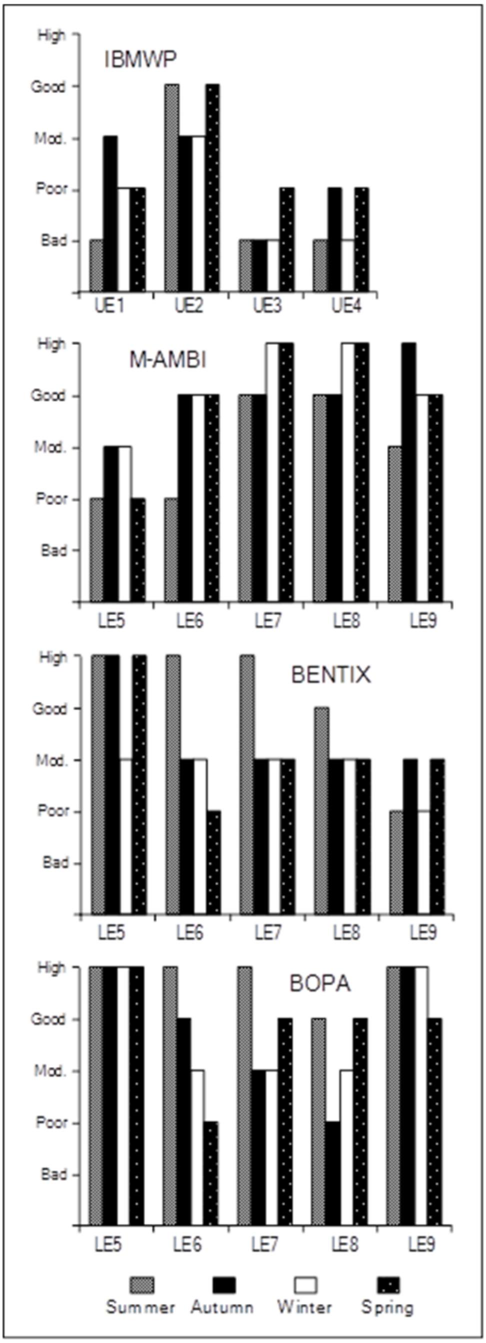

Figure. 3. Ecological status classification of UE and LE stations recorded at each sampling occasion after applying the four different BIs: IBMWP, M-AMBI, BENTIX and BOPA. See Fig. 1 for sampling stations' codification.

Table 4. Significant Spearman correlation coefficients among the UE Hydrological Pressure expressed as the deviation of wedge occurrence probability from probability in natural flow regime conditions, UE Pollution Pressure, community descriptive parameters and the individual metrics ($n = 16$).

	PRIP	H. Pressure (p)	P. Pressure
Shannon-Wiener index (H')	Decrease	−	-0.522[a]
EPT	Decrease	−	-0.662[b]
EPT (%)	Decrease	0.518[a]	-0.605[a]
EPT/oligochaeta	Decrease	−	-0.575[a]
EPT/diptera	Decrease	−	-0.698[b]
EP	Decrease	−	-0.666[b]
EP/total taxa (%)	Decrease	0.542[a]	−
EPTCBO	Decrease	−	-0.599[a]

PRIP, predicted response to increasing perturbation; EPT, ephemeroptera, plecoptera and trichoptera; EP, ephemeroptera and plecoptera; EPTCBO, ephemeroptera, plecoptera, trichoptera, coleoptera, bivalvia and odonata.

[a] $p < 0.05$.

[b] $p < 0.01$.

Table 5. Significant Spearman correlation coefficients among the LE Hydrological Pressure expressed as the deviation of wedge occurrence probability and as deviation of permanence time from natural flow regime conditions, LE Pollution Pressure and the individual metrics (*n* = 20).

	PRIP	H. Pressure (p)	H. Pressure (days)	P. Pressure
Richness (S)	Decrease	0.609[b]	0.639[b]	-0.578[b]
Number of Families	Decrease	0.581[b]	–	–
Number of Genera	Decrease	0.548[a]	–	–
Margalef index (d)	Decrease	0.629[b]	0.686[b]	-0.584[b]
Pielou's evenness (J')	Decrease	0.450[a]	0.511[a]	–
Shannon-Wiener index (H')	Decrease	0.604[b]	0.654[b]	–
Simpson index (1-λ')	Decrease	0.542[a]	0.577[a]	–
Predators (%)	Decrease	0.585[b]	0.637[b]	-0.559[a]
Bentix_GI (%)	Decrease	-0.534[a]	-0.558[a]	0.503[a]
Bentix_GIII (%)	Increase	0.597[b]	0.519[a]	–
Bentix_Tolerant (%)	Increase	0.566[b]	0.596[b]	-0.498[a]
BOPA_Amphip.	Decrease	-0.496[a]	-0.596[b]	0.609[b]
BOPA_Polych.	Increase	0.591[a]	0.651[b]	-0.474[a]
AMBI_GI (%)	Decrease	–	0.470[a]	-0.488[a]
AMBI_GII (%)	Decrease	–	0.504[a]	–
AMBI_GIII (%)	Increase	–	-0.509[a]	0.487[a]
AMBI_GIV (%)	Increase	0.510[a]	0.583[b]	–
AMBI_GV (%)	Increase	0.513[a]	–	–
AMBI_Sensitive (%)	Decrease	–	0.495[a]	-0.469[a]

PRIP, predicted response to increasing perturbation.
[a] $p < 0.05$.
[b] $p < 0.01$.

DISCUSSION

4.1 Anthropogenic pressures and environmental condition of Ebro estuary bottom

Currently, main anthropogenic pressures affecting Ebro estuary are nutrient enrichment and alteration of the natural flow regime. Nutrient loadings of Ebro River are consequence of the inputs of wastewater mainly from agriculture and urban areas on the whole Ebro basin (Lacorte *et al*., 2006; Terrado *et al*., 2006). Water analysis revealed similar nutrient concentrations as recorded by Sierra *et al*. (2002) and Falcó *et al*. (2010). Despite of severe eutrophication episodes occurring in the recent past

(Ibáñez *et al*., 1995), during the last decade the chemical status of Ebro estuary has clearly improved. Nutrient inputs have considerably decreased in the whole basin (Sierra *et al*., 2002; Ibáñez *et al*., 2008) limiting the primary production and therefore the sedimentary input of organic matter throughout the halocline by entrainment processes, alleviating the impact over estuary bottom (Ibáñez *et al*., 1995).

Conversely, the hydrological alteration produced by regulation could be considered the most important anthropogenic pressure affecting lower Ebro River and therefore its estuary. Regulation maintains a decreasing tendency of annual mean flow due constant increment on water demand, mainly for hydroelectric power generation and agricultural irrigation (Ibáñez *et al*., 1996, 2008; Sierra *et al*., 2004; Falcó *et al*., 2010). Moreover, natural variations of river discharge are homogenized through the year buffering the Mediterranean seasonality (Muñoz and Prat, 1989).

The worst impact caused by regulation over estuary ecology, excluding sediment retention by damming and the associated habitat loss and delta regression (Ibáñez *et al*., 1996), is the alteration of salt wedge dynamics. In natural regime flow conditions the salt wedge could advance till its maximum on dry periods or even totally disappear during high flow periods (for more details see Ibáñez *et al*., 1995, 1996; Movellán, 2004). However, the actual homogeneity in river discharges assures the presence of the salt wedge virtually in the same position for long periods (Ibáñez *et al*., 1995; Sierra *et al*., 2002; Falcó *et al*., 2010). This fact was corroborated by results obtained in this study (Fig.1); Ebro estuary remained divided in two contrasting stretches (UE and LE) according with the physicochemical parameters recorded, and supported by the results obtained after analysis of macro-invertebrate community (Nebra *et al*., 2011).

Coupled to long periods of salt wedge the water quality is worsening below the halocline due low water renewal rate; since freshwater flushing events are important for removing the accumulated pollutants and materials (Pierson *et al*., 2002). The entrainment processes between layers allows sedimentation of suspension particles and died organisms from the upper layer to the bottom (Lewis, 1997). This input of organic matter together with the sewage effluents discharged by two urban areas located downstream (Fig.1) and suspension materials coming from the sea undergo a progressive accumulation towards the null point by frontal convergent circulation (Largier, 1993). The chemical reactions at the sediment surface releasing nutrients and organic matter decomposition (Stanley and Nixon, 1992; Pierson *et al*., 2002) could explain nutrient and oxygen concentrations found at LE5-LE6 stations (Table 2). The accumulation of diverse materials could promote eutrophication and oxygen depletion through microbial consumption (Stanley and Nixon, 1992; Largier 1993; Pierson *et al*., 2002) as occurred in the 90's decade. Despite water anoxia was not recorded at any station of LE stretch, certain hypoxia with a declining gradient in DO from river mouth to null point was identified during study period; conversely, oxygen values for UE stretch were not a limiting factor. Benthic macro-invertebrates were more exposed to high nutrient levels and low dissolved oxygen concentrations from mouth to null point, benthic condition improved upstream once overcome null point. In summary, the Ebro estuary is recovering its chemical status approaching it to 'chemical reference conditions', on the other hand regulation is distancing the Ebro estuary from 'hydrological reference conditions', whose for this type of estuaries means great hydrological variation, this fact is especially relevant due to Mediterranean climate seasonality.

The novelty of this study lies in the way to quantify anthropogenic pressures; pollution pressure was estimated by PCA analysis including organic enrichment related variables, expressed as a synthetic index by PCA factor scores extraction. Estimation of hydrological alteration was based in historical data (period from 1913; to 1963), that we assume as 'hydrological reference conditions' for Ebro estuary. This way, allowed us to evaluate deviation of salt wedge dynamics from natural condition and therefore its relevance for estuarine ecology. We identify an increase on occurrence probability and permanence time for LE stretch and just the opposite for UE stretch.

4.2 Response of macroinvertebrate community to increasing perturbation

Differences found in macroinvertebrate community within each estuary stretch seemed to be independent from sediment grain-size and salinity. The lack of salinity gradient and the small variations recorded in sediment composition suggested other factors causing changes in community, such as the accumulation of nutrients or pollutants, the degree of exposition, organic matter, oxygen saturation or the water renewal among others (Carvalho *et al.*, 2006). The impoverishment tendency followed by macroinvertebrate community in LE stretch was in accordance with nutrient enrichment and hypoxia gradients described. The variability of the community parameters showed a marked spatial difference among the studied stations. In an overall view richness, density and diversity indices decreased progressively along nutrient enrichment and oxygen depletion gradient from river mouth to the null point; once overcome this stressed zone these parameters increased again upwards (Fig. 2). Regarding LE stretch, those stations with degraded condition benthos (LE5 and LE6) had fewer species, lower abundances and lower diversity values than stations with a healthier benthos (LE7, LE8 and LE9). Thus, the community parameters seemed to characterize the stations coherently with the environmental gradients identified in the estuary bottom. Nevertheless, there are other possible explanations for community impoverishment tendency land- wards; only euryhaline taxa are able to displace simultaneously with salt wedge's tip, its advance and retreat varied during day with discharge fluctuations depending on electric power genera- tion demand. This fact force stenobiontic taxa to disappear. Seawards this stress disappears increasing the stability of abiotic factors; then the complexity of community is recovered as suggested by Sousa *et al.* (2006). Moreover, stations located near mouth (LE7, LE8 and LE9) can be easily recolonized by constant input of species from adjacent marine areas (Teske and Wooldridge, 2001). Also, Josefson and Hansen (2004) pointed out the low velocity of salt water flux into the estuaries as a cause of low richness by regulating larval dispersal from adjacent sea areas. Despite we found long periods of salt wedge's permanence, time enough to assure the colonization of stations LE7 and LE8. These stations demonstrated to be especially complex in composition, showing high richness and density values. Thus 'elasticity' (rapid community recovery), that is promoted by the presence of undisturbed communities in the vicinity of a particular site (Muxika *et al*., 2005), was assured. The option of daily stress at stations close to null point seemed to be plausible and complementary with oxygen-nutrient gradient and not mutually exclusive.

4.3 Biotic indices and metrics suitability

Results obtained in this study evidenced that suitable ecological status assessment of TWs is a really complex task. It is important to consider overall hindrances and limitations concerning TWs, such as description of reference conditions or identification of typologies, as well as the study case's particular ones. In the Ebro estuary case we found assessment difficulties due to its particular characteristics (stratified estuary). With respect to UE stations, the IBMWP showed no correlation with Hydrological Pressure; probably due to IBMWP was designed to assess impact of organic enrichment or because deviations obtained from natural flow regime were quite small to reflect them in a clear impact to freshwater reach community. Nevertheless, results showed that IBMWP and several metrics, *e.g*. Shannon-Wiener index or EPT, responded to Pollution Pressure in the expected way (negatively correlated), according with the PRIP. Despite of the sampling method used was not the most suitable for IBMWP; it showed an acceptable discriminatory ability identifying four ES categories.

Concerning LE stations, the response of community parameters and individual metrics was the expected showing negative correlation with the Pollution Pressure, except for those metrics being part of BENTIX and BOPA (Tables 3 and 5). Nevertheless, this study revealed a paradoxical response to increasing Hydrological Pressure as probability or permanence time; keeping in mind that the expected response for a pressure is a clear negative impact over biological communities (richness loss, enhance high abundance of few species and low diversities), correlation analysis revealed that Richness, diversity indices and M-AMBI showed a positive response to increasing hydrological alteration just the opposite of their PRIP. Thus, hydrological alteration led to an artificial stability of abiotic conditions facilitating the prompt achievement of 'environmental homeostasis', this promote the substitution of macroinvertebrate community by other community best struc-tured, integrated by typical members of coastal areas and with a great complexity comparing with community found in other temperate estuaries (Nebra *et al*., 2011).

A common problem with ES assessment of estuaries is the impossibility of distinguishing natural from anthropogenic stress, both act over biological communities in the same way with the consequent problem of ES underestimate possibility; this was called 'estuarine quality paradox'. Nevertheless, the Ebro estuary is suffering its own 'quality paradox', since the most important anthropogenic pressure identified nowadays is dimming the natural stress associated with hydrological variation of a typical salt-wedge estuary of the Mediterranean region. This problem is causing an overestimation of all community parameters and therefore of the ES.

Regarding BIs ratings, the discrepancies among BIs were great and evident. For the same station and sampling occasion ratings could range between 1 and 3 ES levels depending on the applied BI. Only a small percentage of overlap was observed (Fig. 3). The problem of disagreement among BIs have been documented in many other studies (Reiss and Kroncke, 2005; Labrune *et al*., 2006; Simboura and Reizopoulou, 2008). Furthermore, for the Ebro estuary case the ES classification of the stations was contradictory; M-AMBI ranked stations in contrary way than BENTIX and BOPA. M-AMBI values showed a decreasing tendency towards null point where are located the stations with worst environmental condition and an impoverished benthic commu- nity. Conversely, BENTIX and BOPA gave to these perturbed stations the maximum ratings (Fig. 3); however, this fact was especially surprising for BENTIX based on the same paradigm (Pearson and Rosenberg, 1978). BENTIX showed a clear decreasing tendency towards river mouth, demonstrating that its ratings were opposed to the values of richness, abundance and diversity indices. The mismatch of the BENTIX in the Ebro estuary was not surprising in the manner as Simboura and Zenetos (2002) previously described the limitations of its use in TWs (*i.e.* estuaries and coastal lagoons). Moreover, according to Simboura and Reizopoulou (2008), this could be related to the different design of each index (in BENTIX each ecological group weighted equally, whereas AMBI renders a different coefficient for each one).

Regarding BOPA index, it did not seem to work adequately for either of stations or sampling occasion, it was not able to distinguish among stations and main causes of stress for LE macroinvertebrate communities. Since, applying BOPA most of the stations showed 'High' and 'Good' ES rating. This BI showed a similar tendency of BENTIX giving better ES classification to those stations with highest nutrient values and impoverished macroinvertebrate community. The problem of BOPA lied on its low discrimination ability and bias to overestimate the ES; similar results were reported by other authors in different TWs systems along Mediterranean coasts (Pranovi *et al.*, 2007; Munari and Mistri, 2007; Afli *et al.*, 2008; Blanchet *et al.*, 2008; de-la-Ossa-Carretero *et al.*, 2009). Probably the explana- tion is that this BI was essentially developed to assess hydrocarbon spill impact over benthic invertebrate communi- ties; in the way that amphipods, the main component of BOPA, are recognized to be sensitive to hydrocarbons (Gesteira and Dauvin, 2000, 2005; Dauvin and Ruellet, 2007). Thus, BOPA did not carry the same bias than M-AMBI and BENTIX for its adaptation to natural muddy bottoms (Blanchet *et al.*, 2008). Other, tendency observed in the BIs applied for the LE stations is that their ratings were so high (none of them found 'Bad' ES and in contrast showed elevated percentages of 'High' and 'Good' ES); BIs generally tended to show low resolving power and to overestimate the ES when are applied under different conditions they were develop for (Pranovi *et al.*, 2007; Zettler *et al.*, 2007; Bouchet and Sauriau, 2008; de-la-Ossa-Carretero *et al.*, 2009; Tataranni and Lardicci, 2010).

CONCLUSIONS

Study outcomes suggest that a different approach for the assessment of TWs is necessary; particular characteristics of each study case difficult the use of 'wide-spread' assessment tools, even more when hydrological features are gaining relevance on ES assessment with respect to water and sediment quality. Analysis of community parameters (abundance, biomass, richness and diver- sity indices) and individual metrics seems to be the correct way to a suitable environmental assessment (they are easier to interpret and can be more broadly applicable than BIs). Identify metrics such as Shannon-Wiener index, Richness, Margalef index or EPT that respond to anthropogenic pressures and integrate them in a multimetric index could be a reliable complement to BIs. Finally, this study establishes a baseline approach to cope with the assessment difficulties not only for the Ebro estuary but also for other Mediterranean estuaries suffering hydrological alteration.

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5 GENERAL DISCUSSION

The present discussion aims to give a general perspective of the main outcomes presented in the previous chapters and to justify the extracted conclusions of this dissertation. Since the main characteristics of the Ebro Estuary were already described in the general introduction section, this general discussion was mainly focused on different issues as the current estuarine condition and, the main factors determining estuarine benthos functioning; the effects of hydrological alterations due to water abstraction and regulation; and the relevance of eutrophication diming of river water. This was accomplished by using benthic macroinvertebrate community as the unifying thread of this dissertation. In general, the present thesis demonstrated that the Ebro Estuary ecosystem is hydrologically altered and suffers an important anthropogenic stress, showing a high probability of stratification and salt wedge state. Besides, the stable and optimal water and sediment characteristics influenced the current structure and ecology of the macroinvertebrate community. Finally, the results of the present dissertation suggested that macroinvertebrate community can be used as a suitable bioindicator in the assessment of anthropogenic impacts on the Ebro Estuary and, by extension, in other Mediterranean estuaries.

5.1 Recent changes on estuary environmental condition and its influence on the benthic macroinvertebrate community

The environmental condition of the Ebro Estuary suffered important changes during the last decades mostly as a result of two main causes, the current water regulation and management program, and the improvement of river water trophic state (see

Chapters I, II). On one hand, in river-dominated estuaries, freshwater inflow determines water column and bottom environmental condition (mainly, causing variation in water features and substrate), and therefore, the ecology of benthic communities (Schroeder *et al*., 1990; Kurup *et al*., 1998; Breault *et al*., 1999). Disturbances due to regulation and water abstraction on hydrology, sediment transport and consequently, on the ecology of rivers and estuaries, are well documented (Drinkwater and Frank 1994; Alber, 2002).

In the case of the lower Ebro River, several studies provided important ecological and hydrological data concerning the disturbances caused by water abstraction and regulation on the Ebro Delta-Estuary complex (Guillén *et al*., 1992; Guillén and Palanques, 1992; Ibáñez *et al*., 1995, 1996, 2008; Sierra *et al*., 2004). However, none of them was completely focused on estuary benthic condition and its influence on biological communities. The impacts of flow regulation in the lower Ebro River (*e.g*. sediment retention by damming associated to habitat loss and delta regression and altered hydrodynamics) began in the late 60's, when dams were built in some important tributaries. These impacts continued increasing during the 70's decade, after the construction of great dams in the main channel scarcely 100 km from river mouth. During the last years, the water management scheme in the lower Ebro River conducted to long periods of salt wedge state in the estuary (highly altered salt wedge hydrodynamics) with direct consequences for benthic environmental condition. The Mediterranean seasonality was concealed since water flow pulses were eliminated; thus the ejection of marine intrusion and the formation of 'fluvial estuarine stretch' (Ibáñez, 1993) was unusual and ephemeral, river and marine water mixing was scarce and restricted to halocline, and the reduction in turbulence maintained water column stratified in marine reach stations, moreover, quality of salt wedge water got worsen as consequence of the low water renewal rate as suggested by Drinkwater and Frank, 1994 and by frontal convergent circulation that tend to accumulate suspended materials in the front of the wedge (Largier, 1993). Finally, the removal of accumulated materials and contaminants associated to high flows was also unusual.

Consequently, under these continued conditions, the benthic community could have greater exposure to low dissolved oxygen events (Holland *et al.*, 1987) and higher sediment contaminant levels (Brown *et al.*, 2000) below the halocline. However, paradoxically, the quasi-permanent presence of salt wedge has an advantageous effect on the estuary bottom ecology, and therefore, for the establishment of biological communities. The environmental stability, associated to long periods of salt wedge, reduced the natural hydrological stress (advance, retreat, stratification breakdown, fluvial estuarine stretch formation), promoting the improvement of environmental conditions. Stability benefits counteracted the harmful effects of salt wedge water quality loss, mainly in null point surrounding areas. This fact, together with a constant input of potential food resources and organisms from adjacent areas, provided the possibility of increasing the complexity of the benthic community as suggested by Sousa *et al.* (2006).

On the other hand, during the last decades, the river experienced a significant decrease in nutrient loadings (see Casamayor *et al.*, 2001; Ibáñez *et al.*, 2008, 2012a, b). Essentially, the reduction in phosphate compounds by law and the improvement of water treatment plants, led to the oligotrophication process reducing the river primary production, since phosphorous limits production in freshwater environments (Correl, 1999); and therefore, the organic matter contribution to the estuary bottom (inputs by settlement and flocculation). This oligotrophication together with the lack of sediments retained in the big dams upstream, increased water transparency allowing the penetration of light to marine water layer; the total oxygen depletion or hypoxia events in the salt wedge were reduced to the minimum, not being restrictive for benthic biota. In the 90's decade, the primary production associated to eutrophication in the lower Ebro River created an excess of organic matter input to the estuary bottom due to high nutrient loadings together with high transparency of water since the damming retention provoked low sediment transport. The oxidation of this organic matter by microorganism, consumed the whole available oxygen producing severe anoxic episodes in the salt wedge. Thus, oxygen deficiency was the limiting factor for the establishment of biological communities (Muñoz, 1990; Ibáñez *et al*., 1995). Nowadays, oxygen condition of estuary benthos is not limiting and so, all estuarine aerobic processes are assured; the primary production of both river and sea water layers are within normal limits, and also river water transparency allowed the activity of primary producers in salt wedge. All together assures the oxygenation needed for organic matter processing and biological communities' development, even with organic matter accumulation towards the salt wedge tip, causing some hypoxia events that frequently occur below the pycnocline (Holland *et al*., 1987). Definitely, both oligotrophication and flow regime are the main drivers of the current environmental condition of Ebro Estuary, the first assuring the required water quality for biological processes, and the last one altering salt wedge dynamics which reduces hydrological stress.

Taking into account the environmental quality and the current macroinvertebrate communities recorded in the Ebro Estuary (**Chapter I**), it is noticeable the enhancement of the health of the Ebro Estuary benthos, when comparing with data before the 1990's decade (Ibáñez *et al*., 1995). The shift on benthic condition favored the macroinvertebrate community recovery, from a very poor community dominated by a few tolerant species (Ibáñez *et al*., 1995), to a very complex macroinvertebrate community (**Chapter I**); not only in richness terms but also in trophic structure, suggesting a great amount of resources and interactions of food webs as suggested by Brown *et al*. (2000). The macroinvertebrate community of the Ebro Estuary took advantage of this environmental condition, and colonized this estuary showing greater a complexity when compared to other temperate estuaries (**Chapters I, II**). In addition, after a community disturbance, such as the formation of fluvial estuarine stretch or marine intrusion clear out events (continuous flows over 350-400 m^3s^{-1}), the community elasticity (rapid recovery) is assured by the presence of undisturbed communities in the vicinity, acting as a source pool of species (Muxika *et al*., 2005); that, in the case of the Ebro Estuary are the adjacent marine shallow areas, that show similar environmental condition and host similar species complex (Sardà and Martín, 1993; Cartes *et al*., 2007, 2008; De Juan and Cartes, 2011; Jordana *et al*., 2015). The community inhabiting the Ebro Estuary was similar to communities registered in other temperate estuaries, and typical soft-bottom species complex were found in the Ebro Estuary too (C**hapters I, II**): Moreover, the euryhaline species were dominant surrounding null point (the most stressful area of the estuary). Nevertheless, the species richness in the Ebro Estuary increased due to the contribution of marine species coming from those adjacent shallow areas, since the entrance of marine origin species into the estuary (in the order of 6 kilometers upstream) was possible by the similar environmental conditions found in the salt wedge together with permanence time enough to arrive in spite of the limited dispersal abilities and slow salt wedge velocity upstream.

5.2 Estuarine macrofaunal trends across a stressful riverine-marine boundary

The boundary between freshwater and marine ecosystems at the head of estuaries has received little attention from aquatic ecologists (Rundle *et al*., 1998); despite that, estuaries, because of their heterogeneity, constituted the perfect frame for research on ecological boundaries in aquatic systems. This is the reason why **chapter II** is focusing on the Ebro Estuary riverine-marine boundary. Since, estuaries provide the potential to study benthic ecology of highly dynamic systems, in both physicochemical and biological terms. Additionally, the transition between fresh and marine communities, containing specialized euryoecious taxa, may be of a particular importance in the study of patterns and ecological processes, over a community dominated by species at the extreme of their range of tolerance. Some authors realized about the relevance of ecological boundaries in ecosystems processes (*e.g.* immigration, emigration, species replacement, edge effects) and so they focused part of their research in estuarine boundaries (*e.g.* Attrill and Rundle, 2002; Elliot and Whitfield, 2011; Whitfield *et al*., 2012; Basset *et al*., 2013; Conde *et al*., 2013), in these cases the research was conducted in mixed estuaries. The present thesis studies, for the first time, benthic macroinvertebrates along the whole length of a salt wedge Mediterranean estuary, including freshwater stretch potentially reachable by marine intrusion in natural regime conditions (mainly in summer or drought periods). The transition between fresh and estuarine systems in this kind of estuary represents a unique boundary in temperate estuaries, which is another relevant reason for promoting the Ebro Estuary conservation.

The use of univariate and multivariate statistical analyses allowed to distinguishing different regions within the Ebro Estuary according to their macrofauna and in concordance with environmental scenario previously discussed (see **Chapters I**, **II**). Salinity gradient and sediment grain size are the main abiotic factors affecting ecological, chemical and physical characteristics of estuaries, and consequently biological communities distribution (Remane and Schlieper, 1971; Dauer, 1993; Ysebaert *et al.*, 1993; Attrill and Thomas, 1996; Mannino and Montagna, 1997; Edgar *et al.*, 1999; among others). Well mixed estuaries have a relatively extensive boundary or gradient zone where fresh and marine water encounter, this gradient zone allows the exchange of suspended materials, organisms and obviously salts that creates the salinity gradient; for this reason, this boundary represents composite ecosystems where the exchange of materials and organisms is inherent to the water mixing. However, in highly stratified estuaries, the salt wedge represented an actual barrier between fresh and marine environments; the lack of powerful mixing drivers such as tides, waves, currents and winds, restricts the exchange of materials and organisms. The friction between descending superficial freshwater layer, with ascending deeper and denser marine layer, creates turbulence waves resulting in water mixing; but still, the power of this friction is not enough to break down the stratification or create a salinity gradient; but in turn, allows the formation of an interface layer, the halocline or pycnocline, within this thin layer occur the water mixing and the exchange (mostly by entrainment) of materials and salts.

In the case of the Ebro Estuary, the discharge regulation reduces flow velocity, softening turbulence and so the mixing between fresh and marine water layers. Thus, the halocline in the estuary is well established (Sierra *et al*., 2004). The thickness and depth of the halocline decreases landwards in the same way as the turbulence power (Sierra *et al*., 2004); consequently, the gradient zone in the estuary bottom is a narrow area located in the salt wedge tip (null point) where the halocline meet bottom. The confined gradient area, between the two homogeneous ecosystems identified in the Ebro Estuary (UE and LE stretches), has extreme consequences on the ecology of benthic communities. A sharp spatial change in ecosystem features implies stressful condition for biota, the ecological optimum of species is rapidly exceeded. In general, organisms have two alternatives when encountering stressful conditions: either they migrate to more favorable environments, or they remain trying to accommodate to the changing conditions; nevertheless, only euryoecious species are able to survive due to a remarkable range of physiological adaptations to variable environmental conditions (Brusca and Brusca, 1990). Species inhabiting a specific ecosystem usually have similar tolerance ranges and their ecophysiological constraints prevent the pass from one ecosystem to another if changes are not gradual. The exchange of species between stretches in the estuary is inexistent and the replacement of species is almost total (only a few specialized species are able to withstand this great variation *e.g*. nereid polychaetes). This community distribution is known as turnover pattern, and usually occurs in contrasting boundary ecosystems *e.g.* sea-surface microlayer, lotic-lentic systems. On the contrary, a wider gradient area or extensive boundary (as found in well mixed estuaries) allows species to resist to slight and gradual changes in environmental features; this helps species to colonize a more extensive area within estuaries, and only when the tolerance range of species is gradually exceeded, they disappear. Therefore the exchange of species is greater comparing with salt wedge estuaries. This distribution pattern of overlapped populations *i.e*. nestedness**,** is commonly found in well-mixed estuaries (Attrill and Rundle, 2002) and other merging ecosystems *e.g.* intertidal areas, forest-meadow transition.

Regarding salt wedge estuaries, and more specifically to the Ebro Estuary, there was a lack of functioning of riverine-marine boundary research, for this reason one of the main outcomes of this dissertation, together with the community turnover, was the identification of ecotonal boundary characteristics (abrupt change in environmental conditions, community shift and narrow transition zone between two homogeneous ecosystems) (see **Chapter II**). Probably the ecotonal perception within the Ebro Estuary was exaggerated by the artificial stability in salt wedge hydrodynamics, as a result of anthropogenic activity (mainly water abstraction and regulation) as suggested by Acha *et al*. (2015). Another characteristic of the Ebro Estuary boundary was the richness impoverishment tendency towards the null point; this bias was observed for both fresh and estuarine communities, and it was discussed in detail in **chapters II and III.** Several plausible causes were suggested in these chapters *e.g.* the sharp environmental change, the nutrient enrichment, hypoxia gradients or limited dispersal abilities; among others. As stated in the **chapter III**, these causes seemed to be cumulative and not mutually exclusive; but, in ecological boundary terms, this trend can be attributable to the edge effects concept: referred to changes in population sizes, species richness, or other aspects of the ecology of individuals, populations, or communities at the boundary between two adjacent habitats (Baker *et al*., 2002; Levin, 2009). The edge effects at the riverine-marine boundary of the Ebro Estuary have a negative influence over the biological communities; probably due to the harsh conditions close to salt wedge tip: salinity change, no net water movement (for this reason is denominated null point), sedimentation, pollutants accumulation and water mixing turbulence, converting it in an high stressful area; the stress increasing is a main factor causing simplification in communities (Martin *et al*., 1993; Saiz-Salinas and González-Oreja, 2000; Sousa *et al*., 2006). Additionally, anthropic disturbances can exacerbate the edge effects because they usually intensify the connectivity loss among adjacent habitats (Levin, 2009). Despite the natural-origin of this estuarine boundary and its intrinsic low connectivity (density difference between layers prevail over mixing drivers), the altered hydrodynamics of the salt wedge reduce the natural connectivity between fresh and marine habitats; steady and predictable river discharges reduces the water mixing, the organisms exchange, the possibility stratification break due to flow pulses, the materials and organic matter removal; and therefore, edge effects are more pronounced than those expected from natural regime flows.

Regardless the lacking of a well stablished salinity gradient, the Ebro Estuary shared relevant characteristics that are intrinsic of well mixed estuaries; the macroinvertebrate community impoverishment tendency landwards was similar to those described for other estuaries (Remane, 1934; Rundle *et al*., 1998; Attrill, 2002; Attrill and Rundle, 2002; Whitfield *et al*., 2012; Barros *et al*., 2014); and the community composition has typical estuarine species (as shown in the previous section). These findings evidence that in spite of the special characteristics of the Ebro Estuary and, not only in ecological functioning but also in its influence over macroinvertebrate community, the community response is analogous to those recorded for well mixed estuaries and the only difference is the way that ecological processes occur in the Ebro Estuary *e.g.* sharp changes instead gradual ones or confined instead extensive gradients.

5.3 Assessing the anthropogenic pressures on the Ebro Estuary and the potential of macroinvertebrates as bioindicators in estuarine environments.

As shown in the general introduction section, the estuaries are aquatic ecosystems especially susceptible of suffering many forms of anthropogenic stress. Estuaries together with coral reefs, are considered the most threatened among the coastal ecosystems (Millennium Ecosystem Assessment, 2005). Despite the strong environmental legislation promulgated with the aim to preserve aquatic ecosystems around the world *e.g.* CWA, WFD; they keep deteriorating as a result of human's actions for example dredging, damming or water withdrawal and regulation in the Ebro Estuary. The biological resources (not only biodiversity *per se*) in aquatic ecosystems are still declining, and the aquatic biota has become homogenized by local extinction events, the introduction of alien species, and genetic diversity loss (Karr and Chu, 1999, and references therein). The explanation is simple, humans degrade aquatic ecosystems in numerous ways; in order to illustrate the origin of environmental degradation of Ebro Delta-Estuary complex, an adaptation of Vasconcelos *et al*. (2007) conceptual model (developed for Portuguese estuaries) is presented in the figure 5.1. This conceptual model shows the main pressure sources (Population, Industry, Agriculture, Fishing, Dredging, Port Activities and Regulation) impairing the Ebro Delta-Estuary complex. Together, all these pressures are breaking its ecological balance and disrupting its biological communities (**Chapter III**). As can be seen in the model, the ecological endpoints are caused by the synergistic interaction among pressures and impacts and not only by a one single factor. In fact, this model can be applied for elsewhere estuary in the world, because the enumerated pressures, impacts and effects represented are a widespread issue concerning estuarine ecosystems (Extence *et al*., 1999; Solis-Weiss *et al*., 2004; Marín-Guirao *et al*., 2005; Millennium Ecosystem Assessment, 2005; Muxika *et al*., 2005; Vasconcelos *et al*., 2007; Day *et al*., 2012).

Among the numerous pressures and impacts identified in the figure 5.1, river flow regulation and organic-nutrient enrichment were recognized as the most important disrupting the estuarine condition, and therefore, biological communities (Nebra *et al*., 2011; Rovira *et al*., 2012b). The **chapter III** of this dissertation focused on the screening of macroinvertebrate based indices and metrics that could potentially be used to assess the ES of Mediterranean estuaries. This was done under the guidance of the WFD, *i.e.* analyzing the response of indices and metrics to the main human induced pressures by means the use of biological monitoring procedures, assessment tools based on bioindicators or BQEs, *e.g.* macroinvertebrates. Human life depends on biological systems for food, air, water, climate control, waste assimilation and other essential goods and services (Costanza *et al*., 1997); this is the main reason to assess aquatic ecosystems in terms of their biological condition (not only paying attention to chemical condition), and the unique criteria for judging if an anthropic

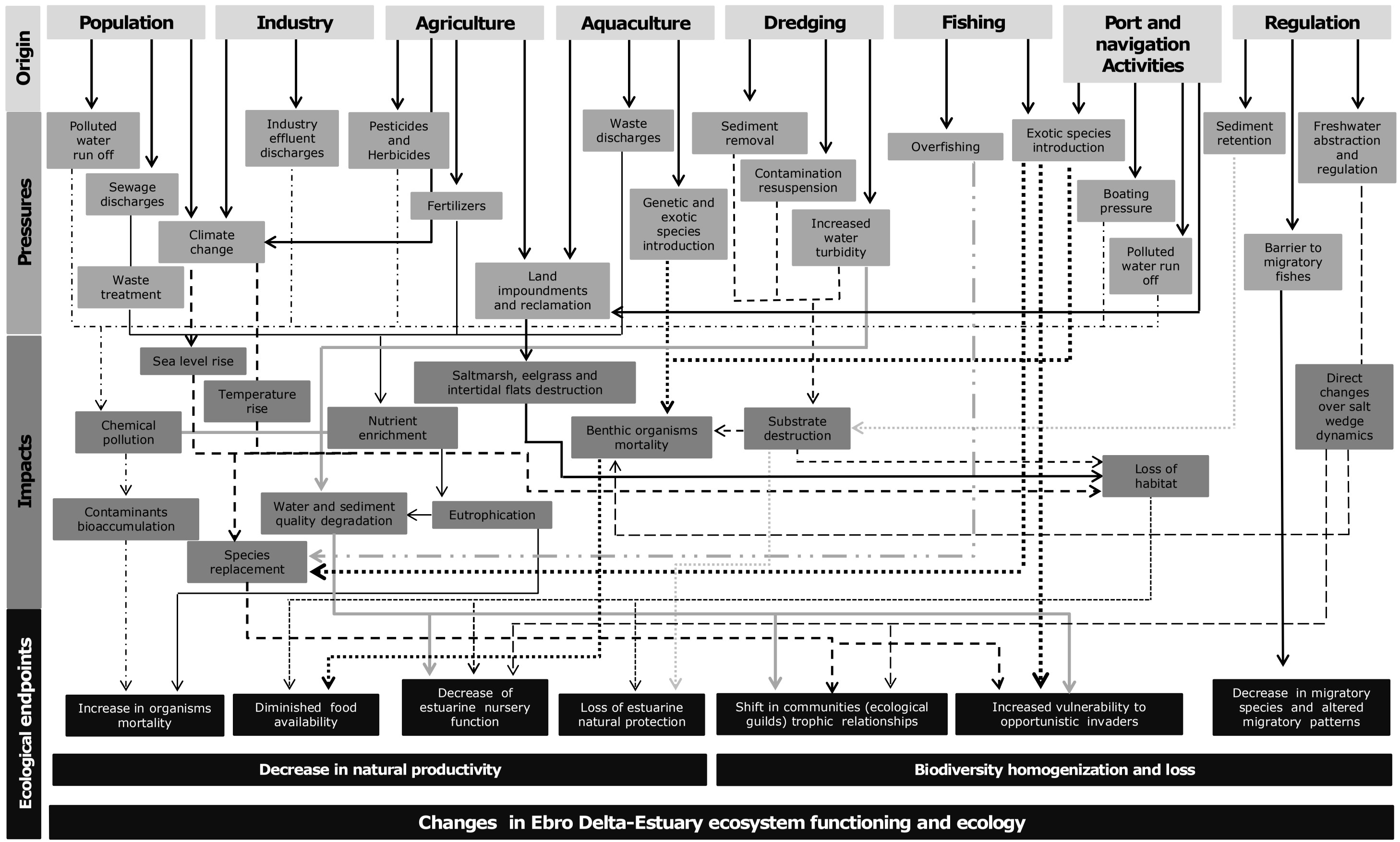

Figure 5.1. Ecological conceptual model for the assessment of the main pressures impairing the Ebro Delta-Estuary complex (modified from Vasconcelos *et al*., 2007)

activity has an impact, must be explicitly biological (Karr and Chu, 1999); in conclusion, biological monitoring is the basis for biological resources protection.

The terms bioindicator or BQE are referred for those species or groups of species that can be used to monitor the environment or ecosystem health. Bioindicators have the ability of reveal the qualitative status of the environment by means of measurable attributes (*e.g*. richness, relative abundance or trophic structure). Macroinvertebrates are usually employed as bioindicators due to their sensitiveness to environmental variability (natural disturbances or human-induced impacts). The results obtained in the **chapter III** confirmed the bioindicator potential of macroinvertebrates; since, the community responses to environmental changes in the Ebro Estuary were evident along spatial and temporal scales, demonstrating their sensitiveness. The impact concept has an intrinsic pejorative or negative mean. However, the human-induced environmental disturbances can cause positive or negative effects over biological communities. According to this, and with the first section of general discussion (*Recent changes on estuary environmental condition and its influence over benthic macroinvertebrate community*), it is expected that a good and suitable bioindicator would be able to reveal both positive and negative effects by means of measurable responses. In the case of Ebro Estuary, an example of measurable positive response is the community richness, abundance and complexity achieved, after long periods of altered salt wedge hydrodynamics (hydrological pressure). On the contrary, community impoverishment (richness and abundance) towards null point is a good example of negative response to impacts or environmental degradation (pollution pressure) (for detailed discussion see **chapter III**).

The main outcomes obtained in the **chapter III** were concerning ES assessment difficulties in estuarine environments due to their particular ecological characteristics. When trying to evaluate the effects of human-induced impacts over macroinvertebrate community, there are numerous biological community attributes that can be measured (quantified mainly as a metrics or BIs) to reveal that effect (Figure 5.2). However, only certain attributes provide useful and reliable information; therefore, the selection of which ones are characterizing properly the impact is a difficult task. For example, several metrics and BIs (*e.g.* richness, abundance, IBMWP and M-AMBI) showed a significant negative correlation with the assessed pressures *e.g.* pollution pressure. At the same time, there is a point related with metrics and BIs suitability, the relevance of previous knowledge about their PRIP in the assessment process (Karr and Chu, 1999). Sometimes, assessment tools, *i.e.* metrics and BIs, can show a significant correlation with any pressure, for example the case of BENTIX and BOPA indices in relation with pollution pressure (see table 3 of **chapter III**). According to their PRIP, it is expected lower ES ratings with increasing pressure, but the results were just the opposite. Both indices gave higher ES ratings to those stations with impoverished macroinvertebrate community, and vice versa. Then, a priori knowledge of their PRIP, allowed identifying the mismatch between expected and final responses, consequently both BIs were discarded for the assessment. For this reason, it is essential for a correct assessment to known metrics and BIs PRIP, with the aim to understand and interpret properly the results that are giving.

Additionally, the PRIP information allowed identifying a relevant and paradoxical result in the case of the Ebro Estuary pressures assessment. The outcomes of **chapter III** revealed that none BQE showed the same sensitiveness to the different pressures disrupting an ecosystem.

Most assessment tools developed in recent studies (Grall and Glémarec, 1997; Weisberg *et al.*, 1997; Borja *et al.*, 2000; Simboura and Zenetos, 2002; Rosenberg *et al.*, 2004) have been based on Pearson and Rosenberg (1978) model; this authors state that macrofaunal communities change in diversity, abundance and species composition according to their intolerance along a gradient of organic enrichment. Dauvin and Ruellet (2007) developed a specific BI to assess the impact of oil spills in marine environments; thus, the great majority of these tools are based on chemical pollution paradigms. However, many of these BIs have been applied indiscriminately to detect a great variety of anthropogenic disturbances; such as, dredging, dumping, engineering works, sewerage plans, gravel extraction, among others (Muxika *et al.*, 2005). Certainly, most BIs respond on any kind of disturbances whether caused by anthropogenic impact or natural processes (Wilson and Jeffrey, 1994), but their suitability must be analyzed carefully.

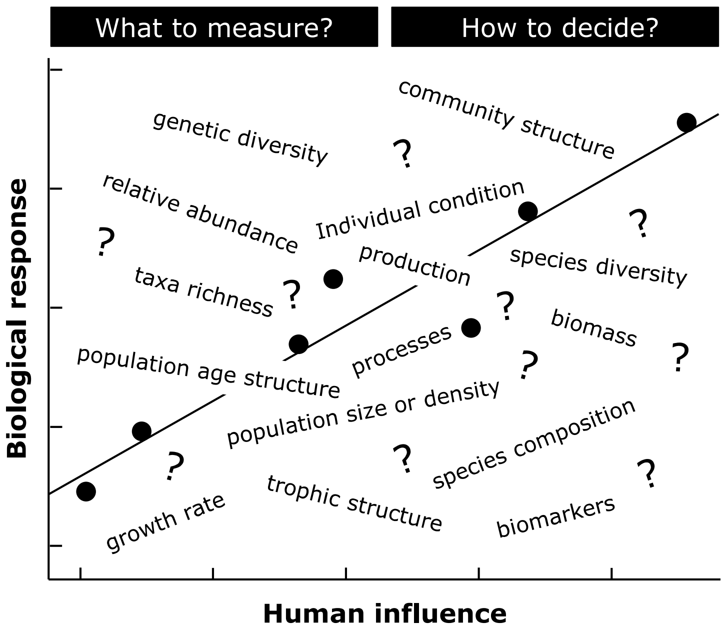

Figure 5.2. Almost any biological attribute can be measured, but only certain ones provide reliable information about biological condition and therefore for a suitable biological assessment (adapted from Karr and Chu, 1999).

In **chapter III**, an attempt to evaluate the impact of hydrological alteration over benthic macroinvertebrates was made by applying generalist BIs, *i.e.* non-specific tools for hydrological pressure assessment. This pressure was estimated as deviation from natural flows historical data (see **chapter III**). The outcomes revealed significant correlations among hydrological pressure and several metrics and BIs; paradoxically, this correlations revealed a positive effect of increasing hydrological alteration over macroinvertebrate community. The increase of salt wedge permanence promoted the substitution of the macroinvertebrate community, by other best structured and integrated by typical members of coastal marine areas; this caused an overestimation of all community parameters and therefore of the ES. Then, the positive effect described was actually a negative effect; since species substitution is a typical impact in hydrological disrupted ecosystems; for example, the river stretch affected by the increase in velocity flow regimes due to hydroelectric power plant activity. The new velocity regimes imply the establishment of rheophilic species or substitution of detritivores by scrapers or filter feeders specialist. Alterations in community structure may occur as a direct consequence of varying flow patterns or indirectly through associated habitat change (Extence *et al.*, 1999), as in the hydroelectric plant or as in the Ebro Estuary examples respectively.

In the Mediterranean region, the associated impacts to hydrological pressures are magnified by the strong seasonality. Thus, human responses to assure water resources for industrial and domestic consumption involve strong flow regulation measures that frequently disrupt aquatic ecosystems (Caiola *et al.*, 2001a, b). Hydrological issues are gaining relevance on ES assessment in relation to water and sediment quality **(chapter III)**, not only by the strong regulation in the basins, but also by the global climate change. Global warming is changing weather patterns causing alterations on hydrological regime, periods of drought are becoming more frequent (Mawdsley *et al.*, 1994) and declines in precipitation is resulting in diminished or disappearing river flows (Extence *et al.*, 1999). The lack of suitable assessment tools to appraise hydrological alteration, adds another limitation on the TWs assessment process. It is important to consider overall hindrances and limitations concerning TWs for achieving a suitable ES assessment. Common issues for TWs assessment are the reference conditions description or typologies identification in this heterogeneous group. Additionally, the particular characteristics of each study case may influence the assessment reliability as demonstrated **chapter III** outcomes. Regarding the biological monitoring, some criticisms were related with the use of BIs, (i) they represent a static expression of ecological status; (ii) they are not explicitly linked to changes in ecological function; (iii) they may not be specific with respect to different kinds of stressors; (iv) they are subject to underlying taxonomic changes across estuarine gradients; (v) they can be labor intensive (*e.g.*, sorting specimens and taxonomic identification); and (vi) they are not applied consistently across biogeographic areas (Racocinski and Zapfe, 2005). In conclusion, the research on estuarine ecosystems, and concretely in salt wedge Mediterranean estuaries, represents an attractive challenge in terms of biological, ecological and preservation perspectives.

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6 CONCLUSIONS

The main conclusions of this dissertation are the following:

1. As a consequence of the altered salt wedge dynamics, the Ebro Estuary can be divided into two separate functional units (upper and lower stretches) in terms of benthic environmental condition and macroinvertebrate community. Upwards the salt wedge, a riverine ecosystem was found without marine influence and colonized by a freshwater community. Conversely, the estuarine ecosystem was represented in the salt wedge stretch, highly influenced by marine intrusion, not only defining benthic environmental condition, but also determining macroinvertebrate community.

2. The long periods of salt wedge state were provoked due to flow regulation and water abstraction in the lower Ebro River. Regulation maintained the river discharge under the limit of marine intrusion penetration, allowing the establishment of the salt wedge and avoiding the formation of the 'fluvial estuarine stretch'.

3. Current macroinvertebrate community composition of the Ebro Estuary differed considerably from the community found in the early 90's, when the anoxic conditions below the halocline, caused by an excess of eutrophic origin organic matter inputs, prevented the establishment of benthic biological communities.

4. The Ebro estuary showed an exceptionally rich and complex macroinvertebrate community (comprising 213 different taxa) when compared to other temperate estuaries. Mollusks, polychaetes and crustaceans were the dominant groups in both richness and abundance.

5. In spite of the obvious ecological differences between well-mixed temperate estuaries and the Ebro Estuary, the species-complex found were similar to those found inhabiting other European estuaries. Close to the null point, the community was dominated by the eurybiontic taxa like *Hediste diversicolor*,

Perinereis cultrifera, Heteromastus filiformis, Corophium orientale and Cyathura carinata; these species are also very common in the oligohaline area of European estuaries.

6. The trophic structure found in the Ebro Estuary was represented by six different trophic guilds; this fact demonstrated that diverse food resources were available.

7. The increasing permanence in salt wedge was related to the increase in richness and abundance in the lower stretch of the estuary; the longer the salt wedge period, the most complex community found. Macroinvertebrates colonization was mainly linked to their dispersal abilities. In spite of being limited by the low marine intrusion ascending velocity, the long periods of salt wedge provideg enough time to assure the colonization of this stretch.

8. The lack of powerful mixing drivers maintained the water column stratified in those sampling stations occupied by marine intrusion. As a consequence, the exchange of materials, salts and organisms was scarce; the lack of a real salinity gradient implied that species must cope with a sudden salinity changes. The salinity was the main driver structuring the benthic macroinvertebrate communities in the Ebro Estuary; there are other environmental parameters causing variation in the community but at smaller scale.

9. The Ebro Estuary riverine-marine boundary fitted with an ecotone model; it showed a narrow transitional interface, characterized by the rapid change between two different homogeneous communities and environments.

10. The Ebro Estuary macroinvertebrate community showed a total replacement or turnover distribution pattern upstream and downstream the salt wedge; only a few eurybiontic species were able to colonize both fresh and marine water environments. These species were dominant in the proximities of the null point, but their presence in distant sampling stations was anecdotal.

11. The null point area of the Ebro Estuary showed the lowest richness and density values; therefore it could be considered analogous to the 'Artenminimum zone' described for well-mixed temperate estuaries. Whereas null point is the most stressful area within Ebro Estuary, the

'Artenminimum zone' is the oligohaline area in mixed estuaries and it represents the most stressful conditions within a mixed estuary.

12. The main anthropogenic pressures affecting the Ebro estuary are the nutrient enrichment and the alteration of its hydrological regime. Nutrient loadings of the Ebro River are consequence of the inputs from agriculture and urban areas on the whole basin; whereas, hydrological alteration is a consequence of the regulatory effect exerted over flows by reservoirs located in the lower Ebro River.

13. During the last decade, the chemical status of the Ebro Estuary has clearly improved, the nutrient inputs, especially phosphorus, have considerably decreased in the whole basin dimming river eutrophication, the organic matter input decrease alleviating the impacts over the estuary bottom; and nowadays, hypoxic events are unusual and so the aerobic processes are not restricted

14. The chemical condition of Ebro estuary improved since the mid 90's decade; as a result, the macroinvertebrate community was able to recolonize the Ebro Estuary benthos. This fact evidence that when environmental condition is improved, the biological communities respond positively aiding to the ecological balance recovery.

15. The flow regulation is disrupting the ecological balance of the lower Ebro River and its estuary in numerous ways: by maintaining a decreasing tendency of annual mean flow due to constant increment on water demand, by homogenizing river discharge and buffering the Mediterranean seasonality and by altering the salt wedge hydrodynamics.

16. Long periods of salt wedge permanence suppressed the water renewal and caused the accumulation of materials and pollutants towards the null point. Consequently, the increase in permanence time provoked a water quality declined upstream, creating a pollution and hypoxia gradient from river mouth to null point.

17. The macroinvertebrates community showed its ability to respond to main stressors affecting the Ebro Estuary. The community attributes declined towards the null point, the most impaired and stressful area within the estuary. Moreover, within the estuarine reach, the community shows a paradoxical response, as hydrological alteration increased, the community achieved a greater complexity due to contribution of marine origin species colonizing the estuarine area.

18. The salt wedge hydrodynamics alteration caused the artificial stability in the environmental conditions; especially, eliminating hydrological stress associated to flow fluctuations. As a consequence, the estuarine community is substituted by other community best structured, integrated by typical marine species with a great complexity.

19. The ecological assessment and biological monitoring of the Ebro Estuary, and by extension, other salt wedge estuaries, should include specific tools for both fresh and marine water; because depending on river discharge the estuary may show different environmental conditions, from fully salt wedge state to fluvial estuarine stretch and all the intermediate situations.

20. The use of classical community parameters (density, abundance, biomass, richness and diversity indices), as complements to BIs assessment, improved the community response interpretation and helped to appraise the suitability and reliability of the BIs.

21. The TWs intrinsic assessment limitations, the discrepancies between BIs and the classification mismatch suggested that a different ES assessment approach for this heterogeneous WB group is necessary; especially in Mediterranean basins, where hydrological issues are gaining relevance on ES assessment with respect to water and sediment quality.

22. The conclusion extracted after the use of BIs, based on pollution paradigms, for the assessment of hydrological pressures related impacts, suggested that this approach is not the most appropriate.

23. The final conclusion is that the Ebro Estuary represents a unique ecosystem in the Mediterranean region, its ecological singularity together with its economic and social relevance imply that its conservation must be an ineludible priority.

7 APPENDIX (ORIGINAL PUBLICATIONS)

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Community structure of benthic macroinvertebrates inhabiting a highly stratified Mediterranean estuary

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SUMMARY: The community composition and spatial distribution of benthic macroinvertebrates were studied along the Ebro estuary, a highly stratified estuary located in the NE Iberian Peninsula. During the last decade the oligotrophication process occurring in the lower Ebro River and its estuary has allowed a complex benthic macroinvertebrate community to become established; these results contrast with the poor community found there in the early nineties. A total of 214 taxa were identified, and polychaetes dominated the community both in abundance and species richness. The results showed spatial differences in the structure and composition of macroinvertebrates, which suggests that there are two distinct communities along the estuary. Each community was found in a specific stretch (upper and lower estuary) in function of the presence of the salt wedge. The macrobenthos of the upper estuary was dominated by freshwater taxa, but some euryhaline species were also found. The lower estuary showed a marine community typical of shallow Mediterranean environments. The transition between these two communities fits an ecotone model. The highest abundances, richness and diversities were recorded at the lower estuarine stations, especially those closer to the river mouth, whereas the lowest values corresponded to the stations adjacent to the tip of the salt wedge.

Keywords: benthic macroinvertebrates, community structure, distribution patterns, salt wedge, highly stratified estuary, Ebro estuary.

RESUMEN: Estructura de la comunidad de macroinvertebrados bentónicos en un estuario mediterráneo altamente estratificado. – La composición de la comunidad y la distribución espacial de los macroinvertebrados bentónicos ha sido estudiada a lo largo del estuario del Río Ebro, un estuario altamente estratificado localizado al NE de la Península Ibérica. El proceso de oligotrofización ocurrido durante la última década en el tramo bajo del río Ebro y su estuario, ha permitido el establecimiento de una compleja comunidad de macroinvertebrados, contrastando con la comunidad encontrada a principios de los noventa. Un total de 214 taxones fueron identificados; los poliquetos constituyeron el grupo dominante en términos de riqueza y abundancia. Los resultados mostraron diferencias espaciales en la estructura y composición de macroinvertebrados, sugiriendo la existencia de dos comunidades diferentes a lo largo del estuario. Cada una de estas comunidades fue encontrada en un tramo específico (alto y bajo estuario) en función de la presencia de la cuña salina. El macrobentos del tramo alto del estuario estaba integrado mayoritariamente por taxones de agua dulce y algunos taxones eurihalinos. Por el contario, el tramo bajo presentó una comunidad marina típica de ambientes mediterráneos someros. La transición entre estas dos comunidades encajó con un modelo ecotonal. Las abundancias, riquezas y diversidades más elevadas fueron registradas en las estaciones del tramo bajo, especialmente en aquellas cercanas a la desembocadura; en cambio, los valores más bajos correspondieron a las estaciones adyacentes al extremo de la cuña salina.

Palabras clave: macroinvertebrados bentónicos, estructura de la comunidad, patrones de distribución, cuña salina, estuario altamente estratificado, estuario del Ebro.

INTRODUCTION

The Ebro estuary (NE, Iberian Peninsula) is a salt wedge or highly stratified estuary (Hansen and Rattray, 1966; Ibáñez *et al*., 1997). The specific characteristics of salt wedge estuaries are: (i) the river discharge controls the marine intrusion mainly due to the low tidal range (usually with an amplitude less than 2 meters); (ii) weak mixing effects cause the water column to be strongly stratified; (iii) the vertical profile of density and salinity shows a marked change with a narrow interface between layers called haloclines; and (iv) the isohalines are arranged horizontally. Although this kind of estuary is well represented along microtidal coasts worldwide (e.g. the Mediterranean Sea and the Gulf of Mexico), there is little research on the macroinvertebrate communities that inhabit them. The Ebro estuary has been extensively studied in relation to its hydrology and salt wedge dynamics (e.g. Ibáñez *et al*., 1997, 1999; Sierra *et al*., 2002, 2004), and some benthic communities of adjacent areas have also been studied (Capaccioni-Azzati and Martín, 1992; Martín *et al*., 2000). A few studies have focused on the biota of the estuary (e.g. Rovira *et al*., 2009), but only one includes a brief description of its macroinvertebrate community (Ibáñez *et al*., 1995). Furthermore, this study was performed when the lower Ebro River and its estuary were under severe eutrophic conditions, very different from the present situation. Highly fluctuating estuarine systems produce strong environmental gradients, which leads to a patchy distribution of organisms that must cope with a wide variety of stresses (Morrisey *et al.*, 1992; Gray and Elliott, 2009) due to both natural and anthropogenic factors (McLusky, 1999; Dauer *et al.*, 2000; Dauvin, 2007; Elliott and Quintino, 2007). Therefore, the benthic invertebrate communities, often used as indicators of the health of an ecosystem, can be very similar in both impacted and non-disturbed estuarine systems. This therefore increases the difficulty of distinguishing natural from anthropogenic stresses. The *Estuarine Quality Paradox* concept (Dauvin, 2007; Elliott and Quintino, 2007) refers to the challenge of detecting anthropogenic impacts in naturally stressed systems using biological assessment methods. In Mediterranean regions and particularly in the Iberian Peninsula, besides the spatial fluctuation there is strong temporal environmental variability in the aquatic systems due to limited water availability during part of the year (Caiola *et al*., 2001; Ferreira *et al*., 2007a). This variability is exacerbated by a long history of human-induced pressures that have led to serious changes in the natural ecological cycles of estuarine systems from this region (Ferreira *et al*., 2007b). Therefore, identifying the factors that structure the benthic macroinvertebrate community of the Ebro estuary will provide a clearer understanding of the ecological functioning of the system both at the spatial and temporal scales. Moreover, it will help to interpret the recent changes in the estuarine system observed during the last two decades (Ibáñez *et al*., 2008). Therefore, this study establishes a robust basis so that macroinvertebrates can be used as indicators of the ecological status of the Ebro estuary.

The purpose of this study was to examine the macroinvertebrate community of the Ebro estuary with regard to species composition, community structure and distribution patterns along spatial and temporal scales and to describe the main abiotic factors affecting benthic communities in this type of estuary.

MATERIALS AND METHODS

Study area

The study was conducted in the Ebro estuary (40º43'10''N, 0º40'30''E) located in the NE of the Iberian Peninsula (Catalonia, Spain) (Fig.1). The Ebro is 910 km long and has a drainage area of 85362 km^2; it is the Spanish river with the highest mean annual flow and one of the most important tributaries to the Mediterranean Sea. The main land use in the basin is agriculture with more than 10000 km^2 of irrigation, corresponding to approximately 90% of the water usage in the basin (Ibáñez *et al*., 2008). The whole basin is strongly regulated by nearly 190 dams (Batalla *et al*., 2004). These affect the mean annual flow, which has decreased greatly since the beginning of the century to the present (Ibáñez *et al*., 1996). The Ebro estuary is highly stratified (30 km long, 240 m mean width and 6-8 m mean depth) and the microtidal amplitude of the Mediterranean Sea, about 20 cm (Cacchione *et al*., 1990), promotes the formation of a salt wedge. The river discharge controls the salt wedge dynamic (advance, retreat and permanence): when the flow exceeds 350-400 m^3 s^{-1} the salt wedge is pushed from the river channel, and the salt wedge reaches its maximum distance upstream (30-32 km from the river mouth) with flows lower than 100 m^3 s^{-1} (Ibáñez *et al*., 1997).

Sampling design and laboratory procedures

Nine sampling stations were established in order to cover the whole estuarine stretch of the Ebro River (Fig. 1). Each station was sampled seasonally (summer 2007 to spring 2008). On each sampling occasion, three sediment samples were collected using a Ponar grab (0.046 m^2). The samples were washed in situ through a 0.5-mm mesh sieve to separate macroinvertebrates from sediment, and the organisms retained were immediately fixed with buffered 10% formalin. Later in the laboratory, all macroinvertebrates were sorted, counted and identified under a stereomicroscope to the lowest possible taxonomic level. Two sediment aliquots of 30 g and 200 g were taken from each grab and stored at -20ºC to estimate the total organic matter (TOM) with the loss on ignition method following Kristensen and Andersen (1987), and grain-size characterization according to Holme and McIntyre (1984). Bottom water samples were collected at each station with a water pump, preserved on ice in the absence of light, transported to the laboratory and stored at -20ºC until analysis. Posterior processing included estimating the total chlorophyll and pheophytin concentration using the colorimetric method (Jeffrey and Humphrey, 1975), the dissolved and total nutrient concentration (PO_4, P_T, NH_4, NO_2, NO_3, N_T and SiO_4) following Koroleff (1977) and the suspended solid concentration (Total suspended solids (TSS, mg l^{-1}) and organic suspended solids (OSS, mg l^{-1})) in compliance with the UNE-EN 872 norm (AENOR, 1996). In addi-

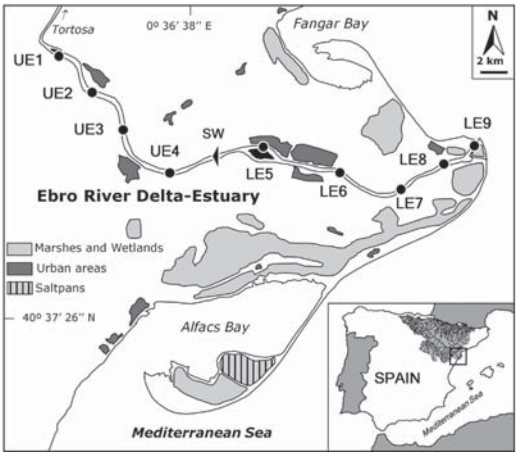

FIG. 1. – Location of the Ebro estuary and its deltaic plain showing the nine sampling stations. UE, upper estuary stations; LE, lower estuary stations; SW, position of the salt wedge tip.

tion, physicochemical and hydromorphological characteristics were recorded on each sampling occasion. A YSI 556 multi-parameter probe was used to measure water temperature (°C), dissolved oxygen (mg l^{-1}), oxygen saturation (%), pH, salinity and conductivity (mS cm^{-1}). Water depth (m) was measured using a Speedtech SM-5 depth-meter sounder. Water flow velocity (m s^{-1}) was recorded with a Valeport m.001 current-meter, and water transparency was estimated using a Secchi disc. The accumulated permanence time (in days) of the salt wedge was calculated using daily mean flow values measured 40 km upstream from the river mouth (Tortosa) by counting the accumulated days before each sampling occasion with mean flow values lower than 350 $m^3 s^{-1}$. This data is available at the Ebro Water Authority (CHE) web site (http://www.chebro.es/).

Data analysis

The following community descriptive parameters were calculated for each station and season (n=36): total abundance (N), density (D, ind m^{-2}), richness (S), Shannon-Wiener's diversity index (H', as $\log_2$), Margalef index (d), Simpson dominance index (1-λ**'**) and Pielou's evenness index (J**'**). In addition, species were classified with the constancy index (Dajoz, 1971) into five categories according to the number of stations in which any given taxa was found in relation to the total number of stations: constant (>76%), very common (51-75%), common (26-50%), uncommon (13-25%) and rare (<12%). Each species was classified into feeding guilds based on the available literature. The feeding guilds included deposit feeders (DF), grazers (G), omnivores (O), parasites (Pa), predators (Pr) and suspension feeders (SF). Appendix 1 provides a list of the taxa, together with their feeding guild, that are mentioned in the text. Non-parametric multivariate techniques were used as described by Field *et al.* (1982) to indentify the possible macroinvertebrate communities. A similarity matrix was computed using the Bray-Curtis coefficient (Legendre and Legendre, 1998) after the four root transformation was applied to the abundance data to downweight the contribution of the most abundant taxa to the similarity (Clarke and Warwick, 2001). All the other statistical analyses were performed using the different routines available in the Multivariate Ecological Research software package PRIMER V6 (Clarke and Gorley, 2006). The stations and taxa were ordered using non-metrical multidimensional scaling (MDS) (Clarke and Warwick, 2001). A similarity percentage analysis (SIMPER) that examines the contribution of each variable to the average resemblances between sample groups was performed. This analysis was also used to identify taxa that contributed to dissimilarity among stations and estuary domains that were pre-determined by ordination analysis. Differences in the community composition were identified using the 1-way analysis of similarities test (ANOSIM) that hypothesizes for differences between groups of samples (defined a priori) through randomization methods on a resemblance matrix. Finally, the relationship between the community structure and environmental variables was investigated with the BIOENV routine, which maximizes a rank correlation (Spearman's coefficient) between resemblance matrices derived from biotic and environmental data, iterating for all possible combinations of environmental variables (Clarke and Warwick, 2001). A Spearman's coefficient value close to 0 indicates a weak relation between the community and environmental variables, whereas a value close to 1 indicates that the environmental variables selected explain the community structure.

RESULTS

Water and sediment features

The Ebro estuary has a sand dominated bottom and a relatively low TOM percentage in both the upper (UE) and lower (LE) parts and throughout the entire year (Table 1). During the study period the salt wedge was only found in the lower estuary stations. At these stations, the accumulated permanence time was different in each season: 55, 143, 257 and 344 days respectively for summer, autumn, winter and spring. The null point (the tip of the salt wedge) was located between UE4 and LE5 in all sampling periods. Nutrient concentrations were higher in the upper estuary stretch (Table 1) except for the ammonia, nitrite, phosphate and silicate concentrations in spring and total phosphorous in summer. The chlorophyll concentrations showed marked differences between the upper and lower estuary; the UE stretch had the highest values during winter and spring, whereas the maximum values in the LE stretch were in summer/autumn. Levels of total pheophytin were lower in the UE stretch except for during the two

TABLE 1. – Sediment characteristics and water physicochemical parameters (seasonal mean±standard deviation, n=4) in the two different stretches. TOM, total organic matter in sediment; Transp., transparency; DO, dissolved oxygen; Cond., conductivity; Sal., salinity; TDS, total dissolved salts; TSS, total suspended solids; OSS, organic suspended solids.

	Upper Estuary				Lower Estuary			
	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring
Mud (%)	11.31±14.59	11.31±14.59	21.08±28.43	1.53±2.08	15.89±10.90	15.89±10.90	25.27±20.78	6.51±5.75
Sand (%)	73.35±23.41	73.35±23.41	57.93±33.30	88.78±16.81	79.84±13.01	79.84±13.01	74.32±21.06	85.36±18.10
Gravel (%)	15.34±27.22	15.34±27.22	20.99±36.79	9.69±17.68	4.27±6.26	4.27±6.26	0.41±0.29	8.13±12.52
TOM (%)	2.67±1.03	2.67±1.03	2.89±1.11	2.45±1.88	4.03±1.22	4.03±1.22	4.36±1.59	3.70±2.21
Depth (m)	3.50±1.73	4.25±1.89	3.75±2.22	4.25±2.06	6.00±1.41	6.80±1.48	6.00±1.58	6.00±1.58
Velocity (m s^{-1})	0.13±0.06	0.17±0.05	0.14±0.05	0.42±0.10	0.06±0.04	0.10±0.09	0.05±0.05	0.25±0.21
Transp. (m)	2.40±0.71	2.68±0.78	1.98±0.73	2.21±0.22	2.37±0.27	1.89±0.23	1.88±0.13	1.60±0.22
T (ºC)	24.26±0.40	22.80±0.07	11.12±0.36	16.32±0.14	22.07±0.19	22.30±0.83	13.27±0.04	15.30±0.51
DO (mg l^{-1})	7.85±0.47	7.89±1.22	13.82±0.69	7.94±0.69	5.25±1.22	6.00±2.4	10.32±0.83	6.72±2.11
DO (%)	94.00±6.26	92.00±14.25	126.28±7.02	81.30±7.24	74.10±17.64	84.78±33.61	123.90±10.11	74.36±16.52
Cond.(mS cm^{-1})	0.95±0.01	1.37±0.00	1.12±0.03	1.04±0.00	51.27±0.53	51.51±0.71	43.21±0.50	25.00±19.78
Sal.	0.47±0.01	0.72±0.00	0.77±0.02	0.62±0.00	35.97±0.31	35.98±0.40	36.89±0.45	20.02±16.27
TDS (g l^{-1})	0.62±0.01	0.93±0.00	0.99±0.02	0.81±0.00	35.30±0.27	35.30±0.34	36.19±0.39	20.09±15.95
Chlorophyll (μg l^{-1})	0.09±0.06	1.16±0.24	1.07±0.91	2.83±2.82	1.01±0.80	2.83±1.06	0.79±0.23	0.69±0.41
Pheophytin (μg l^{-1})	0.05±0.02	1.06±0.10	1.00±0.55	3.43±2.28	0.31±0.18	1.38±0.32	0.66±0.26	0.87±0.50
pH	8.20±0.06	8.25±0.05	7.89±0.09	8.00±0.02	7.98±0.06	8.28±0.15	7.94±0.05	7.83±0.11
PO4 (mg l^{-1})	0.02±0.00	0.03±0.00	0.03±0.01	0.03±0.01	0.01±0.01	0.01±0.02	0.01±0.01	0.03±0.01
P_T (mg l^{-1})	0.08±0.01	0.06±0.01	0.05±0.01	0.05±0.00	0.11±0.02	0.05±0.02	0.02±0.02	0.04±0.02
NH_4 (mg l^{-1})	0.02±0.02	0.04±0.03	0.02±0.02	0.19±0.14	0.05±0.02	0.09±0.12	0.05±0.02	0.20±0.30
NO_2 (mg l^{-1})	0.01±0.00	0.01±0.00	0.02±0.00	0.04±0.01	0.00±0.01	0.00±0.01	0.01±0.00	0.04±0.01
NO_3 (mg l^{-1})	2.08±0.16	1.85±0.34	3.52±0.05	4.45±0.15	0.04±0.03	0.04±0.02	0.10±0.02	3.26±0.89
N_T (mg l^{-1})	2.42±0.08	2.43±0.04	3.52±0.05	5.37±0.08	0.28±0.07	0.20±0.12	0.10±0.02	4.39±1.13
SiO_4 (mg l^{-1})	1.89±0.06	0.85±0.17	1.01±0.15	1.21±0.13	0.42±0.48	0.47±0.33	0.17±0.16	1.28±0.31
TSS (mg l^{-1})	3.05±0.98	3.56±2.68	2.91±1.66	14.69±11.25	20.60±2.17	24.99±3.78	16.91±32.53	5.84±3.51
OSS (mg l^{-1})	1.94±0.52	1.47±0.81	0.99±0.21	3.20±2.31	4.75±1.11	4.71±1.02	1.76±2.27	1.52±0.75
OSS (%)	66.49±18.14	46.07±8.76	43.07±21.78	23.19±2.59	22.92±3.70	18.72±1.53	27.34±12.39	27.04±2.95

last seasons. The UE stretch always had seasonal mean water flow velocities higher than the LE stretch. The values of TSS and OSS were higher in the LE stretch in summer, autumn and winter, whereas in spring the UE stretch showed the maximum values.

Macroinvertebrate abundance, taxa richness and diversity

During one year of seasonal sampling in the Ebro estuary a total of 21805 individuals were collected belonging to 214 different taxa that comprised 151 species, 115 families, 57 orders, 20 classes and 9 phyla (Supplementary material Appendix 1). Annelida was the dominant phylum and accounted for 71.07% of the total abundance. Polychaeta and Oligochaeta contributed with 49.64% and 21.42% respectively. Spionidae was the most abundant family (28.56%) due to the contribution of the most dominant species *Streblospio benedicti* (24.10% of the total abundance). Another dominant phylum was Arthropoda, which contributed 15.56% of the total abundance, with Malacostraca accounting for 10.37% of the total abundance. Mollusca was the third most abundant phylum with 12.09% of the total abundance, and Bivalvia contributed 10.61% of the total abundance. In terms of species richness, Polychaeta contributed with 49 different taxa (40 species) and Bivalvia with 37 taxa (32 sp), followed by Gastropoda with 29 taxa (18 sp) and Insecta with 24 taxa (14 sp). Applying Dajoz's constancy index (considering the 9 stations), 1% of the taxa were found constant, 8% very common, 27% common, 20% uncommon and 44% were rare. Applying the constancy index to UE stations revealed that 9% of the taxa were constant, 14% very common, 19% common, 58% were uncommon and no taxa were rare; whereas in the LE stretch 22% of the taxa were constant, 16% very common, 20% common and 42% were uncommon.

Total density values throughout seasons ranged from 216 to 20022 ind m^{-2} (Table 2). The highest densities were found at the mouth (station LE9) due to the high abundance of the polychaete *S. benedicti*. Intermediate densities were found in the uppermost stations UE1 and UE2 with a large contribution of Tubificidae and the introduced bivalve *Corbicula fluminea*. The lowest densities corresponded to stations UE3, UE4 and LE5 in the middle part of the estuary. Station LE9 had the highest richness values with a maximum of 69 taxa and an annual mean value of 48 taxa; other stations located near the river mouth (LE8 and LE7) also reached high values of richness, whereas stations UE3, UE4 and LE5 showed the lowest richness values (Table 2). Diversity indices showed the same tendency as density and richness, with low values at stations located near the limit of the salt wedge (Table 2). In terms of the trophic structure, the deposit feeders (32%), suspension feeders (29%) and predators (17%) were the dominant feeding guilds in the entire estuary. The contribution of the different feeding guilds in the UE stretch was: deposit feeders (38%), predators (22%), grazers (19%), suspension feeders (14%), omnivores (5%) and parasites (3%). The trophic structure of the LE stretch was dominated by suspension feeders (35%) and deposit feeders (30%).

TABLE 2. – Community descriptive parameters for each sampling station and season. N, total abundance per 0.14 m^2; D, density (ind m^{-2}); S, richness; H'(log_2), Shannon-Wiener diversity index; d, Margalef index; 1-λ', Simpson's index; J', Pielou's evenness; DF (%), deposit feeders; G (%), grazers; O (%), omnivores; Pa (%), parasites; Pr (%), predators; SF (%), suspension feeders. See Figure 1 for sampling station codes.

Station	Season	Density	Community indices					Trophic structure					
			S	H'(log_2)	d	1-λ'	J'	DF	G	O	Pa	Pr	SF
UE1	Summer	2792	11	1.96	1.68	0.67	0.57	54.55	0.00	9.09	9.09	9.09	18.18
UE2	Summer	4820	25	2.16	3.69	0.59	0.47	44.00	16.00	4.00	8.00	12.00	16.00
UE3	Summer	830	6	2.02	1.05	0.72	0.78	33.33	0.00	16.67	16.67	0.00	33.33
UE4	Summer	491	3	1.45	0.47	0.61	0.91	0.00	0.00	33.33	0.00	0.00	66.67
LE5	Summer	216	4	0.63	0.88	0.19	0.31	50.00	25.00	25.00	0.00	0.00	0.00
LE6	Summer	1457	7	1.03	1.13	0.32	0.37	57.14	0.00	14.29	0.00	0.00	28.57
LE7	Summer	2670	23	2.52	3.72	0.67	0.56	47.83	4.35	8.70	0.00	4.35	34.78
LE8	Summer	2583	23	3.18	3.74	0.82	0.70	56.52	0.00	8.70	0.00	8.70	26.09
LE9	Summer	11212	32	0.48	4.22	0.09	0.10	25.00	3.13	15.63	0.00	18.75	37.50
UE1	Autumn	491	13	2.79	2.84	0.80	0.75	30.77	23.08	7.69	0.00	23.08	15.38
UE2	Autumn	2403	23	3.15	3.79	0.80	0.70	34.78	13.04	4.35	0.00	34.78	13.04
UE3	Autumn	1335	8	1.29	1.34	0.42	0.43	62.50	0.00	0.00	0.00	25.00	12.50
UE4	Autumn	505	12	2.57	2.59	0.78	0.72	58.33	0.00	16.67	0.00	16.67	8.33
LE5	Autumn	2020	11	2.00	1.77	0.60	0.58	54.55	0.00	0.00	0.00	0.00	45.45
LE6	Autumn	599	21	3.94	4.53	0.93	0.90	52.38	0.00	4.76	4.76	23.81	14.29
LE7	Autumn	2316	31	3.19	5.20	0.75	0.64	35.48	0.00	6.45	3.23	16.13	38.71
LE8	Autumn	2648	36	2.84	5.93	0.66	0.55	30.56	5.56	5.56	2.78	8.33	47.22
LE9	Autumn	13485	69	2.84	9.03	0.68	0.47	31.88	1.45	7.25	7.25	17.39	34.78
UE1	Winter	9632	21	0.79	2.78	0.18	0.18	33.33	19.05	0.00	4.76	23.81	19.05
UE2	Winter	4906	17	1.57	2.45	0.41	0.38	47.06	17.65	11.76	5.88	5.88	11.76
UE3	Winter	981	4	1.05	0.61	0.48	0.53	50.00	0.00	0.00	0.00	25.00	25.00
UE4	Winter	1522	6	0.99	0.93	0.34	0.38	66.67	0.00	0.00	0.00	16.67	16.67
LE5	Winter	2756	19	2.64	3.03	0.78	0.62	47.37	5.26	0.00	5.26	5.26	36.84
LE6	Winter	4278	27	3.30	4.07	0.86	0.69	51.85	3.70	7.41	3.70	14.81	18.52
LE7	Winter	6934	62	3.81	8.88	0.83	0.64	46.77	3.23	4.84	3.23	16.13	25.81
LE8	Winter	3413	48	4.04	7.63	0.87	0.72	50.00	0.00	8.33	2.08	14.58	25.00
LE9	Winter	20022	58	1.66	7.19	0.35	0.28	37.93	1.72	6.90	5.17	18.97	29.31
UE1	Spring	18319	21	0.98	2.55	0.27	0.22	44.44	22.22	0.00	5.56	11.11	16.67
UE2	Spring	5368	24	1.74	3.48	0.46	0.38	41.67	20.83	8.33	0.00	16.67	12.50
UE3	Spring	1198	9	2.42	1.56	0.78	0.76	55.56	0.00	0.00	0.00	22.22	22.22
UE4	Spring	3802	8	0.34	1.12	0.08	0.11	50.00	12.50	12.50	0.00	12.50	12.50
LE5	Spring	3629	7	0.91	0.96	0.33	0.32	42.86	0.00	14.29	14.29	0.00	28.57
LE6	Spring	1941	28	3.14	4.83	0.80	0.65	46.43	0.00	10.71	0.00	21.43	21.43
LE7	Spring	6486	59	4.15	8.53	0.88	0.71	47.46	1.69	11.86	3.39	15.25	20.34
LE8	Spring	7417	63	4.62	8.94	0.93	0.77	41.27	1.59	12.70	3.17	17.46	23.81
LE9	Spring	1876	33	3.30	5.75	0.81	0.65	48.48	0.00	6.06	3.03	21.21	21.21

Analysis of benthic macroinvertebrate communities

Two different communities were determined according to the ordination of stations and taxa of the MDS analysis based on macroinvertebrate abundance. The ordination showed two definite groups of sampling stations: those corresponding to the upper estuary (UE) and lower estuary (LE) respectively (Fig. 2). The UE group (UE1-UE4) included stations located in the upper estuary stretch and corresponded to a freshwater community, whereas the second group comprised the lower estuary stations (LE5-LE9) and had a community with a large marine influence. In addition, we also applied the MDS analysis considering lower taxonomic categories e.g. genus and family; the results obtained showed the same grouping of stations regardless of the taxonomic level employed in the ordinations. Significant differences in community composition were found between these two groups (ANOSIM r: 0.891, $p<0.001$). Significant differences were also found among stations (ANOSIM global r: 0.694, $p<0.001$) except for the following pairs: UE1-UE3, UE3-UE4, UE4-LE5, LE5-LE6, LE6-LE7, LE6-LE8, LE7-LE8, LE7-LE9 and LE8-LE9, $p>0.05$ (Table 3).

The SIMPER analysis showed that the mean community similarity within the UE group was 32.30%. The taxa that most contributed to the high similarity among stations were *C. fluminea* (27.26%), Tubificidae (18.34%), Naididae (12.02%) and Chironomidae (17.00%). The mean similarity of the LE group was 29.67% with a high contribution from *S. benedicti* (10.44%), *Corophium orientale* (8.56%) and *Caulleriella zetlandica* (6.01%). The similarity contribution of taxa within this group was more balanced than in the UE group, since a total of 35 taxa was necessary to accumulate 90% of the similarity. The mean dissimilarity between these two groups was 96.58% with *C. fluminea*, *S. benedicti*, Tubificidae, *C. orientale*, Naididae, *C. zetlandica*, *Pseudopolydora antennata* and *Armandia cirrhosa* as the taxa with the highest contributions to dissimilarity.

The BIOENV analysis showed that the combination of salinity, dissolved phosphate, total phosphorous, ammonia and the distance from the mouth have a large influence on the structure of the macroinvertebrate communities (ρ=0.741). The combination of salinity, dissolved phosphate, ammonia and nitrate explained the differences in taxa abundance in the upper estuary (ρ=0.308). However, within the community of the lower estuary, the combination of ammonia, total chlorophyll, sand percentage,

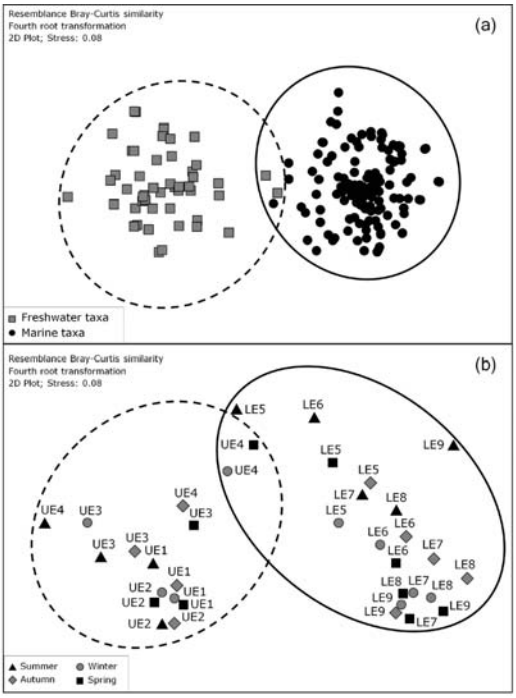

FIG. 2. – Two dimensional MDS plots based on Bray-Curtis similarities of fourth-root transformed macroinvertebrate abundance data: (a) ordination using inter-species resemblance matrix of nine stations; (b) ordination of the nine stations sampled in the Ebro estuary. The dashed line and the solid line encircle the freshwater and marine communities respectively. See Figure 1 for sampling station codes.

TABLE 3. – One-way ANOSIM test to compare the macroinvertebrate communities at different sampling stations. The test results are shown in the lower diagonal of the table. Significant differences between stations (P<0.05) are indicated (*). The R values are shown in bold letters in the upper diagonal of the table. See Figure 1 for sampling station codes.

	UE1	UE2	UE3	UE4	LE5	LE6	LE7	LE8	LE9
UE1		**0.708**	**0.458**	**0.552**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
UE2	0.029*		**0.719**	**0.635**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
UE3	0.057	0.029*		**0.219**	**0.917**	**0.990**	**1.000**	**1.000**	**1.000**
UE4	0.029*	0.029*	0.143		**0.302**	**0.688**	**0.849**	**0.885**	**0.880**
LE5	0.029*	0.029*	0.029*	0.114		**0.219**	**0.542**	**0.667**	**0.604**
LE6	0.029*	0.029*	0.029*	0.029*	0.143		**0.167**	**0.115**	**0.448**
LE7	0.029*	0.029*	0.029*	0.029*	0.029*	0.171		**0.000**	**0.240**
LE8	0.029*	0.029*	0.029*	0.029*	0.029*	0.200	1.000		**0.083**
LE9	0.029*	0.029*	0.029*	0.029*	0.029*	0.029*	0.086	0.229	

TOM and the permanence time of the salt wedge showed the highest correlation and explained the main differences in the macroinvertebrate abundance data (ρ=0.681)

DISCUSSION

The whole Ebro estuary is dominated by sand; however, the percentage of fine deposits such as clay or mud was higher in the lower stretch due to flocculation and settling processes and low velocities recorded at the salt wedge (Sierra *et. al*., 2002). During the study period the bottom water layer of the estuary showed important differences in physicochemical features between the lower and upper estuary stretches. We found freshwater stations (UE1-UE4) that were not exposed to marine intrusions, and saltwater stations (LE5-LE9) that were permanently exposed to marine intrusions and had a well stratified water column. At LE stations, salinity in the salt wedge decreased upstream with small fluctuations but with values always higher than 30, which evidences the weak mixing between water layers. In highly stratified estuaries the salt wedge dynamics are complex and can be explained by a combination of hydromorphological factors, such as the tide amplitude, river channel cross section and flow, and the freshwater runoff is one of the main factors determining the salt wedge regime (Ibáñez *et al*., 1997). Nevertheless, in the lower estuary the salt wedge was present on all sampling occasions and the permanence time almost reached a complete year. Although other long periods of marine intrusion in the Ebro estuary have been recorded before (Ibáñez *et al*., 1995), under natural conditions this period should be approximately 6 months per year (Ibáñez *et al*., 1997). These conditions of the quasi permanent presence of the salt wedge in the lower estuary stretch are exacerbated by the strong flow regulation and the almost total absence of peak flows, which leads to reduced turbulence and therefore to highly stable density-thermal stratification (Ibáñez *et al*., 1995, 1996).

The present conditions of nutrient loading of the Ebro estuary are quite different from the past situation of eutrophication (Ibáñez *et al*., 1995). Under eutrophic conditions, and with long periods of permanence of the salt wedge in the lower estuary at the same time, the water quality was worse below the wedge than above it due to organic matter deposition and low water renewal. This organic enrichment caused oxygen depletion through microbial consumption (Ibáñez *et al*., 1995; Casamayor *et al*., 2001). Recent changes in the nutrient content of the river, especially the reduction of phosphates, have reduced the primary production in the upper layer, whereas in the lower layer it has increased due to higher light penetration (Falco *et al*., 2010); thus, the hypoxic conditions in the lower layer have decreased (Casamayor *et al*., 2001; Ibáñez *et al*., 2008).

Under the present oligotrophication process, the long periods of salt wedge permanence ensure the stability of the water column, which allows the complexity of the benthic communities to increase, as suggested by Sousa *et al*. (2006a). The present situation is very different to that of the early nineties, when a survey conducted in October 1992 showed an impoverished macroinvertebrate community (only seven different taxa were found) due to eutrophication, which caused severe anoxic episodes below the halocline (Ibáñez *et al*., 1995).

The benthic macroinvertebrate community in the Ebro estuary shows considerable spatial and temporal differences, with a complex structure and composition. The multivariate analysis defined two different communities: one from the lower and one from the upper estuary stretch. In contrast, the pattern described in more mixed estuaries (Rundle *et al*., 1998; Ysebaert *et al*., 1998; Sousa *et al*., 2008) supports the idea that these systems work as a continuum of overlapping communities along the salinity gradient, which fits with the ecocline boundary model suggested by Attrill and Rundle (2002). However, the weak longitudinal salinity gradient and the narrow transition zone between fresh and marine water suggest that the Ebro estuary fits much better into an ecotone model, when ecotone is defined as an area of relatively rapid change that produces a narrow ecological zone between two different and homogeneous community types (Van der Maarel, 1990).

The upper stretch of the Ebro estuary was characterized by an impoverished macroinvertebrate community dominated by the non-indigenous bivalve *C. fluminea,* which tends to acquire an invasive pattern (Sousa *et al*., 2006b), together with tolerant taxa such as Tubificidae, Naididae (Oligochaeta) and abundant Chiromidae. The amphipod *C. orientale* was well-represented in number of individuals but its presence was restricted to stations UE3 and UE4 located close to the salt wedge tip due to its euryhaline nature. The salt wedge community was dominated in terms of abundance by the Polychaeta and Malacostraca classes, followed by the phylum Mollusca. Nevertheless, in terms of richness it was dominated by molluscs, polychaetes and crustaceans in this order. This pattern was slightly different from those found in other temperate intertidal areas, where polychaetes are the most diverse group, followed by molluscs and crustaceans (Ysebaert *et al*., 1998; Rodrigues *et al*., 2006). Comparing our results with those from other European estuarine ecosystems we found that the Ebro estuary was colonized in its mouth area by typical marine species associated with the *Abra alba-Lagis koreni* community (colonizing fine sediments rich in organic matter) and with the *Nephtys* spp. community (colonizing sandy sediments). These two communities are widely distributed throughout European estuarine and coastal areas (Dauvin, 2000, 2007; Martín *et al*., 2000; Van Hoey *et al*., 2004; Puente *et al*., 2008). In addition to these communities, we also found tolerant groups dominated by Capitellidae and Spionidae (Polychaeta), together with *Corbula gibba*, which usually colonizes disturbed areas; whereas in the upper stations close to the null point the community was dominated by eurybiontic taxa like *Hediste diversicolor*, *Perinereis cultrifera*, *Heteromastus filiformis*, *C. orientale* and *Cyathura carinata*. These species are also very common in other European estuaries and coastal areas (Marques *et al*., 1993; Ysebaert *et al*., 1998, 2003; Martín *et al*., 2000; Chainho *et al*., 2006; Rodrigues *et al*., 2006; Sousa *et al*., 2006a, 2008).

Currently, the Ebro estuary shows high levels of richness compared with other European estuaries (Rodrigues *et al*., 2006). The trophic structure is well represented with six different trophic guilds. Deposit feeders, suspension feeders and predators are dominant, which suggests that different resources are available (Brown *et al*., 2000). In the upper stretch the diversity and richness decreased seawards, with minimum values found close to the null point because the salinity fluctuation is a physiological barrier for stenohaline freshwater and marine taxa (Mannino and Montagna, 1997). However, diversity and richness at the salt wedge stations declined with increasing distance from the sea, which is a recurring tendency in mixed estuaries (Remane and Schleiper, 1971; Schlacher and Woolbridge, 1996). In the Ebro estuary this impoverishment tendency could be explained by the increase in organic matter, ammonia and total phosphorous towards the tip of the salt wedge in combination with the salinity fluctuations in the same area.

The present study provides baseline data that can be used in future ecological studies on this important Mediterranean estuarine ecosystem, as well as in comparisons with other highly stratified estuaries. Complementary studies are necessary to improve our understanding of the spatial and temporal variability of the macrozoobenthic estuarine community. This knowledge could be an important tool for conserving the biodiversity in the Ebro estuary and could be used to develop biological indices for assessing its ecological status according to the Water Framework Directive of the European Union.

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SUPPLEMENTARY MATERIAL

The following Appendix is available through the web page
http://www.icm.csic.es/scimar/supplm/sm75n3577sm.pdf

APPENDIX 1. – List of the identified taxa that were found at all the stations over the entire study period.

Community structure of benthic macroinvertebrates inhabiting a highly stratified Mediterranean estuary

ALFONSO NEBRA, NUNO CAIOLA and CARLES IBÁÑEZ

Supplementary material

APPENDIX 1. – List of the identified taxa that were found at all the stations over the entire study period. The stations where each taxon was found are also listed. See Figure 1 for sampling station codes; FG, feeding guild (see Table 2 for feeding guild codes); CI, Constancy index; Ct, constant; VC, very common; C, common; UC, uncommon; R, rare.

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
PHYLUM CNIDARIA								
Class Anthozoa								
Diadumene sp.	LE9				Pr	R		UC
PHYLUM PLATYHELMINTHES								
Class Turbellaria								
Dugesia sp.	UE2	UE2		UE2	Pr	R	UC	
Turbellaria indet.		UE2	UE1		Pr	UC	C	
PHYLUM NEMERTINA								
Class Enopla								
Nemertina indet.		LE6,7,9	LE5,6,7,8,9	LE6,7,8,9	Pr	VC		Ct
Prostoma graecense (Böhmig, 1892)		UE2	UE1,2,4	UE2	Pr	C	VC	
PHYLUM NEMATODA								
Nematoda indet.	UE1,2,3	LE9	UE1,2; LE9	UE1	Pa	C	VC	UC
PHYLUM MOLLUSCA								
Class Gastropoda								
Aplysiidae indet.	LE9	LE8			G	UC		C
Bittium reticulatum (da Costa, 1778)		LE8,9	LE7,8,9	LE7,8,9	DF	C		VC
Buccinum sp.	LE9				O	R		UC
Chrysallida sp.		LE6,7,8,9	LE5,6,7,8,9	LE5,7,8,9	Pa	VC		Ct
Eulimella polita (Verrill, 1872)		LE9			Pa	R		UC
Ferrissia clessiniana (Jickeli, 1882)	UE2	UE2		UE2	G	R	UC	
Gyraulus albus (Müller, 1774)				UE1,2,4	G	C	VC	
Haminoea navicula (da Costa, 1778)			LE6,7		G	UC		C
Hinia limata (Chemnitz, 1795)	LE9	LE9		LE9	Pr	R		UC
Hydrobia sp.	LE7		LE5	LE7	G	UC		C
Hydrobia ulvae (Pennant, 1777)			LE7		G	R		UC
Mangelia sp.		LE9			O	R		UC
Melanella polita (Linnaeus, 1758)			LE9		Pa	R		UC
Nassarius mutabilis (Linnaeus, 1758)		LE9	LE9		O	R		UC
Nassarius pygmaeus (Lamarck, 1822)	LE7,8,9	LE9			O	C		VC
Nassarius sp.		LE7,9			O	UC		C
Neverita josephinia Risso, 1826	LE9	LE9			Pr	R		UC
Odostomia conoidea (Brocchi, 1814)				LE8	Pa	R		UC
Odostomia sp.		LE9			Pa	R		UC
Physella acuta (Draparnaud, 1805)	UE2	UE1,2	UE1,2	UE1,2	G	UC	C	
Radix auricularia (Linnaeus, 1758)			UE2		G	R	UC	
Radix peregra (Müller, 1774)	UE2		UE1	UE2	G	UC	C	
Retusa truncatula (Bruguière, 1792)	LE8	LE9	LE7	LE6,7,8	Pr	C		Ct
Rissoa sp.		LE9	LE9		G	R		UC
Rissoa ventricosa Desmarest, 1814				LE8	G	R		UC
Tricolia sp.		LE8			G	R		UC
Turbonilla lactea (Linnaeus, 1758)		LE9	LE7	LE7	Pa	UC		C
Turritella sp.		LE9			SF	R		UC
Class Bivalvia								
Abra alba (Wood, 1802)	LE9	LE5,7,8,9	LE7,8,9	LE7,8,9	SF	C		Ct
Abra nitida (Müller, 1776)		LE9	LE9	LE8	SF	UC		C
Acanthocardia echinata (Linnaeus, 1758)		LE7,8,9			SF	C		VC
Acanthocardia paucicostata (Sowerby, 1841)	LE8	LE6,7	LE7,8,9	LE7,8	SF	C		Ct
Acanthocardia tuberculata (Linnaeus, 1758)				LE9	SF	R		UC
Cerastoderma edule (Linnaeus, 1758)				LE8	SF	R		UC
Cerastoderma glaucum (Poiret, 1789)		LE8	LE5,7		SF	C		VC
Circomphalus casina (Linnaeus, 1758)	LE7	LE8	LE8		SF	UC		C
Corbicula fluminea (Müller, 1774)	UE1,2,3,4	UE1,2,3,4	UE1,2,3,4	UE1,2,3	SF	C	Ct	
Corbula gibba (Olivi, 1792)	LE7,9	LE5,6,7,8,9	LE5,6,7,8,9	LE5,6,7,8	SF	VC		Ct
Donax semistriatus Poli, 1795	LE9	LE9			SF	R		UC
Donax sp.		LE9			SF	R		UC
Donax trunculus Linnaeus, 1758	LE7				SF	R		UC
Donax venustus Poli, 1795		LE8			SF	R		UC
Dosinia lupinus (Linnaeus, 1758)		LE8,9	LE7,8,9	LE8,9	SF	C		VC
Gari fervensis (Gmelin, 1791)		LE9			SF	R		UC
Gastrana fragilis (Linnaeus, 1758)			LE9		SF	R		UC
Glycymeris glycymeris (Linnaeus, 1758)		LE9			SF	R		UC
Laevicardium crassum (Gmelin, 1791)		LE8			SF	R		UC
Lutraria lutraria (Linnaeus, 1758)	LE9				SF	R		UC
Mactra corallina (Linnaeus, 1758)	LE7,9	LE7,8			SF	C		VC
Mactra sp.		LE9	LE9		SF	R		UC
Musculus discors (Linnaeus, 1767)	LE7,8	LE7,9	LE5,7	LE6,7,8	SF	VC		Ct
Mytilus galloprovincialis Lamarck, 1819				LE8	SF	R		UC
Pandora inaequivalvis (Linnaeus, 1758)	LE7,8	LE7,8,9	LE8,9	LE7	SF	C		VC
Pharus legumen (Linnaeus, 1758)		LE9		LE9	SF	R		UC

Appendix 1 (cont.). – List of the identified taxa that were found at all the stations over the entire study period. The stations where each taxon was found are also listed. See Figure 1 for sampling station codes; FG, feeding guild (see Table 2 for feeding guild codes); CI, Constancy index; Ct, constant; VC, very common; C, common; UC, uncommon; R, rare.

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Pitar rudis (Poli, 1795)		LE8	LE8		SF	R		UC
Scrobicularia plana (da Costa, 1778)	LE9			LE8	SF	UC		C
Solemya togata (Poli, 1795)			LE9		SF	R		UC
Solen sp.		LE9	LE5		SF	UC		C
Spisula subtruncata (da Costa, 1778)	LE8,9	LE9	LE7	LE7,8,9	SF	C		VC
Tapes philippinarum (Adams and Reeve, 1850)			LE7	LE7	SF	R		UC
Tapes pullastra (Unspecified)	LE7	LE7,8			SF	UC		C
Tapes sp.		LE8,9	LE6,7,8,9	LE7,8,9	SF	C		Ct
Tellina albicans (Gmelin, 1791)		LE8,9	LE7,9	LE7	SF	C		VC
Tellina sp.		LE8,9	LE7,9	LE6,9	SF	C		Ct
Tellina tenuis da Costa, 1778		LE9	LE5,8,9	LE7	SF	C		Ct
Class Scaphopoda								
Antalis novemcostata (Lamarck, 1818)				LE8	Pr	R		UC
Antalis sp.		LE8,9	LE9	LE7,8	Pr	C		VC
PHYLUM ANNELIDA								
Class Hirudinea								
Helobdella stagnalis (Linnaeus, 1758)	UE2				Pr	R	UC	
Piscicola geometra (Linnaeus, 1758)	UE2				Pa	R	UC	
Class Oligochaeta								
Haplotaxidae indet.			UE3		DF	R	UC	
Lumbricidae indet.	UE2			UE1	DF	UC	C	
Naididae indet.	UE1,2	UE1,2,3	UE1,2,4; LE5	UE1,2,3,4	DF	VC	Ct	UC
Tubificidae indet.	UE1,2,3; LE5	UE1,2,3,4	UE1,2,3	UE1,2,3	DF	VC	Ct	UC
Class Polychaeta								
Ampharete grubei Malmgren, 1865	LE6,7,8	LE6,7,8,9	LE6,7,8,9	LE6,7,8	DF	C		Ct
Aricidea sp.	LE8,9	LE6,7,8,9	LE7,8,9	LE6,7,8,9	DF	VC		Ct
Armandia cirrhosa Filippi, 1861	LE6,7,8	LE5,6,7,9	LE5,6,7,9	LE7,8	DF	VC		Ct
Capitella capitata (Fabricius, 1780)	LE6		LE5,6,7,8,9	LE6,8,9	DF	VC		Ct
Capitellidae indet.	LE8	LE7			DF	UC		C
Caulleriella zetlandica (McIntosh, 1911)	LE8	LE6,7,8,9	LE6,7,8,9	LE6,7,8,9	DF	C		Ct
Cirratulus cirratus (Müller, 1776)			LE7,8,9	LE7,9	DF	C		VC
Clymenura clypeata (Saint-Joseph, 1894)			LE7	LE7,8	DF	UC		C
Diopatra neapolitana Delle Chiaje, 1841			LE8,9	LE7,9	DF	C		VC
Eteone picta Quatrefagues, 1865		LE6,7,9	LE7,9	LE7	Pr	C		VC
Euclymene oerstedi (Claparède, 1863)			LE7,8,9	LE6,7,8	DF	C		Ct
Eunice harassii Audouin & Edwards, 1834		LE8,9	LE7		DF	C		VC
Ficopomatus enigmaticus (Fauvel, 1923)	LE6				SF	R		UC
Glycera sp.		LE6,9	LE6,7,8,9	LE6,7,8,9	Pr	C		Ct
Glycera tesselata Grube, 1840				LE9	Pr	R		UC
Glycera tridactyla Schmarda, 1861	LE8,9	LE6,7			Pr	C		Ct
Harmothoe sp.		LE6,9	LE7,8,9		Pr	C		Ct
Hediste diversicolor (Müller, 1776)	LE7,8	LE6,7,8	LE7,8	LE5,6,7,8	O	C		Ct
Heteromastus filiformis (Claparède, 1864)		LE5,6	LE7,8,9	LE6,7,8,9	DF	VC		Ct
Hydroides norvegicus Gunnerus, 1768		LE7			SF	R		UC
Lagis koreni Malmgren, 1866	LE9		LE6,9		DF	UC		C
Laonice cirrata (Sars, 1851)			LE8,9		DF	UC		C
Lepidonotus squamatus (Linnaeus, 1758)			LE7,9	LE8	Pr	C		VC
Lumbrineris sp.		LE7,8,9	LE6,7,8	LE7,8	Pr	C		Ct
Magelona papillicornis Müller, 1858		LE9		LE7,9	DF	UC		C
Melinna palmata Grube, 1870		LE6,7,8,9	LE5,7,8,9	LE6,7,8	DF	VC		Ct
Micronephthys maryae San Martín, 1982	LE7	LE7,8,9	LE7,8,9	LE6,7,8,9	Pr	C		Ct
Neosabellides oceanica (Fauvel, 1909)			LE6,7,8	LE7	DF	C		VC
Nephtys assimilis Örsted, 1843				LE9	Pr	R		UC
Nephtys cirrosa (Ehlers, 1868)	LE9				Pr	R		UC
Nephtys hombergii Lamarck, 1818			LE9		Pr	R		UC
Nephtys sp.				LE6,8	Pr	UC		C
Nereididae indet.			UE4; LE6,7	UE4	DF	C	UC	C
Notomastus sp.		LE9	LE7,8,9		DF	C		VC
Oriopsis armandi (Claparède, 1864)	LE7,8,9	LE5,7,9	LE5,6,9	UE4; LE5,8	SF	VC	UC	Ct
Paradoneis lyra (Southern, 1914)		LE7,9	LE6,7,8,9	LE7,8,9	DF	C		Ct
Perinereis cultrifera (Grube, 1840)	LE5,6	UE4	LE6	UE4; LE8	O	VC	UC	VC
Phyllodoce mucosa Örsted, 1843	LE9	LE9	LE9		Pr	R		UC
Phylo foetida (Claparède, 1869)			LE8	LE7	DF	UC		C
Pista cristata (Müller, 1776)	LE7,8	LE6,7,8,9	LE7,8,9	LE7,8	DF	C		Ct
Prionospio malmgreni Claparède, 1869		LE7,8,9	LE7,8,9	LE7,8,9	DF	C		VC
Pseudopolydora antennata (Claparède, 1869)	LE7,8	LE5,6,9	LE5,6,7,8,9	LE6,7,8,9	DF	VC		Ct
Sabella pavonina Savigny, 1822			LE8	LE7,8	SF	UC		C
Sabellidae indet.	LE6		LE8,9	LE6	SF	C		VC
Serpula vermicularis Linnaeus, 1767		LE5,6,7,8	LE7		SF	C		Ct
Sigambra parva (Day, 1963)		LE9	LE7,8,9	LE7,8	Pr	C		VC

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Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Spio filicornis (Müller, 1776)	LE8	LE5,6,7,9	LE5,6,7,8,9	LE6,7,8,9	DF	VC		Ct
Streblospio benedicti Webster, 1879	LE7,8,9	LE5,6,7,8,9	LE5,6,7,8,9	LE5,6,8,9	DF	VC		Ct
Syllidia armata Quatrefages, 1866			LE6,7,8,9	LE6,7,8,9	Pr	C		Ct
PHYLUM ARTHROPODA								
Class Arachnida								
Acaridida indet.			UE1		Pr	R	UC	
Halacaridae indet.				UE3	Pr	R	UC	
Hydrozetes sp.		UE2			Pr	R	UC	
Lebertia sp.	UE2	UE2,4			Pr	UC	C	
Sperchon sp.		UE2		UE2	Pr	R	UC	
Torrenticola sp.		UE2,3	UE3		Pr	UC	C	
Class Pantopoda								
Nymphon gracile Leach, 1814				LE7	O	R		UC
Class Branchiopoda								
Daphnia longispina (Müller, 1776)	LE5				G	R		UC
Eurycercus lamellatus (Müller, 1776)				UE1	G	R	UC	
Ilyocryptus sordidus (Liévin, 1848)			UE1		G	R	UC	
Simocephalus exspinosus (Koch, 1841)		UE1			G	R	UC	
Simocephalus vetulus (Müller, 1776)				UE1	G	R	UC	
Class Ostracoda								
Cyprideis torosa (Jones, 1850)			LE7	LE8	DF	UC		C
Fabaeformiscandona fabaeformis (Fischer, 1851)		UE2	UE1,2	UE1,4	DF	C	VC	
Herpetocypris brevicaudata (Kaufmann, 1900)		UE3			DF	R	UC	
Herpetocypris sp.		UE4			DF	R	UC	
Class Copepoda								
Acanthocyclops latipes (Lowndes, 1927)			UE1		SF	R	UC	
Canuella furcigera Sars, 1903			LE7		SF	R		UC
Centropages chierchae Giesbrecht, 1889			LE6		SF	R		UC
Cyclops sp.				UE1,3	SF	UC	C	
Eucyclops serrulatus (Fischer, 1851)			UE1		SF	R	UC	
Macrocyclops albidus (Jurine, 1820)		UE1,2	UE1	UE1	SF	UC	C	
Class Malacostraca								
Ampelisca brevicornis (Costa, 1853)			LE9		SF	R		UC
Ampelisca sp.			LE7		SF	R		UC
Ampelisca typica (Bate, 1856)	LE8,9	LE9	LE9		SF	UC		C
Apseudes latreillii (Milne-Edwards, 1828)	LE9	LE8,9			DF	UC		C
Bathyporeia sp.				LE9	DF	R		UC
Bodotria arenosa Goodsir, 1843				LE9	DF	R		UC
Corophium orientale Schellenberg, 1928	LE5,6,7,8,9	UE4; LE5,6,9	UE4; LE5,6	UE3,4; LE5,6	DF	Ct	C	Ct
Corophium rotundirostre Stephensen, 1915			LE7,8,9	LE7,8,9	DF	C		VC
Cumopsis goodsir (Van Beneden, 1861)		LE9			DF	R		UC
Cyathura carinata (Krøyer, 1847)	LE7		LE7,8	LE7,8	DF	UC		C
Decapoda indet.	LE9			LE7,9	O	UC		C
Diastylis sp.		LE8	LE8	LE6,7,8	DF	UC		C
Echinogammarus longisetosus Pinkster, 1973	UE1,3,4	UE4	UE2	UE2	O	C	Ct	
Gammarus aequicauda (Martyinov, 1931)				LE8	O	R		UC
Iphinoe sp.	LE9	LE9		LE7,8	DF	UC		UC
Lembos sp.					DF	R		UC
Lembos spiniventris (Stebbing, 1895)				LE7	DF	R		UC
Leptocheirus pilosus Zaddach, 1844	LE9		LE7	LE8	SF	C		VC
Leucothoe incisa (Robertson, 1892)	LE9		LE7,8	LE7,8	O	C		VC
Liocarcinus corrugatus (Pennant, 1777)			LE9		O	R		UC
Medorippe lanata (Linnaeus, 1767)			LE9		O	R		UC
Microprotopus sp.		LE9			O	R		UC
Monoculodes acutipes Ledoyer, 1983	LE9		LE8	LE6,7,8,9	O	C		Ct
Pariambus typicus (Kroyer, 1844)		LE8	LE8,9	LE6,7,8	O	C		Ct
Perioculodes longimanus (Bate & Westwood, 1868)				LE7,8	DF	UC		C
Phtisica marina Slabber, 1769			LE6,7	LE7,8	O	C		VC
Praunus flexuosus (Müller, 1776)	LE9				SF	R		UC
Pseudocuma longicorne (Bate, 1858)	LE8	LE9			DF	UC		C
Sphaeroma serratum (Fabricius, 1787)				LE8	O	R		UC
Synchelidium haplocheles (Grube, 1864)	LE7	UE4			DF	UC	UC	UC
Synchelidium sp.	LE8				DF	R		UC
Upogebia pusilla (Petagna, 1792)	LE9				SF	R		UC
Upogebia sp.		LE9			SF	R		UC
Class Insecta								
Baetis fuscatus (Linnaeus, 1761)	UE2	UE2,3		UE2	DF	UC	C	
Baetis pavidus Grandi, 1949	LE7		LE6,7		DF	UC		C
Caenis luctuosa (Burmeister, 1839)	UE1,2,3	UE1,2	UE1,2	UE1,2	DF	C	VC	

APPENDIX 1 (cont.). – List of the identified taxa that were found at all the stations over the entire study period. The stations where each taxon was found are also listed. See Figure 1 for sampling station codes; FG, feeding guild (see Table 2 for feeding guild codes); CI, Constancy index; Ct, constant; VC, very common; C, common; UC, uncommon; R, rare.

Taxa	Summer	Autumn	Winter	Spring	FG	CI	CI UE	CI LE
Ceraclea dissimilis (Stephens, 1836)				UE2	DF	R	UC	
Ceraclea sobradieli (Navás, 1917)	UE2			UE2	DF	R	UC	
Chironomus sp.	LE7,8	UE4	UE1	UE1,3; LE6	DF	VC	VC	VC
Choroterpes picteti (Eaton, 1871)	UE2				DF	R	UC	
Coenagrion pulchellum (Van der Linden, 1825)				UE1	Pr	R	UC	
Coenagrionidae indet.		UE1	UE1		Pr	R	UC	
Drypos sp.		UE1	UE1		G	R	UC	
Ecnomus tenellus (Rambur, 1842)	UE2	UE1,2	UE2	UE2	O	UC	C	
Ephoron virgo (Olivier, 1791)	UE1,2,3,4			UE2	SF	C	Ct	
Hydropsyche exocellata Duföur, 1841	UE2	UE2	LE5	UE2	SF	UC	UC	UC
Hydroptila sp.	UE2	UE2		UE2	G	R	UC	
Mystacides azurea (Linnaeus, 1761)	UE2				DF	R	UC	
Orthotrichia angustella (McLachlan, 1865)			UE2		G	R	UC	
Pseudocloeon atrebatinus Eaton, 1870	UE2	UE2	UE2		DF	R	UC	
Psychomyia pusilla (Fabricius, 1781)				UE2	DF	R	UC	
Sf. Orthocladiinae indet.	UE1,2; LE7	UE1,2,4	UE1,2,4; LE5,6,7	UE1,2; LE5,7,8	DF	Ct	VC	Ct
Sf. Tanypodinae indet.	UE1	UE1,2,3,4	UE1	UE1,2,3,4	Pr	C	Ct	
Simulium erithrocephalum (De Geer, 1776)	UE2		UE2		SF	R	UC	
Tr. Chironomini indet.	UE1	UE4	UE2	UE2	DF	C	VC	
Tr. Tanytarsini indet.	UE1,2	UE2,3	UE1,2	UE1,2,3	DF	C	VC	
Trithemis annulata (Palisot de Beauvois, 1807)		UE1			Pr	R	UC	
PHYLUM PHORONIDA								
Class Phoronida								
Phoronis ovalis Wright, 1856				LE6	SF	R		UC
Phoronis psammophila Cori, 1889		LE5,7,8,9	LE6,7,8	LE6,7,8	SF	VC		Ct
PHYLUM ECHINODERMATA								
Class Holothuroidea								
Thyone sp.			LE7	LE7,8	DF	UC		C
Class Ophiuroidea								
Amphipholis squamata (Delle Chiaje, 1828)	LE9	LE9	LE7,8	LE8	DF	C		VC
Amphiura chiajei Forbes, 1843			LE9	LE7	DF	UC		C
Class Echinoidea								
Fibulariidae indet.	LE9				DF	R		UC
Echinocardium sp.		LE9			DF	R		UC

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Towards a suitable ecological status assessment of highly stratified mediterranean estuaries: A comparison of benthic invertebrate fauna indices

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ABSTRACT

Biotic indices developed to assess the ecological status of coastal waters according to the European Water Framework Directive (WFD) often show discrepancies when they are applied in transitional environments. Although several indices have been widely used in transitional waters throughout Europe, there is still a lack of knowledge about their suitability assessing ecological status. We evaluated the performance of most common used biotic indices and community parameters (e.g. Multivariate AZTI's Marine Biotic Index (M-AMBI), BENTIX, Benthic Opportunistic Polychaetes Amphipods index (BOPA), diversity indices, species richness, abundance) that have been proposed in the scope of WFD, using data of macroinvertebrate community coming from a special case of transitional water body, the highly stratified Ebro estuary. Additionally, we tested their ability to respond to the main pressures affecting the Ebro estuary, the hydrological alteration due to regulation and the pollution pressure due to nutrient enrichment. Estimation of hydrological alteration was based on flow historical data (period from 1913 to 1963), that we assumed as 'hydrological reference conditions' for Ebro estuary. Pollution pressure was estimated by means of PCA analysis including organic and nutrient enrichment related variables, expressed as a synthetic index by PCA factor scores extraction. All the community parameters were able to detect changes in macrofauna composition along the estuarine gradients and were able to differentiate between the impoverished stations and the healthier ones. Regarding indices, the ratings were contradictory and only M-AMBI classified the stations in the correct way. Strong significant correlations were found between indices and metrics and the calculated pressures; nevertheless, these correlations showed a paradoxical result, since increasing hydrological alteration benefited the macrofauna, achieving great complexity. Other identified limitations of biotic indices were the opposite classifications, overestimation of ecological status and low resolution ability. We conclude that for transitional water ecosystems, where each water body has particular characteristics, is difficult the use of 'common biological' assessment tools as the results of this study, among others (more details in discussion section), have demonstrated. Nevertheless, M-AMBI seemed to work in the correct way, so further investigation about its use for transitional waters is necessary. The development of new strategies such as the use of historical data, the use of metrics as a complement for the assessment could be a reliable alternative.

1. Introduction

Estuaries are interface systems between rivers and sea, characterized by unstable hydrological, morphological and chemical conditions, resulting in stressful habitats where biological communities are structured along strong environmental gradients (Day et al., 1989; Dauvin, 2007). Nevertheless, these complex ecosystems are largely recognized by their high productivity and their importance, from both economic and conservation perspectives (Ysebaert et al., 1998; Edgar et al., 1999; McLusky, 1999; Pierson et al., 2002; Russell et al., 2006).

The rapid human population growth during the last century has increased the pressures over these areas threatening their ecological integrity, their economic value and even affecting

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public health (Schlacher and Wooldridge, 1996; Edgar et al., 1999; McLusky, 1999; Dauer et al., 2000; Dauvin, 2007; Elliott and Quintino, 2007; Gray and Elliott, 2009). The main anthropogenic pressures affecting estuaries are industrial wastewater, urban sewage effluents, agriculture and farmland runoff, fish farming and harbors (Justic et al., 1995; McLusky and Elliott, 2004 Zaldívar et al., 2008; Gray and Elliott, 2009). These activities cause an excess of nutrients in Water Bodies (WBs), increase the organic matter loads and even promote the accumulation of dangerous pollutants in the sediment such as heavy metals, toxic compounds and hydrocarbon substances (Boynton et al., 1995; Day et al., 1997; Cantillo, 1998; Nedwell et al., 1999; Navarro-Ortega et al., 2010). High nutrient loads produce direct ecological impacts over biological communities (Karlson et al., 2002), mostly associated with eutrophication processes (Bock et al., 1999; Wang et al., 1999; Hänninen et al., 2000). Besides, organic enrichment causes episodes of hypoxia and low redox potential values. These facts disturb composition, trophic structure and biomass of the biological communities (Pearson and Rosenberg, 1978; Grebmeier et al., 1988; Díaz, 2001).

In Mediterranean aquatic ecosystems, the impacts produced by these pressures are magnified by the strong seasonal and interannual hydrological variability (Caiola et al., 2001a; Ferreira et al., 2007a, b). Moreover, human responses to this hydrological fluctuation involve flow regulation measures, such as reservoirs, that frequently disrupt aquatic ecosystems, producing accentuated environmental changes (Caiola et al., 2001b).

The European Union reacted to the severe ecological decline of aquatic ecosystems by proclaiming Water Framework Directive (WFD) in 2000 (European Parliament, 2000). The WFD provides a basis for the conservation, protection and improvement the ecological integrity of all WBs, including groundwater, inland surface water, coastal and transitional waters. According to the WFD the estuaries are classified as Transitional Waters (TWs); defining them as: bodies of surface water in the vicinity of river mouths which are partly saline in character as a result of their proximity to coastal waters but which are substantially influenced by freshwater flows.

Ecological quality assessment of a water body must be based on the status of different biological quality elements (e.g. benthic invertebrate fauna or aquatic flora) and endorsed by hydromorphological and physicochemical quality elements. The status of these elements is determined by the deviation they exhibit from the type-specific reference conditions, at undisturbed or nearly undisturbed situations (WFD, 2000/60/EC -Annex V). Benthic invertebrates have been identified as key biological element for Ecological Status (ES) assessment of TWs; they play important roles in the ecology of aquatic ecosystems and respond to anthropogenic stress (Pearson and Rosenberg, 1978; Dauer, 1993; Grall and Glemarec, 1997 Dauer et al., 2000; Simboura and Zenetos, 2002; Bustos-Baez and Frid, 2003; Perus et al., 2007). Nevertheless, within estuarine ecosystems it is difficult to establish a stressor-response relationship using Biotic Indices (BIs) since they are naturally stressed ecosystems; this difficulty was coined as the term 'Estuarine Quality Paradox' (Dauvin, 2007; Elliott and Quintino, 2007).

Moreover, in highly stratified estuaries, like the study case, obtaining such a response is even more difficult because both natural and anthropic hydrological variations (spatial and temporal) produce rapid and abrupt changes in biological communities (Nebra et al., 2011). Therefore, establishing reference conditions for these systems (the basis for the development of BI according to the WFD criteria) is a challenging task.

Since the apparition of the WFD in the year 2000, some BIs based on soft-bottom benthic invertebrate communities such as the AZTI's Marine Biotic Index (AMBI) (Borja et al., 2000), the multivariate AMBI (M-AMBI) (Borja et al., 2004; Muxika et al., 2007), BENTIX (Simboura and Zenetos, 2002) and Benthic Opportunistic Polychaetes Amphipods index (BOPA) (Dauvin and Ruellet, 2007) have proved to be very useful tools in assessing the ES of coastal and TWs, especially regarding nutrient and organic enrichment. However, the estuarine systems where these indices were developed correspond to 'well-mixed' type, which are

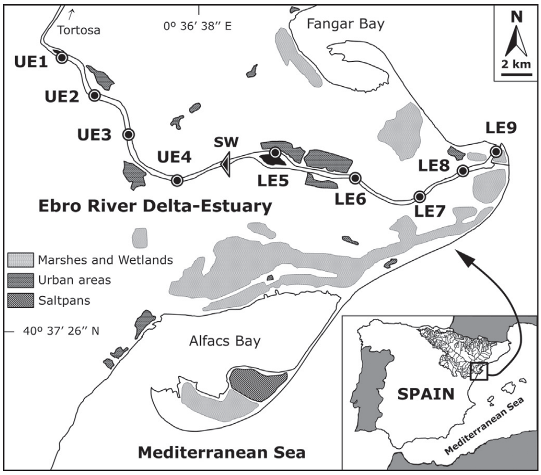

Fig. 1. Map of the Ebro River basin and its delta showing the studied estuary with the position of the nine sampling stations. UE, upper estuary stations; LE, lower estuary stations; SW, null point position.

Table 1
Biotic indices value ranges and ES boundaries used in this paper to estimate the sampling stations ES categories.

Biotic index	Index value	ES	Index requirements
IBMWP	$100 < \text{IBMWP}$	High	PRIP: decrease
	$61 \leq \text{IBMWP} < 100$	Good	TR: family
	$35 \leq \text{IBMWP} < 61$	Moderate	MSP: D-frame net 500 μm
	$15 \leq \text{IBMWP} < 35$	Poor	Qualitative
	$0 < \text{IBMWP} < 15$	Bad	FA: freshwater
M-AMBI	$0.77 < \text{M-AMBI} \leq 1.00$	High	PRIP: decrease
	$0.53 < \text{M-AMBI} \leq 0.77$	Good	TR: usually genus or species level
	$0.39 < \text{M-AMBI} \leq 0.53$	Moderate	MSP: grab, replicates, >1 mm
	$0.20 < \text{M-AMBI} \leq 0.39$	Poor	Quantitative
	$0.00 < \text{M-AMBI} \leq 0.20$	Bad	FA: coastal and transitional waters
BENTIX	$4.5 \leq \text{BENTIX} < 6.0$	High	PRIP: decrease
	$3.5 \leq \text{BENTIX} < 4.5$	Good	TR: usually genus or species level
	$2.5 \leq \text{BENTIX} < 3.5$	Moderate	MSP: grab, replicates, >1 mm
	$2.0 \leq \text{BENTIX} < 2.5$	Poor	Quantitative
	$0.0 \leq \text{BENTIX} < 2.0$	Bad	FA: coastal and transitional waters
BOPA	$0.00000 \leq \text{BOPA} \leq 0.04576$	High	PRIP: increase
	$0.04576 < \text{BOPA} \leq 0.13966$	Good	TR: genus and species level
	$0.13966 < \text{BOPA} \leq 0.19382$	Moderate	MSP: grab, replicates, >1 mm
	$0.19382 < \text{BOPA} \leq 0.26761$	Poor	Quantitative
	$0.26761 < \text{BOPA} \leq 0.30103$	Bad	FA: coastal and transitional waters

PRIP, predicted response to increasing perturbation; TR, taxonomic resolution required; MSP, methodology and sample processing; FA, field of application.

systems with different ecological dynamics compared with ‘highly stratified’ estuaries like the Ebro estuary.

The present study analyzes the performance of M-AMBI, BENTIX and BOPA indices developed under the scope of the WFD, to the main anthropic pressures on the Ebro estuary, a highly stratified Mediterranean estuary. It is expected that results obtained could assist on the development of a suitable ES assessment approach for salt wedge estuaries.

2. Methods

2.1. Study site

The Ebro estuary (Fig. 1) is a highly stratified or salt-wedge estuary located at the NE of the Iberian Peninsula (40°43′10″N, 0°40′30″E). The microtidal regime of the Mediterranean Sea about 20 cm (Cacchione et al., 1990), promotes the formation of a salt wedge whose dynamics (advance, retreat and permanence) is controlled by the river discharge. Continuous river flow values exceeding 350–400 $m^3 s^{-1}$ pushes the salt wedge from the river channel and the estuary becomes a river. Conversely, when the river discharge is lower than 100 $m^3 s^{-1}$, the salt wedge reaches its maximum distance upstream 30–32 km from the river mouth (Ibáñez et al., 1997); intermediate flows together with the bathymetry of river-bed placed the salt wedge in different positions (Ibáñez et al., 1996). The main land use in the basin (85,362 km^2) is agriculture with more than 10,000 km^2 of irrigation, corresponding to approximately 90% of the water usage in the basin (Ibáñez et al., 2008). The main human impacts in the lower Ebro river and therefore its estuary are the strong flow regulation in the whole basin by nearly 190 dams (Batalla et al., 2004) and the nutrient enrichment of river water due to the input of agricultural and urban sewage effluents (Sierra et al., 2002; Terrado et al., 2006; Falcó et al., 2010). Nevertheless, during the last 15 years, an improvement of urban sewage treatment together with the restriction in the use of phosphate-based compounds dimmed the eutrophication process (Ibáñez et al., 2008, 2012a, b).

2.2. Sampling design and laboratory procedures

In order to cover the whole study area nine sampling stations were established from the river mouth to 37 km upstream. This stretch included the estuarine freshwater reach potentially accessible by salt wedge (Fig. 1). Each station was sampled seasonally (July and October 2007; January and April 2008) for benthic macroinvertebrates, in each sampling occasion three sediment samples were collected using a Ponar grab (0.046 m^2), sediment grain size and total organic matter (TOM), dissolved oxygen (DO), chlorophyll a, pheophytin, total suspended solids (TSS), organic suspended solids (OSS), nutrient loadings: phosphate (PO_4), total phosphorous (P_T), ammonia (NH_4), nitrite (NO_2), nitrate (NO_3), total nitrogen (N_T) and hydromorphological characteristics (depth, flow velocity and water transparency) (see Nebra et al., 2011 for detailed sampling and analysis procedures).

2.3. Biotic indices computation

The benthic macroinvertebrates of the Ebro estuary were structured in two contrasting communities associated with the upper estuary (UE) and lower estuary (LE) stretches, fresh and saltwater respectively; regardless of the sampling season due to maintained flows throughout the year (Nebra et al., 2011). Therefore, the sensitivity of the BIs and metrics to human disturbance was analyzed separately, using specific BIs and metrics for these two stretches. For the UE, the applied BI was the Iberian Biological Monitoring Working Party (IBMWP) (Alba-Tercedor et al., 2002) adapted for WFD requirements by Catalan Water Agency (ACA, 2006) see Table 1 for ES Boundaries and further information. Some commonly used freshwater macroinvertebrate metrics were also computed; these were: the percentages of functional feeding groups (grazers, deposit feeders, parasites, predators and suspension feeders) and the number and ratios (total and relative) of invertebrate orders comprising sensitive taxa (Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, Bivalvia and Odonata). Regarding LE stations M-AMBI, BENTIX and BOPA were applied (Table 1); the computation of the three marine indices (M-AMBI, BENTIX and BOPA) is based on the frequencies of functional or ecological groups that are considered as metrics. In these cases, besides the BI score, the metrics' individual scores were also analyzed. The descriptions, codes and predictable response to human pressures of the computed indices and metrics are summarized in Tables 3, 4 and 5. Moreover, the number of taxonomic ranks (families and genera), Shannon–Wiener diversity, Margalef diversity, Simpson dominance and Pielou's evenness indices as well as community

Table 2
Water physic-chemical parameters (annual mean and standard deviation) and sediment characteristics of the 9 sampling stations.

	Upper estuary stretch				Lower estuary stretch				
	UE1	UE2	UE3	UE4	LE5	LE6	LE7	LE8	LE9
Mouth distance (km)	37.00	34.00	30.00	25.00	18.00	12.00	6.00	3.00	0.00
Salinity	0.65 ± 0.13	0.64 ± 0.13	0.64 ± 0.13	0.65 ± 0.13	26.98 ± 17.58	34.79 ± 2.66	35.34 ± 2.00	35.57 ± 2.20	28.40 ± 16.35
DO (mg l^{-1})	9.43 ± 2.92	8.90 ± 2.92	9.18 ± 3.06	9.99 ± 3.23	6.21 ± 3.59	6.36 ± 3.36	6.82 ± 2.42	7.55 ± 2.20	8.41 ± 1.56
DO (%)	98.68 ± 21.50	93.18 ± 19.77	96.63 ± 21.50	105.10 ± 20.85	73.50 ± 34.82	80.90 ± 37.53	88.10 ± 26.72	98.48 ± 25.85	105.45 ± 17.76
PO_4 (mg l^{-1})	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.01 ± 0.02
P_T (mg l^{-1})	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.02
NH_4 (mg l^{-1})	0.04 ± 0.05	0.05 ± 0.06	0.06 ± 0.03	0.13 ± 0.18	0.30 ± 0.30	0.06 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.07
NO_2 (mg l^{-1})	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.03	0.01 ± 0.02	0.01 ± 0.01	0.01 ± 0.02
NO_3 (mg l^{-1})	3.07 ± 1.19	2.94 ± 1.26	3.01 ± 1.32	2.89 ± 1.20	1.08 ± 1.98	0.87 ± 1.63	0.85 ± 1.6	0.49 ± 0.88	1.01 ± 1.93
N_T (mg l^{-1})	3.40 ± 1.36	3.43 ± 1.41	3.45 ± 1.39	3.47 ± 1.41	1.53 ± 2.57	1.38 ± 2.37	1.17 ± 1.93	0.78± 1.20	1.36 ± 2.44
SiO_4 (mg l^{-1})	1.26 ± 0.39	1.15 ± 0.52	1.20 ± 0.55	1.35 ± 0.40	1.03 ± 0.48	0.64 ± 0.64	0.39 ± 0.55	0.40± 0.38	0.46 ± 0.55
Chlorophyll (μg l^{-1})	2.65 ± 3.08	0.75 ± 0.47	0.87 ± 0.70	0.88 ± 0.72	0.91 ± 0.52	0.87 ± 0.95	1.56 ± 1.51	1.94 ± 1.45	1.37 ± 1.04
Pheophytin (μg l^{-1})	2.43 ± 3.02	0.99 ± 0.86	1.01 ± 0.85	1.12 ± 1.13	1.18 ± 0.73	0.78 ± 0.47	0.77 ± 0.56	0.61 ± 0.43	0.69 ± 0.32
TSS (mg l^{-1})	9.21 ± 14.52	2.83 ± 2.25	4.85 ± 2.62	7.32 ± 4.07	14.43 ± 9.34	13.06 ± 10.69	12.52 ± 10.61	13.35 ± 12.87	32.06 ± 30.53
OSS (mg l^{-1})	2.41 ± 2.75	1.40 ± 0.85	1.59 ± 0.33	2.20 ± 0.75	3.43 ± 2.30	2.92 ± 2.18	2.61 ± 1.91	2.76 ± 2.35	4.21 ± 2.29
OSS (%)	48.05 ± 22.93	59.66 ± 26.71	37.27 ± 13.08	33.83 ± 13.72	25.87 ± 6.56	27.2 ± 9.76	23.94 ± 5.1	23.39 ± 4.13	19.62 ± 9.70
Mud (%)	12.36 ± 6.46	0.18 ± 0.11	31.56 ± 24.48	1.13 ± 0.88	1.91 ± 1.20	13.22 ± 6.04	16.69 ± 10.34	15.27 ± 0.54	32.36 ± 21.25
Sand (%)	86.69 ± 6.56	43.66 ± 16.41	66.53 ± 24.69	96.55 ± 2.70	97.86 ± 1.11	84.33 ± 4.24	82.47 ± 9.99	69.41 ± 12.75	65.13 ± 19.93
Gravel (%)	0.95 ± 0.11	56.16 ± 16.31	1.91 ± 0.21	2.33 ± 1.82	0.23 ± 0.08	2.45 ± 1.80	0.85 ± 0.35	15.33 ± 12.21	2.51 ± 1.91
TOM (%)	3.37 ± 0.76	1.30 ± 0.28	3.55 ± 0.24	2.47 ± 1.43	2.09 ± 0.24	4.86 ± 0.54	3.64 ± 1.30	5.07 ± 1.79	4.47 ± 1.04

structure and abundance descriptors (total abundance, density and taxa richness) were calculated.

2.4. Hydrological alteration

Prior to river regulation in the early sixties, big floods were common in the lower Ebro (Ibáñez et al., 1996); the suppression of such floods together with minimum flow conditions in summer and autumn led to an altered salt wedge dynamics in the Ebro estuary. For this reason an estimation of hydrological alteration was made with the aim of quantifying the effect over the biological communities. The Hydrological Pressure for the Ebro estuary was expressed as the deviation of the salt wedge dynamics from the 'expected natural dynamics' that it would correspond to each sampling occasion. For this, the dynamics of the salt wedge (position, probability of occurrence and permanence time in days) was calculated for each sampling station and occasion as a function of the daily average river flows. The position was estimated following Ibáñez et al. (1996), permanence time in days was estimated by counting the accumulated days before each sampling occasion with mean flow values lower than 350 $m^3 s^{-1}$ according to Ibáñez (1993), data available at Ebro Basin Authority web site (station 9027: Tortosa). Then, the dynamics of salt wedge (position, presence probability and permanence in days) was computed for each month and each sampling station during the period from 1913 to 1963 using Ebro Water Authority daily average flow values at Tortosa station. This time series represents the natural flow period, i.e. period before the construction of the two dams responsible for the lower Ebro regulation. The Hydrological Pressure was finally expressed as the absolute value of the deviation of the salt wedge presence (expressed as probability and time) from the monthly average probability and number of days of the salt wedge during natural flows period.

Table 3
Significant Spearman correlation coefficients among the BI scores, hydrological pressure expressed as the deviation of wedge occurrence probability and as deviation of permanence time from natural flow regime conditions, pollution pressure, environmental parameters and the community descriptive parameters (UE stretch $n = 16$; LE stretch $n = 20$).

Calculated pressures	IBMWP	M-AMBI	BENTIX	BOPA
H. pressure (p)	–	0.624[b]	−0.570[b]	0.530[a]
H. pressure (days)	–	0.660[b]	−0.599[b]	0.554[a]
P. pressure	−0.505[a]	−0.484[a]	0.520[a]	−0.474[a]
Environ. parameters				
DO (mg l^{-1})	–	–	−0.489[a]	–
NH_4 (mg l^{-1})	–	−0.490[a]	0.464[a]	–
P_T (mg l^{-1})	0.580[a]	–	–	–
TSS (mg l^{-1})	–	–	–	−0.476[a]
OSS (mg l^{-1})	–	−0.458[a]	–	−0.512[a]
TOM (%)	−0.648[b]	0.529[a]	−0.552[a]	0.502[a]
Community parameters				
Richness (S)	0.940[b]	0.902[b]	−0.838[b]	0.516[a]
Number of families	0.957[b]	–	–	–
Number of genera	0.933[b]	–	–	–
Density (ind m^{-2})	0.523[a]	0.501[a]	−0.588[b]	–
Margalef index (d)	0.942[b]	0.933[b]	−0.825[b]	0.582[b]
Pielou's evenness (J')	–	0.629[b]	–	0.663[b]
Shannon–Wiener index (H')	–	0.863[b]	–	0.742[b]
Simpson index (1-λ')	–	0.758[b]	–	0.707[b]
Deposit feeders (%)	–	–	0.570[b]	–
Grazers (%)	0.816[b]	–	–	–
Predators (%)	–	0.748[b]	−0.887[b]	0.589[b]
Suspension feeders (%)	−0.573[a]	–	–	–

DO, dissolved oxygen; P_T, total phosphorous; TSS, total suspended solids; OSS, organic suspended solids; TOM, total organic matter in sediment.
[a] $p < 0.05$.
[b] $p < 0.01$.

Table 4
Significant Spearman correlation coefficients among the UE Hydrological Pressure expressed as the deviation of wedge occurrence probability from probability in natural flow regime conditions, UE Pollution Pressure, community descriptive parameters and the individual metrics ($n = 16$).

	PRIP	H. pressure (p)	P. pressure
Shannon–Wiener index (H')	Decrease	–	−0.522[a]
EPT	Decrease	–	−0.662[b]
EPT (%)	Decrease	0.518[a]	−0.605[a]
EPT/oligochaeta	Decrease	–	−0.575[a]
EPT/diptera	Decrease	–	−0.698[b]
EP	Decrease	–	−0.666[b]
EP/total taxa (%)	Decrease	0.542[a]	–
EPTCBO	Decrease	–	−0.599[a]

PRIP, predicted response to increasing perturbation; EPT, ephemeroptera, plecoptera and trichoptera; EP, ephemeroptera and plecoptera; EPTCBO, ephemeroptera, plecoptera, trichoptera, coleoptera, bivalvia and odonata.
[a] $p < 0.05$.
[b] $p < 0.01$.

2.5. Data analysis

Principal Component Analysis (PCA) was performed separately for each estuarine stretch (UE and LE) with the organic pollution related variables (DO, nutrients, chlorophyll a, pheophytin and organic matter in sediment and in suspension). Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was used to assess the usefulness of a PCA. KMO ranges from 0 to 1 and should be >0.5 if variables are sufficiently interdependent for PCA to be useful (Tabachnick and Fidell, 2001). Once obtained the final PCAs, the two first factors of each PCA were merged by summing (inverting the values if their trends were opposed) as a combined index of Pollution Pressure. One way ANOVA followed by Post-hoc (LSD) test were carried out among stations for testing environmental parameters differences. To test the response of the BIs and metrics to the anthropogenic disturbance gradient, a correlation analysis was carried out with the Pollution Pressure index and with Hydrological Pressure index (probability and time). The measured variables were log or square root transformed (for absolute values and percentages, respectively) because homoscedasticity and linearity were clearly improved. Statistical analyses were performed using STATISTICA 8 software.

3. Results

3.1. Benthic environmental condition

During the study period daily mean river discharge was always below 350 $m^3 s^{-1}$ allowing the penetration of the salt wedge. This fact divided the estuary in two different stretches 'upper and lower estuary' (UE and LE); the UE (stations UE1-UE4) had freshwater (0.65 $g l^{-1} \pm 0.005$) (Table 2); whereas the LE five stations (LE5-LE9) had marine water ranging from (26.98 $g l^{-1} \pm 17.58$) to (35.57 $g l^{-1} \pm 2.20$), ANOVA revealed no significant differences for salinity within each stretch. LE stations permanence time of salt wedge was different for each sampling occasion 52, 140, 254 and 341 days for summer, autumn, winter and spring, respectively. The null point or salt wedge's tip was always located at the same position, between UE4-LE5 stations (25–18 Km from river mouth). Regarding nutrient concentrations, in general terms were higher in river water than in sea water (Table 2); especially for phosphates, nitrates, total nitrogen and silicate. Regarding LE stretch, LE5 showed the highest nutrient values such as PO_4 (0.03 $mg l^{-1} \pm 0.01$), P_T (0.06 $mg l^{-1} \pm 0.03$) and NH_4 whose concentration value was five times greater than values reported for other stations (0.30 $mg l^{-1} \pm 0.30$). Regarding LE stations, ANOVA ($P < 0.05$) revealed significant differences among LE5 and the rest of stations for PO_4 (post-hoc test, $P < 0.0307$ to 0.0438), P_T (post-hoc test,

$P < 0.0297$ to 0.0448) and NH_4 (post-hoc test, $P < 0.0099$ to 0.0229). The other nutrients showed an increase tendency from LE9 to LE5, from river mouth to null point. DO values were higher in UE stretch ranging from (8.90 ± 2.92) to (9.99 ± 3.23); whereas in LE stations oxygen concentrations decreased upstream, the lowest values were recorded at stations close to the null point (Table 2). Chlorophyll a concentrations were slightly higher in LE stations but without significant differences between UE and LE stations ($0.25\,\mu g\,l^{-1} \pm 0.10$ to $0.47\,\mu g\,l^{-1} \pm 0.07$, respectively). Pheophytin levels in the UE stations were close to $1.00\,\mu g\,l^{-1}$; except for the station UE1 in which the pheophytin largely exceeded the $2.00\,\mu g\,l^{-1}$. The LE stations presented pheophytin levels ranging from $0.61\,\mu g\,l^{-1} \pm 0.43$ to $1.18\,\mu g\,l^{-1} \pm 0.73$. The percentage of organic matter in sediment was slightly higher in LE stations ranging from (2.09 ± 0.24) to (4.86 ± 0.54); ANOVA ($P < 0.05$) revealed significant differences in TOM content, among stations UE1-UE2 (post-hoc test, $P < 0.0041$), UE2-UE3 (post-hoc test, $P < 0.0024$) and LE5 and the rest of stations (except LE7) (post-hoc test, $P < 0.0019$ to 0.0089).

The first two axes of the PCA for the UE explained 87.17% (62.00% and 25.17%, respectively) of the total variation. KMO (0.561) indicated the usefulness of the PCA; NO_2, NO_3 and NH_4 were positively correlated with the first PCA axis. In contrast, TOM, OSS were positively correlated with second axis. The first and second axis of PCA for the LE explained 53.4% and 23.89% respectively of the total variation. KMO (0.692) indicated the usefulness of the PCA; NO_2, NO_3 and PO_4 were positively correlated with the first PCA axis; the second axis was mainly related with NH_4 and OSS.

3.2. Macroinvertebrate abundance, taxa richness and diversity

A total of 21,805 individuals were collected belonging to 214 different taxa; for more detailed community results see Nebra et al.

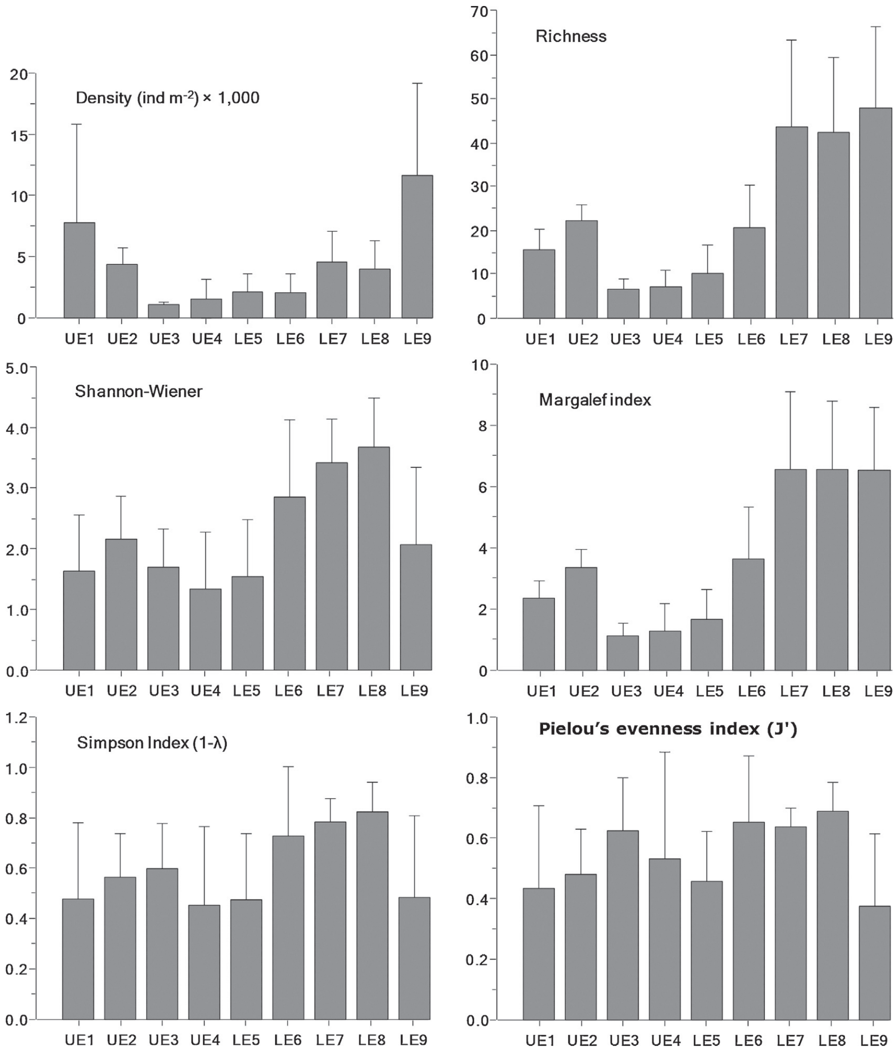

Fig. 2. Community descriptive parameters (annual mean ± standard deviation bars, $n = 4$) recorded at each station. See Fig. 1 for sampling stations' codification.

(2011). Higher densities were found at LE stretch (Fig. 2), especially at those stations near river mouth. LE9 station showed an annual mean of 11,650 ind m^{-2}. Intermediate density values were found in upper estuary stations due to the contribution of Tubificidae and the non-indigenous taxon *Corbicula fluminea*. The lowest densities corresponded to stations UE3, UE4, LE5 and LE6 located around null point. This pattern was similar in taxa richness terms (Fig. 2); station LE9 showed the highest richness values around 50 different taxa of annual mean. Stations located near river mouth LE8 and LE7 reached high values of richness too; whereas stations UE3, UE4 and LE5 showed the lowest values. Regarding diversity indices, all of them showed higher values in LE stations (Fig. 2) with a decreasing tendency towards null point.

3.3. Benthic biological indices and individual metrics

Due to the dynamic of the Ebro estuary specific BIs and metrics were computed for each stretch (UE and LE).

3.3.1. IBMWP

All the families found in UE stations computed for IBMWP calculation. Concerning UE stations, 12.4% were classified as ‘Good’, 18.8% as ‘Moderate’, 31.3% as ‘Poor’ and 37.5% as ‘Bad’. There wasn't any station achieving ‘High’ ES. The worst ES ratings corresponded to stations UE3 and UE4 which ranged between ‘Bad’ and ‘Poor’ (Fig. 3); UE2 ranged between ‘Moderate’ and ‘Good’ achieving this category in summer and spring. Station UE1ratings ranged from ‘Bad’ to ‘Moderate’. Spearman correlation coefficient reported significant correlations among IBMWP and 10 analyzed variables (Table 3). The Pollution Pressure showed strong negative correlation with IBMWP. Regarding community parameters richness measures, density and Margalef index showed significant and strong positive correlation with IBMWP. Total organic matter in sediment showed significant negative correlation with IBMWP.

3.3.2. M-AMBI

The percentage of non-scoring taxa in LE stations was very low (0.14% ± 0.30). results showed that 25.00% of LE stations were classified as ‘High’, 45.00% as ‘Good’, 15.00% as ‘Moderate’ and 15.00% as ‘Poor’; there were no ‘Bad’ ES rating (Fig. 3). Worst ES ratings corresponded to LE5 which ranged between ‘Poor’ and ‘Moderate’ (Fig. 3); LE6 ranged between ‘Poor’ and ‘Good’ reaching this category in the last three sampling occasions. LE7-LE8 showed ‘Good’ and ‘High’ ratings in the two first and two last sampling occasions respectively. The LE9 ES ranged between ‘Moderate’ and ‘High’.

Spearman correlation reported significant correlations among M-AMBI and 13 analyzed factors (Table 3). The Hydrological Pressure as probability and time reported strong positive correlation with this index; conversely the Pollution Pressure showed negative correlation. Regarding community parameters, all of them showed significant and strong positive correlation with M-AMBI. Ammonium was negatively correlated with M-AMBI.

3.3.3. BENTIX

Similarly to M-AMBI, BENTIX index showed similar percentages of non-scoring taxa 0.18% ± 0.30. Within LE stretch, the 25.00% of stations were classified as ‘High’, 5.00% as ‘Good’, 55.00% as ‘Moderate’ and 15.00% as ‘Poor’; there were no ‘Bad’ ES ratings. Contrary to M-AMBI, best ES ratings corresponded to LE5 which ranged between ‘Moderate’ and ‘High’; this rating was achieved in three of the sampling occasions (Fig. 3). LE6 ranged between ‘Poor’ and ‘High’ achieving ‘Moderate’ rating in two sampling occasions. LE7-LE8 showed similar pattern with ‘Moderate’ category in the three last sampling occasions; station LE9 ratings ranged between ‘Poor’ and ‘Moderate’. Spearman correlation coefficient reported

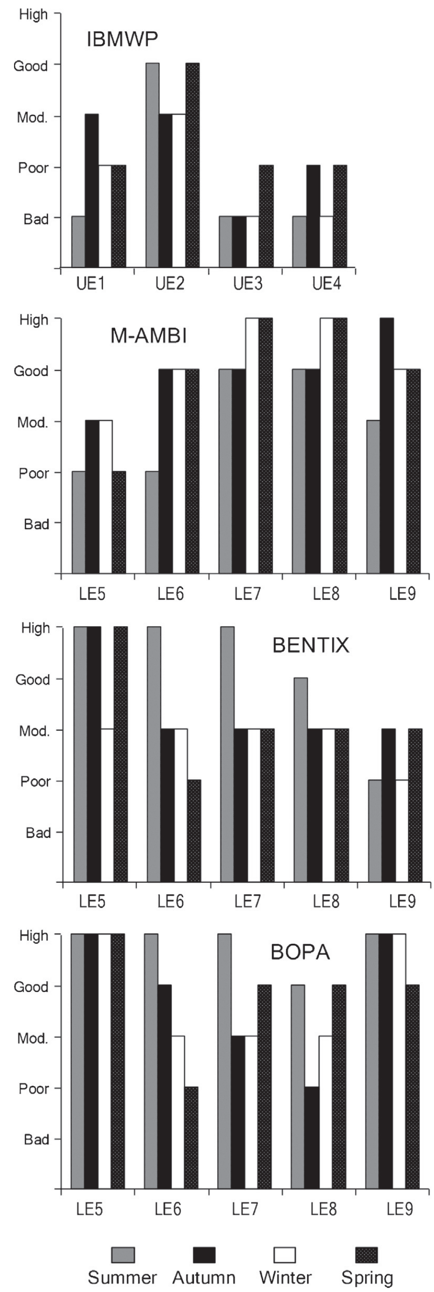

Fig. 3. Ecological status classification of UE and LE stations recorded at each sampling occasion after applying the four different BIs: IBMWP, M-AMBI, BENTIX and BOPA. See Fig. 1 for sampling stations' codification.

significant correlations among BENTIX and 11 analyzed variables (Table 3). Both measures of Hydrological Pressure showed strong negative correlation with BENTIX, nevertheless the Pollution pressure was positively correlated. Richness and Margalef index showed significant and strong negative correlation with BENTIX.

Regarding environmental parameters, DO showed negative correlation with BENTIX.

3.3.4. BOPA

According to this index, the benthic estuarine condition ranged between ‘High’ and ‘Poor’ ES categories; there were no ‘Bad’ ES rating. A 45.00% of LE stations were classified as ‘High’, 25.00% as ‘Good’, 20.00% as ‘Moderate’ and 10.00% as ‘Poor’. Best ES ratings corresponded to station LE5 which reached ‘High’ ES in all sampling occasions; following LE9 with ‘High’ category in three of the four sampling occasions (Fig. 3). LE6 ranged between ‘High’ and ‘Poor’ ES and together with LE8 were the only ones reaching ‘Poor’ rating with BOPA. LE7 ratings ranged between ‘Moderate’ and ‘High’ and showed ‘Moderate’ category in two sampling occasions; LE8 was the only station that did not reach ‘High’ ES rating with BOPA.

Spearman correlation analysis indicated significant correlations among BOPA and 12 analyzed variables (Table 3). BOPA was positively correlated with Hydrological Pressure and negatively with Pollution Pressure. With regard to community descriptive parameters, richness, Margalef index, Pielou's evenness, Shannon–Wiener's diversity index and Simpson index showed significant strong positive correlations with BOPA; TSS and OSS showed negative correlation with BOPA.

3.3.5. Individual metrics

Spearman correlation coefficients indicated strong significant negative correlations among Pollution Pressure for UE stretch and metrics such as Shannon–Wiener index and EPT (see Table 4 for data and abbreviations). In contrast, Hydrological pressure for UE reach was strong and positively correlated with the percentage of EPT (%) and EP/Total (%), despite of the predicted response to increasing perturbation (PRIP) for these two metrics. Spearman correlation coefficients indicated strong significant correlations among Pollution Pressure for LE stretch and six metrics in the expected way of their PRIP. Nevertheless, four metrics e.g. BENTIX-GI (%) a sensitive species group, showed a correlation not according with their PRIP. Hydrological pressure for LE stretch showed strong correlations with the great part of the metrics; nevertheless these correlations were not according with their PRIP (Table 5).

4. Discussion

4.1. Anthropogenic pressures and environmental condition of Ebro estuary bottom

Currently, main anthropogenic pressures affecting Ebro estuary are nutrient enrichment and alteration of the natural flow regime. Nutrient loadings of Ebro River are consequence of the inputs of wastewater mainly from agriculture and urban areas on the whole Ebro basin (Lacorte et al., 2006; Terrado et al., 2006). Water analysis revealed similar nutrient concentrations as recorded by Sierra et al. (2002) and Falcó et al. (2010). Despite of severe eutrophication episodes occurring in the recent past (Ibáñez et al., 1995), during the last decade the chemical status of Ebro estuary has clearly improved. Nutrient inputs have considerably decreased in the whole basin (Sierra et al., 2002; Ibáñez et al., 2008) limiting the primary production and therefore the sedimentary input of organic matter throughout the halocline by entrainment processes, alleviating the impact over estuary bottom (Ibáñez et al., 1995).

Conversely, the hydrological alteration produced by regulation could be considered the most important anthropogenic pressure affecting lower Ebro River and therefore its estuary. Regulation maintains a decreasing tendency of annual mean flow due constant increment on water demand, mainly for hydroelectric power generation and agricultural irrigation (Ibáñez et al., 1996, 2008; Sierra et al., 2004; Falcó et al., 2010). Moreover, natural variations of river discharge are homogenized through the year buffering the Mediterranean seasonality.

The worst impact caused by regulation over estuary ecology, excluding sediment retention by damming and the associated habitat loss and delta regression (Ibáñez et al., 1996), is the alteration of salt wedge dynamics. In natural regime flow conditions the salt wedge could advance till its maximum on dry periods or even totally disappear during high flow periods (for more details see Ibáñez et al., 1995, 1996; Movellán, 2004). However, the actual homogeneity in river discharges assures the presence of the salt wedge virtually in the same position for long periods (Ibáñez et al., 1995; Sierra et al., 2002; Falcó et al., 2010). This fact was corroborated by results obtained in this study (Fig. 1); Ebro estuary remained divided in two contrasting stretches (UE and LE) according with the physicochemical parameters recorded,

Table 5
Significant Spearman correlation coefficients among the LE Hydrological Pressure expressed as the deviation of wedge occurrence probability and as deviation of permanence time from natural flow regime conditions, LE Pollution Pressure and the individual metrics ($n = 20$).

	PRIP	H. pressure (p)	H. pressure (days)	P. pressure
Richness (S)	Decrease	0.609[b]	0.639[b]	−0.578[b]
Number of families	Decrease	0.581[b]	–	–
Number of genera	Decrease	0.548[a]	–	–
Margalef index (d)	Decrease	0.629[b]	0.686[b]	−0.584[b]
Pielou's evenness (J')	Decrease	0.450[a]	0.511[a]	–
Shannon–Wiener index (H')	Decrease	0.604[b]	0.654[b]	–
Simpson index (1−λ')	Decrease	0.542[a]	0.577[a]	–
Predators (%)	Decrease	0.585[b]	0.637[b]	−0.559[a]
BENTIX_GI (%)	Decrease	−0.534[a]	−0.558[a]	0.503[a]
BENTIX_GIII (%)	Increase	0.597[b]	0.519[a]	–
BENTIX_Tolerant (%)	Increase	0.566[b]	0.596[b]	−0.498[a]
BOPA_Amphip.	Decrease	−0.496[a]	−0.596[b]	0.609[b]
BOPA_Polych.	Increase	0.591[a]	0.651[b]	−0.474[a]
AMBI_GI (%)	Decrease	–	0.470[a]	−0.488[a]
AMBI_GII (%)	Decrease	–	0.504[a]	–
AMBI_GIII (%)	Increase	–	−0.509[a]	0.487[a]
AMBI_GIV (%)	Increase	0.510[a]	0.583[b]	–
AMBI_GV (%)	Increase	0.513[a]	–	–
AMBI_Sensitive (%)	Decrease	–	0.495[a]	−0.469[a]

PRIP, predicted response to increasing perturbation.
[a] $p < 0.05$.
[b] $p < 0.01$.

and supported by the results obtained after analysis of macroinvertebrate community (Nebra et al., 2011).

Coupled to long periods of salt wedge the water quality is worsening below the halocline due low water renewal rate; since freshwater flushing events are important for removing the accumulated pollutants and materials (Pierson et al., 2002). The entrainment processes between layers allows sedimentation of suspension particles and died organisms from the upper layer to the bottom (Lewis, 1997). This input of organic matter together with the sewage effluents discharged by two urban areas located downstream (Fig. 1) and suspension materials coming from the sea undergo a progressive accumulation towards the null point by frontal convergent circulation (Largier, 1993). The chemical reactions at the sediment surface releasing nutrients and organic matter decomposition (Stanley and Nixon, 1992; Pierson et al., 2002) could explain nutrient and oxygen concentrations found at LE5-LE6 stations (Table 2). The accumulation of diverse materials could promote eutrophication and oxygen depletion through microbial consumption (Stanley and Nixon, 1992; Largier 1993; Pierson et al., 2002) as occurred in the 90 's decade. Despite water anoxia was not recorded at any station of LE stretch, certain hypoxia with a declining gradient in DO from river mouth to null point was identified during study period; conversely, oxygen values for UE stretch were not a limiting factor. Benthic macroinvertebrates were more exposed to high nutrient levels and low dissolved oxygen concentrations from mouth to null point, benthic condition improved upstream once overcome null point. In summary, the Ebro estuary is recovering its chemical status approaching it to 'chemical reference conditions', on the other hand regulation is distancing the Ebro estuary from 'hydrological reference conditions', whose for this type of estuaries means great hydrological variation, this fact is especially relevant due to Mediterranean climate seasonality.

The novelty of this study lies in the way to quantify anthropogenic pressures; pollution pressure was estimated by PCA analysis including organic enrichment related variables, expressed as a synthetic index by PCA factor scores extraction. Estimation of hydrological alteration was based in historical data (period from 1913; to 1963), that we assume as 'hydrological reference conditions' for Ebro estuary. This way, allowed us to evaluate deviation of salt wedge dynamics from natural condition and therefore its relevance for estuarine ecology. We identify an increase on occurrence probability and permanence time for LE stretch and just the opposite for UE stretch.

4.2. Response of macroinvertebrate community to increasing perturbation

Differences found in macroinvertebrate community within each estuary stretch seemed to be independent from sediment grain-size and salinity. The lack of salinity gradient and the small variations recorded in sediment composition suggested other factors causing changes in community, such as the accumulation of nutrients or pollutants, the degree of exposition, organic matter, oxygen saturation or the water renewal among others (Carvalho et al., 2006). The impoverishment tendency followed by macroinvertebrate community in LE stretch was in accordance with nutrient enrichment and hypoxia gradients described. The variability of the community parameters showed a marked spatial difference among the studied stations. In an overall view richness, density and diversity indices decreased progressively along nutrient enrichment and oxygen depletion gradient from river mouth to the null point; once overcome this stressed zone these parameters increased again upwards (Fig. 2). Regarding LE stretch, those stations with degraded condition benthos (LE5 and LE6) had fewer species, lower abundances and lower diversity values than stations with a healthier benthos (LE7, LE8 and LE9). Thus, the community parameters seemed to characterize the stations coherently with the environmental gradients identified in the estuary bottom. Nevertheless, there are other possible explanations for community impoverishment tendency landwards; only euryhaline taxa are able to displace simultaneously with salt wedge's tip, its advance and retreat varied during day with discharge fluctuations depending on electric power generation demand. This fact force stenobiontic taxa to disappear. Seawards this stress disappears increasing the stability of abiotic factors; then the complexity of community is recovered as suggested by Sousa et al. (2006). Moreover, stations located near mouth (LE7, LE8 and LE9) can be easily recolonized by constant input of species from adjacent marine areas (Teske and Wooldridge, 2001). Also, Josefson and Hansen (2004) pointed out the low velocity of salt water flux into the estuaries as a cause of low richness by regulating larval dispersal from adjacent sea areas. Despite we found long periods of salt wedge's permanence, time enough to assure the colonization of stations LE7 and LE8. These stations demonstrated to be especially complex in composition, showing high richness and density values. Thus 'elasticity' (rapid community recovery), that is promoted by the presence of undisturbed communities in the vicinity of a particular site (Muxika et al., 2005), was assured. The option of daily stress at stations close to null point seemed to be plausible and complementary with oxygen-nutrient gradient and not mutually exclusive.

4.3. Biotic indices and metrics suitability

Results obtained in this study evidenced that suitable ecological status assessment of TWs is a really complex task. It is important to consider overall hindrances and limitations concerning TWs, such as description of reference conditions or identification of typologies, as well as the study case's particular ones. In the Ebro estuary case we found assessment difficulties due to its particular characteristics (stratified estuary). With respect to UE stations, the IBMWP showed no correlation with Hydrological Pressure; probably due to IBMWP was designed to assess impact of organic enrichment or because deviations obtained from natural flow regime were quite small to reflect them in a clear impact to freshwater reach community. Nevertheless, results showed that IBMWP and several metrics, e.g. Shannon–Wiener index or EPT, responded to Pollution Pressure in the expected way (negatively correlated), according with the PRIP. Despite of the sampling method used was not the most suitable for IBMWP; it showed an acceptable discriminatory ability identifying four ES categories.

Concerning LE stations, the response of community parameters and individual metrics was the expected showing negative correlation with the Pollution Pressure, except for those metrics being part of BENTIX and BOPA (Tables 3 and 5). Nevertheless, this study revealed a paradoxical response to increasing Hydrological Pressure as probability or permanence time; keeping in mind that the expected response for a pressure is a clear negative impact over biological communities (richness loss, enhance high abundance of few species and low diversities), correlation analysis revealed that Richness, diversity indices and M-AMBI showed a positive response to increasing hydrological alteration just the opposite of their PRIP. Thus, hydrological alteration led to an artificial stability of abiotic conditions facilitating the prompt achievement of 'environmental homeostasis', this promote the substitution of macroinvertebrate community by other community best structured, integrated by typical members of coastal areas and with a great complexity comparing with community found in other temperate estuaries (Nebra et al., 2011).

A common problem with ES assessment of estuaries is the impossibility of distinguishing natural from anthropogenic stress, both act over biological communities in the same way with the consequent problem of ES underestimate possibility; this was called 'estuarine quality paradox'. Nevertheless, the Ebro estuary is suffering its own 'quality paradox', since the most important anthropogenic pressure identified nowadays is dimming the natural stress associated with hydrological variation of a typical salt-wedge estuary of the Mediterranean region. This problem is causing an overestimation of all community parameters and therefore of the ES.

Regarding BIs ratings, the discrepancies among BIs were great and evident. For the same station and sampling occasion ratings could range between 1 and 3 ES levels depending on the applied BI. Only a small percentage of overlap was observed (Fig. 3). The problem of disagreement among BIs have been documented in many other studies (Reiss and Kroncke, 2005; Labrune et al., 2006; Simboura and Reizopoulou, 2008). Furthermore, for the Ebro estuary case the ES classification of the stations was contradictory; M-AMBI ranked stations in contrary way than BENTIX and BOPA. M-AMBI values showed a decreasing tendency towards null point where are located the stations with worst environmental condition and an impoverished benthic community. Conversely, BENTIX and BOPA gave to these perturbed stations the maximum ratings (Fig. 3); however, this fact was especially surprising for BENTIX based on the same paradigm (Pearson and Rosenberg, 1978). BENTIX showed a clear decreasing tendency towards river mouth, demonstrating that its ratings were opposed to the values of richness, abundance and diversity indices. The mismatch of the BENTIX in the Ebro estuary was not surprising in the manner as Simboura and Zenetos (2002) previously described the limitations of its use in TWs (i.e. estuaries and coastal lagoons). Moreover, according to Simboura and Reizopoulou (2008), this could be related to the different design of each index (in BENTIX each ecological group weighted equally, whereas AMBI renders a different coefficient for each one).

Regarding BOPA index, it did not seem to work adequately for either of stations or sampling occasion, it was not able to distinguish among stations and main causes of stress for LE macroinvertebrate communities. Since, applying BOPA most of the stations showed 'High' and 'Good' ES rating. This BI showed a similar tendency of BENTIX giving better ES classification to those stations with highest nutrient values and impoverished macroinvertebrate community. The problem of BOPA lied on its low discrimination ability and bias to overestimate the ES; similar results were reported by other authors in different TWs systems along Mediterranean coasts (Pranovi et al., 2007; Munari and Mistri, 2007; Afli et al., 2008; Blanchet et al., 2008; de-la-Ossa-Carretero et al., 2009). Probably the explanation is that this BI was essentially developed to assess hydrocarbon spill impact over benthic invertebrate communities; in the way that amphipods, the main component of BOPA, are recognized to be sensitive to hydrocarbons (Gesteira and Dauvin, 2000, 2005; Dauvin and Ruellet, 2007). Thus, BOPA did not carry the same bias than M-AMBI and BENTIX for its adaptation to natural muddy bottoms (Blanchet et al., 2008). Other, tendency observed in the BIs applied for the LE stations is that their ratings were so high (none of them found 'Bad' ES and in contrast showed elevated percentages of 'High' and 'Good' ES); BIs generally tended to show low resolving power and to overestimate the ES when are applied under different conditions they were develop for (Pranovi et al., 2007; Zettler et al., 2007; Bouchet and Sauriau, 2008; de-la-Ossa-Carretero et al., 2009; Tataranni and Lardicci, 2010).

5. Conclusions

Study outcomes suggest that a different approach for the assessment of TWs is necessary; particular characteristics of each study case difficult the use of 'wide-spread' assessment tools, even more when hydrological features are gaining relevance on ES assessment with respect to water and sediment quality. Analysis of community parameters (abundance, biomass, richness and diversity indices) and individual metrics seems to be the correct way to a suitable environmental assessment (they are easier to interpret and can be more broadly applicable than BIs). Identify metrics such as Shannon–Wiener index, Richness, Margalef index or EPT that respond to anthropogenic pressures and integrate them in a multimetric index could be a reliable complement to BIs. Finally, this study establishes a baseline approach to cope with the assessment difficulties not only for the Ebro estuary but also for other Mediterranean estuaries suffering hydrological alteration.

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