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1 Introduction

Despite the technological progress applied in recent years to the various production phases of Mediterranean Marine Fish Farming (MMFF), from reproduction to nutrition, several welfare and health issues of farmed fish still represent relevant limiting factors for sustainable production. In particular, the attempts to increase and diversify MMFF productions have been often accompanied by the emergence or reoccurrence of transmissible diseases strongly conditioned by management and environmental factors with consequent significant economic losses and repercussions on fish welfare and health parameters.

The experience gained in the attempt to control diseases has amply demonstrated that the treatment (when applicable) is often not conclusive, highlighting that the "disease event" is the result of many interacting factors linked to the pathogen, the host and the environment (intended as production system and surrounding ecosystem), which underly the occurrence of disease and influence morbidity and mortality rates.

For this reason, primary prevention measures have to be aimed at avoiding or limiting the contact between pathogen and host, ensuring optimal water quality, adequate nutritional intake, and implementing good management practices, supporting host's resistance/immunity to pathogens and complying with high standards of fish welfare, as addressed in the already issued deliverable D3.4 "Prophylactic practices for Mediterranean farmed fish" and in the forthcoming D3.5 "New vaccines" and D.3.6 "Methodology for assessing welfare in MMFF".

It is evident that an integrated approach is required to prevent and control diseases of farmed fish, which means adopting a holistic view to take into consideration the integrated manner all the different factors involved in the development of a disease and in fish health management (**Figure 1**).

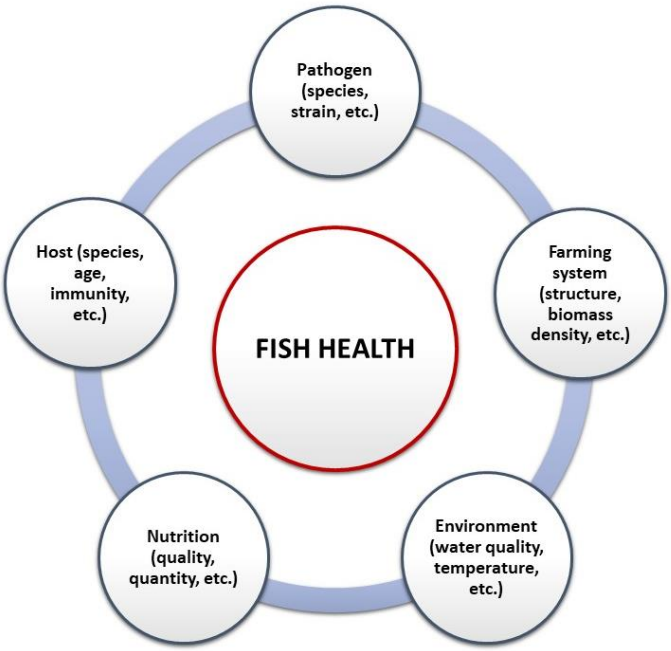

Figure 1. Different factors to be considered in fish health management.

Based on this concept, the project PerformFISH has developed in WP3 an integrated network of research activities aiming to provide the Mediterranean finfish aquaculture with effective tools to prevent, mitigate and control the most relevant diseases of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*).

Substantially, the diseases indicated as most relevant in the majority of Mediterranean aquaculture facilities by the members of the Industry Technical Experts Committee (ITEC; fish producers associations, fish health experts, etc.) of PerformFISH in a preliminary consultation have been: VNN by Betanodavirus and Vibriosis by *Vibrio anguillarum* and other Vibrios for European sea bass, Sparicotylosis by *Sparicotyle chrysophrii* and Enteromyxosis by *Enteromyxum leei* for gilthead sea bream and Photobacteriosis due to *Photobacterium damselae* subsp. *piscicida* for both species.

Some pathogens listed by the ITEC members were not considered in this Deliverable as they concerned only few facilities or specific areas (i.e. the crustacean *Lernanthropus kroyeri*). However, some additional pathogens were considered only at later stages of the project implementation as emerging (i.e. reassortant strains of Betanodavirus and *Enterospora nucleophila* in gilthead sea bream).

In the already issued Deliverable 3.2 “Epidemiological status of Mediterranean farmed fish”, an updated overview of the main diseases of European sea bass and gilthead sea bream has been provided, starting from the dawn of this sector and arriving at the current situation. Based on this analysis, it can be concluded that in the last decades there has been a substantial improvement in the knowledge on the diseases affecting these two species although some aspects have not yet been fully clarified as already done for most salmon and salmonids diseases.

Among these aspects, a reliable estimate of their impact (i.e. all the direct and indirect losses induced by them) and a further development of diagnostic techniques (both advanced and of practical utility) still show big room for improvement that could be useful to develop a higher awareness of European sea bass and gilthead sea bream diseases and to support the implementation of surveillance and biosecurity measures, respectively.

2 Importance of diagnosis for controlling diseases in aquaculture

2.1 Diagnosis as a key element for the prevention and control of fish diseases

“A correct diagnosis is three-fourths the remedy” is a famous sentence enunciated by Mahatma Gandhi to indicate that there is no cure without a diagnosis first. In addition to the cure of a specific disease, the diagnosis is therefore a key element for several biosecurity-related aspects, which are fundamental for the prevention and control of diseases.

In fact, as previously indicated, prevention should be considered the core and basis for the healthcare in aquaculture and management of aquatic systems. One of the main axis of any health and disease prevention system is the establishment of powerful integrated surveillance programs within the companies and farms, well designed and coordinated togeher with official surveillance programs and also precise diagnostic systems allowing accurate and efficent strategies and decisions. And the key for that is the availability of reliable, precise and efficient diagnostic methods allowing decision makers to adopt the best decisions (OIE-Office

International des Epizoozies/World Organization for Animal Health, 2019 https://www.oie.int/standard-setting/aquatic-manual/access-online/).

This deliverable was written in the historically exceptional moment of the SARS-CoV-2 pandemic that has shaken the whole world and particularly many European countries and from this extraordinary situation, we all learnt how relevant were, still are and will be the diagnostics for the affected people, health care services and in general, for all those involved in the management, control and supervision of the measures intended for the protection of the population. In this context, it is clearly indentifiable the relevance of the different diagnostic tests (fast tests, qPCR, serological tests) and diagnostic protocols (at clinical level but also at epidemiological level) in the Covid-19 management. The absence of available tests and realiable diagnostic protocols, together with the undervaluation of the risks and the lack of realistic prevention measures mainly during the early phases of the pandemic expansion may explain why the pandemic was not controlled in certain countries and why other countries with much more investment in prevention and control succeed in this control.

Going back to fish and aquaculture, the aquatic environment and fish farming activities have particular characteristics that turn this activity in an activity particularly sensitive to diseases. Moreover, the huge number of fish managed by the farms (the highest in any other vertebrate farming activities) substantially restrict the reaction capacity and response time. These are the main reasons why aquaculture, and also the Mediterranean finfish farming activity requires intensive and accurated disease and pathogen surveillance programs based in robust and efficient diagnostic systems.

2.2 Diagnostic capacity in Med Aquaculture

As strongly encouraged by the EU Commission, the consortia of the two projects funded within the same topic of Horizon 2020, MedAID (Mediterranean Aquaculture Integrated Development) and PerformFISH (Integrating Innovative Approaches for Competitive and Sustainable Performance across the Mediterranean Aquaculture Value Chain), agreed on collaborating for the improvement of the diagnostics of diseases of European sea bass and gilthead sea bream. The task was not shared only between partners of the two consortia, but also with all laboratories/ichthyopathologists involved in diagnostics of diseases of these two species in Mediterranean area.

Therefore, mutual efforts were undertaken to build up - 15 years after the document issued by CIHEAM “Mediterranean Aquaculture Diagnostic Laboratories”, Options méditerranéennes, série B: Etudes et Recherches, numéro 49 - an updated database of the different actors involved in diagnostics of sea bass and sea bream diseases together with an evaluation of diagnostic capacities.

With the main aims to set up a database/repository of laboratories and ichthyopathologists working in diagnostics of marine fish diseases in Mediterranean aquaculture, to facilitate communication and collaboration actions, and to define diagnostic capacities and methodologies applied for the most relevant pathogens in order to give the basis for optimization of diagnostic techniques, a questionnaire-based “*Survey on laboratories and ichthyopathologists working in the diagnostics of European seabass and gilthead seabream diseases*” was performed.

The online questionnaire was composed of set of questions divided into 5 sections:

1) General information on each laboratory;
2) methods applied to diagnose parasitic diseases;
3) methods applied to diagnose bacterial diseases;
4) methods applied to diagnose viral diseases;
5) other methods applied to diagnose all groups of aforementioned pathogens, such as histology, immunohistochemistry or other immunological methods, *in situ* hybridization and NGS.

Some additional questions about the prevalence of the most important parasitic, bacterial, viral or other health problems were included at the end of each chapter (still to be analysed).

The questionnaire was created using google forms and is still active on the following URL: https://docs.google.com/forms/d/1_cb2cX4lCiS8jxgFpZciKvVgpo_UjxaRXmVxRZauFQg/edit

Questionnaire on diagnostic capacities in Mediterranean basin

MedAID & PerformFISH

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A complete version of the questionnaire is reported in Annex 1.

123 actors were identified as involved in diagnostics of sea bass and sea bream diseases throughout the Mediterranean basin (European and non-European Mediterranean countries), as well as two specific laboratories from European non-Mediterranean countries, Denmark and UK, who are partners in MedAID and PerformFISH project respectively. Readiness to participate in the survey was given by almost half (59) of the contacted laboratories and eventually 55 laboratories filled in the questionnaire. Submitted answers were analysed and a scoring of laboratory methods was made according to the following agreed criteria:

Capacity to diagnose parasitic diseases – Basic: fresh mount/staining techniques; Advanced: basic plus molecular methods or electron microscopy (EM); Specialized: basic methods plus molecular methods plus EM;

Capacity to diagnose bacterial diseases – Basic: isolation with biochemical identification and sensitivity testing; Advanced: basic plus molecular methods and/or serology and/or MALDI-TOF; Specialized: basic plus all other techniques;

Capacity to diagnose viral diseases – Basic: molecular methods; Advanced: basic plus the isolation of viruses in cell cultures; Specialized: advanced plus additional methods such as TEM, serology;

Use of other techniques – Basic: histology; Advanced: basic plus other methods such as immunohistochemistry (IHC); Specialized: advanced plus *in situ* hybridization, immunology or NGS.

Results of the questionnaires have been jointly analysed by MedAID and PerformFISH partners involved in diagnostics and preliminarily presented at the Open workshop "Bottlenecks in diagnostics of Mediterranean fish diseases" held in September 9th, 2019 during the 19th EAFP Conference in Porto, Portugal, jointly organized by PerformFish, MedAid and ParaFishControl. Complete results are presented in the Section 2.2 of the Deliverable 4.2 "Report on diagnostic capacities and harmonizing methods" issued by MedAID and available at http://www.medaid-h2020.eu/index.php/2019/09/19/deliverable-d4-2/ , to which we therefore refer as the result of a shared action.

3 Diagnostic techniques in Med aquaculture

Diagnostics in Mediterranean finfish farming requires visual examination on the diseases and disease-related pathogens but also requires a careful evaluation and assessment of the surrounding conditions related to the aquatic environment where fish are reared (environment, "in farm" aquatic conditions) and also conditions related to the management. These conditions are much more complex and play a much higher role in the fish health than in terrestrial farming and should also be controlled and tracked in close association with the diseases and pathogens. Some of the recommendations on the follow up of these conditions are addressed particularly in D3.4 "Prophylactic practices for Mediterranean farmed fish" but also in WP7, focused on the data management related to KPI.

Concerning diagnostics in disease control of Mediterranean finfish aquaculture, it is necessary to take into account the wide spectrum of pathogens, diseases and reared species in the Mediterranean. In the PerformFISH deliverable 3.2 "Epidemiological status of Mediterranean farmed fish" there is a complete and updated overview of the current situation of the disease problems in the Mediterranean aquaculture, mainly focused on European seabass and gilthead seabream production. As described in deliverable 3.2, PerformFISH **run** an internal survey amongst the associations and farms selecting the most relevant ones in term**s** of impact **o**n the industry and from these most relevant diseases, WP3 members did an internal review and study on the diagnostic techniques used at farm and laboratory level, including those techniques widely used but also the techniques still in development. The information of this review has been compiled in the chapter 4 of this deliverable. Together with this study, the different members of the WP **run** an exercise of evaluation of their expertises and capabilities to develop new or improved diagnostic methods, techniques or protocols. In this evaluation, we anal**y**sed each of the current available methodologies, exploring advantages but also weaknesses at the same time. Amongst others, we explored different aspects such as quickness, feasibility, cost, availability, accessibility, standardization, accuracy, sensitivity, specificity, precision and resolution. Together with this assessment, we also did a TRL (Technology Readiness Level) assessment of the different techniques **to explore whether** this TRL could be improved. From all these analyses, we designed the different diagnostic developments described in the last section of this deliverable.

4 Diagnostic techniques for the most relevant diseases of European sea bass and gilthead sea bream: state of the art

4.1 Viral encephalopathy and retinopathy (VNN) by *Betanodavirus*

4.1.1 Presumptive diagnosis

The viral encephalopathy and retinopathy (VER) otherwise known as viral nervous necrosis (VNN), caused by the infection of viruses included in the genus *Betanodavirus*, family *Nodaviridae*, has been described in several fish species, mainly marine (OIE, 2019).

The disease generally occurs with outbreaks of acute mortality; clinical signs consist mainly in behavioural abnormalities such as erratic swimming, whirling, belly-up at rest, lying down at the tank bottom and fast swimming in circles or straight-ahead (OIE, 2019). Despite lesions such as hyperinflation of the swim bladder (mainly in larvae) or traumatic haemorrhages/erosions in the oral part of the body (mainly in bigger fish) has been described in diseased fish (OIE, 2019), they are not always present and most of the time fish involved in VNN outbreaks have no gross lesions or do not display evident clinical signs.

Based on phylogenetic analysis, betanodaviruses have been clustered into four genotypes: Striped jack nervous necrosis virus (SJNNV), Tiger puffer nervous necrosis virus (TPNNV), Barfin flounder nervous necrosis virus (BFNNV) and Redspotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997; Thiéry et al., 2012). In the Mediterranean basin, the infection is generally due to the RGNNV genotype that is associated with outbreaks in European sea bass (*Dicentrarchus labrax*) occurring at high temperature (about 25°C) (Doan et al., 2017). However, other genotypes have been associated with VER outbreaks in Southern Europe, including the SJNNV genotype and the reassortant strains SJNNV/RGNNV and RGNNV/SJNNV and involving other species such as gilthead sea bream (*Sparus aurata*), Senegalese sole (*Solea senegalensis*) and common sole (*Solea solea*) (Olveira et al., 2009; Panzarin et al., 2012; Volpe et al., 2020). These outbreaks occur in environmental conditions, different from those generally described for RGNNV infection in sea bass such as in the case of mortality episodes observed at about 19-21°C in gilthead sea bream and European sea bass larvae (Toffan et al., 2017; Volpe et al., 2020).

Definitely, the circulation of several betanodavirus strains and the involvement of different fish species makes it necessary to investigate the presence of betanodavirus by laboratory tests in mortality outbreaks observed in several environmental conditions.
Microscopic findings such as vacuolation and necrosis of nervous cells of the spinal cord, brain and/or retina are typically observed in diseased fish and the observation of these lesions can make suspect betanodavirus infection. However, cases of infection without these typical microscopic lesions were reported mainly in adult fish, so confirmatory diagnosis cannot be based only on microscopic lesion observation (OIE, 2019).

4.1.2 Confirmatory diagnosis and target surveillance

The confirmatory diagnosis has to be based on a test that can confirm the presence of the virus and its effects, if possible, in a suspected sample; on the other hand, the target surveillance has to be based on techniques able to detect the virus in the stock, focusing mainly on the presence of asymptomatic carriers.

Many methods have been developed and used to detect and study betanodavirus infection; however, the practical application of most of them is restricted due to several limitations.

The use of permissive cell culture followed by immunological or molecular identification represents the reference (Gold standard) method due to its high sensitivity and the ability to provide a viral isolate for further characterization (OIE, 2019). Sensitivity assays showed a limit of detection in the range of $10^{1.55}$-$10^{1.8}$ TCID50/ml for virus titration on cell cultures (Dalla Valle et al., 2005; Panzarin et al., 2010). However, this method is time-consuming and requires a great experience and a specialized laboratory to manage cell culture (Doan et al., 2017). Virus isolation is mainly based on the use of two cell lines (SSN-1; E-11) available through the European Collection of Cell Cultures (ECACC) and permissive for the four genotypes (OIE, 2019), however several other cell lines were developed and used for research and diagnostic purposes (**Table 1**).

Table 1. Cell lines permissive for the isolation of betanodaviruses

Name	Origin	Reference	Permissiveness
SSN-1	Striped snakehead (*Channa striatus*)	Frerichs et al., 1996	RGNNV; SJNNV; TPNNV; BFNNV
E-11	Clone of SSN-1 cell line	Iwamoto et al., 2000	RGNNV; SJNNV; TPNNV; BFNNV
GF-1	Grouper (*Epinephelus coioides*)	Chi et al., 1999	GNNV (RGNNV)
GB	Grouper (*Epinephelus awoara*)	Lai et al., 2001	YGNNV (RGNNV)
SF	Barramundi/Asian sea bass (*Lates calcarifer*)	Chang et al., 2001	GNV (RGNNV)
SAF-1	Gilthead seabream (*Sparus aurata*)	Bejar et al., 1997; Bandin et al., 2006	RGNNV; SJNNV; BFNNV
SISK	Asian sea bass (*Lates calcarifer*), kidney	Sarath Babu et al., 2013	RGNNV
SISS	Asian sea bass (*Lates calcarifer*), Spleen	Sarath Babu et al., 2013	RGNNV
DLB-1	European sea bass (*Dicentrarchus labrax*), brain	Chaves-Pozo et al., 2019	RGNNV; SJNNV; TPNNV; BFNNV
SGA	Freshwater mosquitofish (*Gambusia affinis*), skin	Jyotsna et al., 2019	RGNNV; SJNNV
BGA	Freshwater mosquitofish (*Gambusia affinis*), bone	Jyotsna et al., 2020	RGNNV; SJNNV

Antigen-detection methods, such as indirect fluorescent antibody test, usually used to identify viruses isolated in cell culture, can also be applied directly to the tissue; however, their limited sensitivity makes these methods applicable only to clinically affected fish and unreliable for surveillance (Nuñez-Ortiz et al., 2016; OIE, 2019). Methods based on antigen detection have been developed using both polyclonal or monoclonal antibodies (**Table 2**); due to the presence of three serotypes among betanodaviruses, specificity of these tests depends mainly on serum used for the assay (Panzarin et al., 2016).

Molecular methods applied directly to tissue samples can provide an excellent alternative to the use of cell culture for confirmatory diagnosis and also for target surveillance. PCR-based methods, in fact, have short processing time, and high sensitivity and specificity, and are therefore suitable tools for the rapid detection of betanodavirus in both clinical and subclinically infected fish. However, they need full validation through inter-laboratory proficiency tests to

ascertain their performance (OIE, 2019). As part of the collaborative activity between MedAID and PerformFISH, some PerformFish partners participated to the 2nd NNV Interlaboratory proficiency tests (ILPTs) organized by the IZSVE in the framework of WP4 of the MedAID project. The NNV ILPT was organized to assess the capacity of laboratories to diagnose VNN using real-time RT-PCR (rRT-PCR) or endpoint RT-PCR and genotype detected viruses and it showed that there is room for improvement, particularly in the ability of laboratories to genotype different genotypes of NNV. Results of the 2nd NNV ILPTs are reported into the Section 3.2 of the Deliverable 4.2 "Report on diagnostic capacities and harmonizing methods" issued by MedAID available at http://www.medaid-h2020.eu/index.php/2019/09/19/ deliverable-d4-2/.

Table 2. Antigen-detection based techniques developed to detect betanodaviruses

Method	Reference	Virus	Sample	Fish species	Sensitivity
Direct ELISA	Arimoto et al., 1992	SJNNV	Eggs, larvae, brood stocks	Striped jack Pseudocaranx dentex	Detection limit virus antigens: 5 ng/well
Fluorescent antibody technique (FAT)	Nguyen et al 1996	SJNNV	FFPE tissue	Striped jack Pseudocaranx dentex larvae	Positivity 12 hours post infection
Indirect fluorescent antibody test (IFA)	OIE, 2019	Depending on Ab used Polyclonal/monoclonal Abs	Brain imprints /frozen sections or FFPE tissue	Depending on serum used	Recommended only in clinically affected fish/Presumptive diagnosis
Immunohistochemistry (IHC)	OIE, 2019	Depending on serum used	FFPE brain and retina	Depending on serum used	Presumptive diagnosis

Several PCR-based techniques have been developed targeting both RNA1 and RNA2 genome segments (**Table 3**). Despite genome-based techniques are prone to produce false negative results due to genetic variability, the selection of highly conserved genome regions can overcome this problem. Conventional RT-PCR has been widely applied for confirmatory diagnosis, however sequencing has been recommended to avoid false positive results (OIE, 2019).

The use of a nested-PCR approach can further provide a more sensitive and specific test all together (Nishizawa et al., 1994; Thiéry et al., 1999; Dalla Valle et al., 2000; Volpe et al., 2017).

Further improvement in the use of molecular techniques to detect the virus directly in tissue samples has been provided by the real time PCR technology otherwise known as qPCR. Particularly, these assays are less time-consuming than classical approaches. Furthermore, real time PCR methods decrease significantly cross-contamination occurring during post-amplification procedures compared with conventional PCR. For this reason, qPCR is the only molecular method proposed by OIE, for target surveillance (OIE, 2019). Actually, two real time PCR protocols have been fully validated by the OIE Reference Laboratory, one targeting the

RNA2 (Panzarin et al., 2012) and the other the RNA1 (Baud et al. 2015). Moreover, some companies set up pre-mixed real time PCR kit for nervous necrosis virus (NNV) detection, however, they don't provide details regarding primers and probe used.

Table 3. Example for PCR protocols developed to detect betanodavirus

Method (primers)	Reference	Virus	Target RNA
F2-R3	Nishizawa et al., 1994	RGNNV; SJNNV; TPNNV; BFNNV	RNA2 (T4 variable region)
VNNV1-VNNV2	Dalla Valle et al., 2000	Isolates of Mediterranean origin	RNA2
F2-R3	Thiéry et al., 1999	Isolates of Mediterranean origin	RNA2
AH95-F1-AH95-R1	Grotmol et al., 2000	BFNNV	RNA2
S6-S7	Ciulli et al., 2006	Dicentrarchus encephalitis virus, RGNNV	RNA2 (whole coding region)
VNNV5-VNNV6	Toffolo et al., 2007	RGNNV; SJNNV; BFNNV	RNA1

Recently, the need of economic, rapid, sensitive and efficient methods to diagnose fish disease directly in the field has raised interest in techniques that require less time and experience than those required by PCR-based methods. Actually, few rapid methods for the field diagnosis have been set up to detect betanodavirus, however some relevant limits with regard to their sensitivity has been raised (Zrnčić et al., 2020). On the other hand, biomolecular methods such as loop-mediated isothermal amplification (LAMP), cross-priming isothermal amplification (CPA) and nucleic acid sequence based amplification (NASBA) revealed high sensitivity coupled with the advantage to conduct the whole reaction rapidly under an isothermal condition (Biswas and Sakai, 2014). Some techniques using isothermal amplification have yet been developed and tested to detect betanodavirus in fish (**Table 4**).

Table 4. Isothermal amplification methods developed to detect Betanodavirus

Method	Reference	Virus	Target RNA	Host
NASBA	Starkey et al., 2004	RGNNV	RNA2	Sea bass, grouper, striped jack, cod
RT-LAMP	Sung and Lu, 2009	RGNNV	RNA2	Grouper
LAMP	Xu et al., 2010	RGNNV	RNA2	Grouper
Microfluidic LAMP	Wang et al., 2011	RGNNV	RNA1	Grouper
RT-LAMP	Suebsing et al., 2012	RGNNV, SJNNV	RNA2	Olive flounder
LAMP	Mekata et al., 2015	RGNNV, TPNNV	RNA1	Not tested
CPA+LFD	Su et al., 2015	RGNNV	RNA1	Half-smooth tongue sole
LAMP	Hwang et al., 2016	RGNNV	RNA2	Grouper

Abbreviations: NASBA = nucleic acid sequence based amplification; LAMP = loop-mediated isothermal amplification; RT-LAMP = reverse transcriptase-LAMP; CPA = cross-priming isothermal amplification; LFD = lateral flow dipstick.

These methods show generally a good speed, sensitivity and cost-effectiveness, however, their efficiency in detection of different viral strains circulating in the field has not always been tested. Anyway, regardless of the technology used, the development of an ubiquitous assay detecting all viral species would be desirable, but because of the high genetic diversity of betanodavirus it remains a big challenge. Furthermore, the co-circulation of different genotypes and strains makes it particularly pressing the need for a rapid and reliable genotyping method to identify the viral genotype, which can help to know strain features (i.e. host range, optimal temperature) and to arrange the best control strategy (Zrnčić et al., 2020). Now, sequencing and analysis of both viral genome segments (RNA1 and RNA2) is the solely method available to genotype viral strains, however this is time-consuming and in addition only few laboratories are able to apply this method to genotype efficiently the different NNV genotypes as pointed out during the 2nd NNV Interlaboratory proficiency tests (ILPTs).

4.1.3 Indirect diagnosis

An indirect ELISA for the detection of anti-NNV antibodies was recently developed and evaluated to detect specific antibodies from naturally exposed fish (Jaramillo et al., 2015; Jaramillo et al., 2016); median estimates of the diagnostic sensitivity and specificity calculated using RT-qPCR as a second test of the VNN ELISA were 81.8% and 86.7%, respectively. Despite the use of VNN ELISA cannot rule out the occurrence of false positives/negatives, especially when asymptomatic fish are tested, this technique offers the advantage of non-lethal test and it was fit for the purpose of identifying animals in naturally exposed populations. Moreover, with further evaluation in larger populations this test might be used for estimating infection prevalence to facilitate risk analysis. However, further application of this technique showed the lack of association between sero-status in broodstock and the subsequent occurrence of VER disease in their progeny indicating that ELISA tests for anti-NNV antibodies are not suitable for the purpose of preventing vertical transmission of NNV in barramundi (Jaramillo et al., 2017). ELISA test aiming to detect antibobies has been applied also in European sea bass broodstock screening, however it needs to be repeated more than once to give reliable outcomes (Zrnčić et al., 2020).

4.2 Vibriosis by *Vibrio anguillarum* and *Vibrio harveyi*

4.2.1 Vibrio anguillarum

Vibrio anguillarum, also known as *Listonella anguillarum*, is the causative agent of vibriosis, a deadly haemorrhagic septicaemic disease affecting various marine and fresh/brackish water fish, bivalves and crustaceans.

At present, 23 O serotypes (O1–O23) within *V. anguillarum* are distinguished, each displaying a different pathogenicity and host specificity. Of these, only serotypes O1, O2 and to a lesser extent O3 are causative agents of fish mortality (Tiainem *et al*., 1997; Pedersen *et al*., 1998 Toranzo *et al*., 2017). While serotype O3 affects mainly eel and ayu cultured in Japan and Atlantic salmon reared in Chile, serotypes O1 and O2 have a wide distribution of host species and geographical region (Toranzo *et al*., 2017), including the Mediterranean area.

Rapid identification of pathogens is crucial for effective disease control in aquaculture; detection of pathogens is important not only in infected fish (clinically and subclinically), but also in the environment, e.g. between harvesting and restocking, and as an early warning system (Toranzo *et al*., 2004).

Culture, Staining and Biochemical Phenotype

Vibrio anguillarum is a Gram-negative, comma shaped rod bacterium, belonging to the family Vibrionaceae. It is polarly flagellated, non-spore forming, halophilic and facultatively anaerobic (Madigan *et al*., 2000; Buller 2004).

V. anguillarum can be easily isolated from internal organs of selected diseased fish mainly from peracute and acute cases developing septicaemia and after 24-48h of incubation at temperatures around 22°C by using media such as trypticase soy agar (TSA) supplemented with 1-2% sodium chloride (NaCl), marine 2216 agar (MA), marine salt agar with blood (MSA-B) and blood agar (BA), or selective media, such as thiosulphate citrate bile salts sucrose agar medium (TCBS) were produce yellow colony or green, *V. anguillarum* medium (VAM) and CHROMagar Vibrio (CHROMagar Microbiology, Paris, France) (Bolinches *et al.*, 1988). *V. anguillarum* shows sensitivity to the Vibriostatic 0/129 (150 µg and 10 µg).

API system: The use of the miniaturized system API-20E (Biomerieux, France) modified for marine isolates (Grisez *et al*., 1991) is valuable for a rapid presumptive identification of the bacterium although the pathogen is not included in the API-20E code index, the system and Buller (2014) report different code (**Table 5**).

Table 5. API 20E codes by Buller (2014*)*

API 20E CODE	API 20E CODE	API 20E CODE	API 20E CODE	API 20E CODE
304452456	304752476	324632657	324752656	324752757
304572557	304752657	324712677	324752657	324752777
304652456	324472757	3247524	324752756	324772656
304752456	324562757	324752557	324752756	324772657

Complete and accurate microbiological identification usually relies on a battery of biochemical or serological tests. These methods require the subculture of numerous single isolates, and phenotypic variability of strains could make identification questionable in some cases; routine processing is thus time consuming and expensive, with poor or low levels of sensitivity.

Molecular techniques

Several molecular methods have been developed for the specific detection of *V. anguillarum* (**Table 6**).

16S rRNA PCR: Amplification of 16S rDNA was used by several authors (Kita-Tsukamoto *et al.,* 1993; Avsever *et al*., 2015) but it is not precise for the discrimination of closely related species (e.g. *V. mimicus* and *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus*, *V. fluvialis* and *V. furnissii*, *V. anguillarum* and *V. ordalii*) as they share nearly identical 16S rRNA gene sequences.

Several genes were useful for identification of *V. anguillarum* such as *vah amiB rpoS rpoN empA groEL* (Hirono *et al*., 1996; Gonzalez et al., 2003; Demircan and Candan, 2006; Hong *et al*., 2007; Kim *et al*., 2008; Xiao *et al.,* 2009 Kim *et al*., 2010).

Multiplex PCR: several multiplex PCR (mPCR) assays were used for simultaneous detection of more than one *Vibrio* species in a single test (Rodkhum *et al*., 2006) or simultaneous detection of several bacterial pathogens (Ferreira Pinto *et al*., 2017; Nishiki *et al*., 2018. A multiplex PCR and a DNA microarray was used for detection of *V. vulnificus*, *Listonella anguillarum*, *Photobacterium damselae* subsp. *damselae*, *Aeromonas salmonicida* subsp. *salmonicida*, and *V. parahaemolyticus*. The array was composed of nine short oligonucleotide probes complementary to seven chromosomal loci (*cyt*, *rpoN*, *gyrB*, *toxR*, *ureC*, *dly*, and *vapA*) and two plasmid-borne loci (fatA and A.sal). Nine primer sets were designed to amplify short fragments of these loci (100 to 177 bp) in a multiplex PCR. PCR products were subsequently labeled by nick translation and hybridized to the microarray (González *et al*., 2004a). Kim *et al*. (2019) to identify the *Vibrio* from cage-cultured marine fish, they designed specific primers targeting the RNA polymerase sigma factor (*rpoD*), recombination protein F (*recF*), transcriptional regulator (*toxR*) genes (known as housekeeping genes), and two multiplex PCR sets (PCR set 1 for *V. anguillarum, V. gigan*tis, *V. atlanticus*, *V. harveyi,* and *V. scophthalmi*; and PCR set 2 for *V. lentus, V. splendidus*, *V. alginolyticus, Photobacterium piscicola* and *P. damselae*).

Real Time PCR (RT-PCR): Crisafi *et al*. (2011) developed a real-time PCR assays for the rapid detection of *V. anguillarum* based on the amplification of 16S and *toxR* genes.

loop-mediated isothermal amplification method (LAMP). Successfully developed a LAMP assay to detect *V. anguillarum* based on the *amiB* gene and the *empA* gene, respectively (Kulkarni *et al*., 2009; Gao *et al*., 2010).

Duplex LAMP assays were developed for detecting *V. anguillarum* and *V. alginolyticus* by targeting the *groEL* gene of and the fklB gene respectively (Siddique *et al.,* 2019).

Multiplex LAMP: A triplex loop-mediated isothermal amplification (triLAMP) method was developed by Yu *et al*., (2013) to detect three major fish pathogens, i.e., *Vibrio harveyi*, *Vibrio anguillarum* and *Vibrio alginolyticus*. The LAMP primers were designed on the sequences of the *Vhh*P2 gene of *V. harveyi,* the *ToxR* gene of *V. anguillarum*, and the collagenase gene of *V. alginolyticus*. The sensitivity of the triLAMP method was 10^2 to 10^3 times higher than the conventional PCR methods, which was equal to the monoplex LAMP method.

on-chip LAMP: Zhou *et al*. (2014) developed a real-time fluorogenic loop-mediated isothermal amplification assay integrated on a microfluidic disc chip (on-chip LAMP), which was capable of simultaneously detecting 10 pathogenic bacteria in aquatic animals, i.e., *Nocardia seriolae*, *Pseudomonas putida*, *Streptococcus iniae*, *Vibrio alginolyticus*, *V. anguillarum*, *V. fluvialis*, *V. harveyi*, *V. parahaemolyticus*, *V. rotiferianus*, and *V. vulnificus*.

Multilocus sequence analysis (MLSA): Steinum *at al.* (2016) performed a MLSA study focused on the identification of *V. anguillarum* and *V. ordalii* based on sequences of eight housekeeping genes, *atpA*, *ftsZ*, *gapA*, *gyrB*, *mreB, rpoA*, *topA*, and *pyrH*.

MALDI-TOF MS: has been described as a fast alternative to sequence analysis and biochemical tests for the identification and classification of bacterial isolates based on their protein composition.

Several studies have been carried out by MALDI-TOF MS to classify *Vibrio* isolates including *V. anguillarum* (Dieckmann *et al*., 2010; Erler *et al.,* 2015). This method was applied with success in PerformFish project for an accurate identification of clinical *V. anguillarum* isolates from European sea bass and gilthead sea bream (Kazazić *et al*., 2019).

Table 6. Molecular Methods for the identification of Vibrio anguillarum

ASSAY	TARGET GENE	PRIMER SEQUENCE (5'-3')	PRODUCT (bp)	SPECIFICITY	REFERENCE
PCR	*16S*	vacl F GTGAGGTAATGGCTCACCAAG vacl R CTCTGGATGTCAAGAGTAGGTAAGGT	749	*V. anguillarum* (Cross-reaction with *V. ordalii)*	Avsever *et al.*, 2015
PCR	*vah1*	VAH-P1- ACCGATGCCATCGCTCAAGA VAH-P2-GGATATTGACCGAAGAGTCA	490	*not detecting V. anguillarum strains that did not possess the vah1 gene - not able to differentiate between V. anguillarum and V. ordalii*	Hirono *et al.*, 1996
PCR	*amiB*	van-ami8- ACATCATCCATTTGTTAC van-ami417 -CCTTATCACTATCCAAATTG	429	*V.anguillarum*	Hong *et al.*, 2007
PCR	*rpoS*	VARPO1-AGACCAAGAGATCATGGATT ARPO2-AGTTGTTCGTATCTGGGATG	689	*V.anguillarum*	Kim *et al.*, 2008
PCR	*rpoN*	rpoN-ang 5 GTTCATAGCATCAATGAGGAG rpoN-ang 3 GAGCAGACAATATGTTGGATG	519	*V.anguillarum*	Gonzalez et al., 2003; Demircan and Candan, 2006
PCR	*empA*	empAF- CAGGCTCGCAGTATTGTGC empAR- CGTCACCAGAATTCGCATC	439	*V.anguillarum*	Xiao *et al.*, 2009
PCR	*groEL*	TATCACTGTTGAAGAAGGTCAAGCACTG CGCTTCAAGTGCAGGAAGCAG	195	*V.anguillarum*	Kim *et al.*, 2010
Multiplex PCR + DNA Microarray	*vapA* *A.plas* *rpoN* *fatA* *ureC* *dly* *gyrB* *toxR* *cyt*	F-A.sal-1 ATTAGCCCGAACGACAACAC R-A.sal-2 GTCGTTGAATTGGCCTTCAC P-A.sal-vapA AACTAAGCAGCCGGTACTGGACTTC F-A.sal-3 TCCGTTGGATATGGCTCTTC R-A.sal-4 TTATCGAGGCAGCCAACAAT P-A.sal-plas TCGACACAAAATTCAAATTTAACCCC F-V.ang-1 CCAGCAAGAGATCCAAGAGG R-V.ang-2 ACACCTCAGCACTGGCTTCT P-V.ang-rpoN CGCTGATGTTCATAGCATCAATGAG F-V.ang-3 GTCCGCAAGATGGAATGAAT R-V.ang-4 ACTGCTGCCACTTCCTTTGT P-V.ang-fatA AGTTCAGCAAACCTTCCCACAATTT F-P.dam-1 CACCAGGGGTCTGGAATATG R-P.dam-2 GCTCCAGCTTCAATTTGCTC P-P.dam-ureC CTGGAAGCCGTTGATGACTTACCTA F-P.dam-3 GCAATTGTTGGTGAACGATG R-P.dam-4 CGTCGCATGAAATGATCTTG P-P.dam-dly GTCAATATGGCCCAGATTGTTTT F-V.par-1 GCTAAGCAGGGTCGTAATCG R-V.par-2 GACCGATACCACAGCCAAGT P-V.par-gyrB CGCAAGAAGTTGCAACGCTTATTAC F-V.par-3 CTTGGATTCCACGCGTTATT R-V.par-4 TGATTTGCGGGTGATTTACA P-V.par-toxR ATCTCAGTTCCGTCAGATTGGTGAG F-V.vul-1 TTCATTCGAGCGTGAATTTG R-V.vul-2 ATCAAATACCCAGCCACTGC P-V.vul-cyt CCAAGAGCTTGGATGCTATTTCACC	177 101 125 137 127 137 145 147 100	*A.salmonicida* *V. anguillarum* *P.damselae subsp. damselae* *V.parahaemolyticus* *V. vulnificus*	Gonzalez et al., 2004a
	vah 1 *vah 2*	VAH-P1- ACCGATGCCATCGCTCAAGA VAH-P2-GGATATTGACCGAAGAGTCA ATGAACGAAGATAACCCCCAGA TCACTCTTCTGCTATCACTGG	490 876	*V. anguillarum. multi-plex PCR method also*	

Multiplex PCR	*vah 3* *vah 4* *vah 5*	ATGACTTCTTCTAAATTTTCGTTATGTGCG GATAGAGCGGACTTTGCTTG ATGAAAACCATACGCTCAGCATCT TCACGCTTGTTTTTGGTTTAAATGAAATCG ATGCTCACGATAAGCCCTTTTAGAT TCAAGGGTTAGGCGCGTGAT	1128 603 1758	*showed amplicons of vah2 and vah 3 when tested with V. ordalii*	Rodkhum *et al.*, 2006
Multiplex PCR	*etfD* *val* *rpoN* *vhh*	EtdF AGCGCAGCTAACGGTAAAGT EtfA_R TGTAACCGTGTTGGCGTAAG ValF CTCTCCCAATTCAGCCCTCTA ValR GACTCTTCACAACAGAACTC rpoN-ang5 GTTCATAGCATCAATGAGGAG rpoN-ang3 GAGCAGACAATATGTTGGATG VhF ACGCTTGATGGCTACTGGTGGAG VhR CTTCGCACCTGCATCGG	426 773 519 606	*E. tarda* *V. alginolyticus* *V. anguillarum* *V. harveyi*	Ferreira Pinto *et al.*, 2017
Multiplex PCR	*SufB* *HlyA* *ureC* *16S*	VH298-F-AGCTATTATTCCGCGCCATCTTG VH842-R-AGGACGATCACTTCTACCACCG VA1186-F-CGCATTAACCCGATTGGTTACGC VA1535-R-TCATCACAGCTTGAGGCAGAGAG PD785-F-CCATTGGTGATCGTGTTATCCACGTA PD1671-R-CTCTGTGGCTGGCTCACAAGTTA CCTGGTAGTCCACGCCGTAA CGAATTAAACCACATGCTCCA	545 350 887 168	*V. harveyi* *V. anguillarum* *P. damselae subsp. damselae*	Nishiki *et al.*, 2018
Multiplex PCR	*rpoD* *toxR* *recF* *rpoB* *rpoD* *toxR* *rpoD*	Vuni-rpoD- CTTTYGCTTCACCGATAGACAT Vuni-rpoD-F.4 CAAGGCTATCTGACCTACGC Van-rpoD-F.7 CAGACARCAAGAAGAAGACATTCG Vgi-tox-R GGCATGATGAAAGCGATAAGCAGT Vuni-tox-F CCWAARCGCGGTTAYCAAYTKAT Vat-recF-F.2 ATTTGAGAGTTCACTCGCGGGC Vat-recF-R.3 GCTTAGTTCTCGATAGTGTGTTGC VH-4F GTGATGAAGAAGCTTATCGCGATT VH-7R CGCCTTCTTCAGTTAACGCAGGA Vsc-rpoD-F.2 CCAAGTTCAAAACGCCGTTGCA Vle-rpoD-F.3 GAACGGTAATCGTCGCTCAGTCC Vspl-tox-R.1 GTTGTTGCTGGTTCCACTTCAAC Vuni-tox-F CCWAARCGCGGTTAYCAAYTKAT php-rpoD-F.6 AGTACGCACTAGCGAGCGTCTTA Val-rpoD-F AATGAAATGATGCTAGACGTATTCCG Phd-rpoD-F.2 GATGGTGACAGCAGCGACG	1075 125 292 372 601 740 113 218 279 371 519	*Vibrio* spp. *V. anguillarum* *V. gigantis* *V. atlanticus* *V. harveyi* *V. scophthalmi* *V. lentus* *V. splendidus* *P. piscola* *V. alginolyticus* *P. damselae*	Kim *et al.*, 2019
RT-PCR RPA assay	*16S* *toxR*	16SF- CCACGCCGTAACGATGTCTA 16SR-CCAGGCGGTCTACTTAACGCGT 16SF- TGCCAGCGAGTCATGTCG 16SR-CGTAAGGGCCATGATGACTTG 16SF- AGCACCGGCTAACTCCGTG 16SR- TGCGCTTTACGCCCAGTAAT 16SF-CCTGGTAGTCCACGCCGTAA 16SR-CCAGGCGGTCTACTTAACGC ToxR . F- ACACCACCAACGAGCCTGA ToxR. R TTGTCTCTTCGGGTTGCGA Tox R1. F-CACCACCAACGAGCCTGAA Tox R1. R-TTGTCTCTTCGGGTTGCGA Tox R2. F-GCCTGAAGAGGAACCGTTACTG Tox R2. R-CTGTCGTCACGGTTTGGGAT Tox R3. F-GAGCCTGAAGAGGAACCGTTAC Tox R3. R-CTGTCGTCACGGTTTGGGAT		*V. anguillarum*	Crisafi *et al.*, 2011
LAMP	*empA*	empA-BIP GCATGATGGCGCAGCAAATTTAACCGCCT-GCTTTTGTTC empA-FIP CGTAAACTTG GCGATACCTTTCTTTACCGGTCTTTAATACG TCAG empA-B3 GGTAATGCGTAAGTGC AATA empA-F3 CCAGCAGATGTAT ATGGT		*V. anguillarum*	Gao *et al.*, 2010
	groEL (V. anguillarum)	groEL-F3 CACTTGAAGCGGTAGCAA groEL-B3 TCAAGACCGATCTCTTCAGA groEL-FIP ACAATGCCACGCATGTTGTTC-*CTCGAG*-GTGGCGGAAGATGTTGAA F2 GTGGCGGAAGATGTTGAA F1c ACAATGCCACGCATGTTGTTC groEL-BIP GCCGTGAAAGCACCTGGT-*CTCGAG*-AGTACCGCCCGTTAGG		*V. anguillarum*	

Duplex LAMP	*fklB* (*V. alginolyticus*)	B2 AGTACCGCCCGTTAGG B1c GCCGTGAAAGCACCTGGT groEL-LoopF ACAACCAGCGTCGCCAAT groEL-LoopB AGCGATGCTACAAGACATTGCT fklB-F3 AGCACTACAAGAACTTCACA fklB-B3 GTGAACGCGAACTGAGCTTTCT fklB-FIP CGCTTCGCCTTCTGCTGCC-*CTCGAG*-CTCGTGCAGAAACAGCTCGC F2 CTCGTGCAGAAACAGCTCGC F1c CGCTTCGCCTTCTGCTGCC fklB-BIP ACCTGAAGTTAACGTTCTAGAC-*CTCGAG*-CTGTAGGAACTGCACCAGTA B2 CTGTAGGAACTGCACCAGTA B1c ACCTGAAGTTAACGTTCTAGAC klB-LoopF GCAATTTTTGCTGCTTCTTG fklB-LoopB TCTTCAGTACGAAATCATTTC		*V. alginolyticus*	Siddique *et al.,* 2019
Tri LAMP	*vhh* P2(*V. harveyi*) *toxR* (*V. anguillarum*) Collagenase (*V. alginolyticus*)	Vhhp2-F3-CAATTCGAAACAGGCGTG Vhhp2-B3-AGTAAAGCTTGCCACACG Vhhp2-FIP-CGCCACCACCATATCCATCG-GAATTCGGTTAGTCAATGGTGGAACA (*Eco*RI) Vhhp2-BIP-GGATGTAAATGAGTTTGGCTTTCCG-GGATCCTTGTCCTATGTTATACGGGTTG (*Bam*HI) ToxR-F3-ATTCGTTAAAACCGTGCC ToxR-B3-TCCCTATTTTTACTTTCGCG ToxR-FIP-TCGTTGGTGGTGTTTTTTTGTGA-TTTTCGAGGTTATCAAATGATCGC ToxR-BIP-GGAACCGTTACTGGCCAGTG-GGATCCTTGGGATGTTGTCTCTTCG (*Bam*HI) ValC-F3-GCGTACTTACCGCAAGTGA ValC-B3-CCCTTAGAGCAAAATCGCCT ValC-FIP-ACACCATTAACCGCATTGCGC-GAATTCCAGTGGCTTACACGTTGGA (*Eco*RI) ValC-BIP-GTGGGCAGTGGAACGAGCAA-TTTTAAGCTTTGGCAAGGTCTGT		*V. harveyi* *V.anguillarum* *V. alginolyticus*	Yu *et al.,* 2013
on-chip LAMP	*empA*	Van-F3 CCAACGGGGAATGTGCTAG Van-B3 GGCGACAATCCCACGAAG Van-FIPGCTGGTTGAGCTTGCGACCGttttGGGTTACAAGGTACGCGAA VanBIPTCGATCAGTTTGGGCTCAGGTGttttAAGTGGTTGGTTTGCTGC Van-LF AGGTATAGAAGGCTTCGGAG Van-LB GCTGATTTGTATGTCAAAGCC		*V. anguillarum*	Zhou *et al.,* 2014

Serological techniques*:*

DAPI: fluorescently labelled monoclonal antibody/DAPI (4',6-diamidino-2-phenylondole) double staining technique was developed to detect *V. anguillarum* in water environments without the need for a cultivation step (Miyamoto and Eguchi 1997).

Latex agglutination-based assay (BIONOR Mono-Va-kit). Agglutination tests based on preculturing of sample. The test reagents consist of monodisperse particles coated with specific antibodies against fish pathogenic bacteria. Although this kit contains latex particles coated with monoclonal antibodies against the three pathogenic serotypes O1, O2 and O3, some strains of the environmental serotypes O4, O5 and O7 were found to lead to false-positive results. Furthermore, cross-reactions with *Vibrio splendidus* and motile *Aeromonas* strains are frequently observed (Romalde *et al*., 1995a; Gonzalez *et al.,* 2004b). The sensitivity assay howed a difference of about 1 to 2 log units among the titers obtained with the Mono-Vakit (10^{8} bacteria ml^{-1}) and rabbit antisera (10^{7} bacteria ml^{-1}).

ELISA-based (Bionor AQUARAPID-Va test) (Gonzalez *et al.,* 2004b). A manual system based on Enzyme Immuno Assay principle. Available in 40 tests per kit.

Magnetic particle enzyme immunoassay (Bionor AQUAEIA-Va test) (Gonzalez *et al*., 2004b). Semiautomated system based on Magnetic Particle Enzyme Immuno Assay principle. Available in 50 tests per kit. The AQUARAPID-Va and the AQUAEIA-Va systems were able to detect 5 x 10^6 and 5 x 10^7 bacteria g^{-1} of fish tissue, respectively. The simplicity, effectiveness and speed of the AQUARAPID-Va system confirmed this method as the most suitable serological test for the detection of *V. anguillarum* in field analysis and small-scale laboratory. These commercial kits suffer from the same limitations as more conventional techniques, i.e. cross-reaction with other serotypes or related bacteria. In addition, antibody-based identification techniques are known to have limited sensitivity, limiting their use in analysing environmental samples (Lievens *et al.,* 2005).

Antibiotic sensitivity

Antibiotic sensitivity has always been closely associated with diagnostics for bacterial diseases. In fact, for clinical routine practice in human and animal medicine, identification plus antibiogram are closely associated. For bacterial diseases, the information concerning the susceptibility of the pathogen against different antibacterials is sometimes even more relevant than a precise identification of the pathogen itself. Antibiotic sensitivity methods (antibiograms) used in fish disease diagnostics are mainly based on the same methods used in other animal species. These methods are:

- Disk diffusion method (Kirby-Bauer), using disks containing a certain concentration of a specific antibiotic, that are placed onto a plate with media (almost always Müeller-Hinton agar) where bacteria are growing;

-Stokes method, similar to disk diffusion method but using a second bacterial strain as control;

-Etest, a commercial test based on the same diffusion methodology but using a strip with a gradient of antibiotic;

- Minimum inhibitory concentration (MIC) determination by agar and/or broth dilution methods.

In finfish disease routine microbiological practice, and also for gilthead seabream and European seabass bacterial diseases including those due to *Vibrio anguillarum*, but also other *Vibrio* species, *Photobacterium damselae* subsp. *pisicicida* and other bacterial species, disk diffusion method has always been applied, being cost- and time-effective, as the main antibiotic sensitivity method used. Only in few cases (mainly in research), more accurate dilution methods are used. This still imposes a real handicap for an efficient therapeutic management, as the quality of the information concerning the susceptibility/resistance of the isolated fish bacteria to antibiotics is quite low. This is due to different facts, among which the most relevant is that currently clinical breakpoints are not available for antibiotics against bacterial pathogens of fish (except for *Aeromonas salmonicida* in salmon, VET03-04-S2 document, CLSI, 2014), so the criteria about “sensitivity” or “resistance” have been widely borrowed from those defined for humans. Moreover, to overcome the lack of breakpoints for antibiotics against bacterial pathogens of fish it could be possible to define and apply the Epidemiological cut-off values (ECVs for CLSI or ECOFFs for EUCAST). Unfortunately, this is not currently feasible as MICs values defined for specific antibiotics against bacterial pathogens of fish (including European sea bass and gilthead sea bream) following international standard procedures are very scarce both in the literature and in the diagnostic routine in which the disk diffusion method is still widely used. In

fact, as known, data obtained by Kirby-Bauer cannot be converted into MIC values with enough precision and reliability.

In addition to this problem, it is also very important to stress that for an efficient antibacterial treatment in a fish stock, speediness in the delivery of the medicated feeds is paramount. This necessity is widely explained in the PerformFISH deliverable (D.3.3) on therapeutics under the concept of methaphylaxis in fish therapeutics. For this reason, it is so relevant to obtain precise and reliable information about the different MIC of the isolates for specific antibiotics in order to immediately select the most suitable antibiotic and treatment protocol. Unfortunately, nowadays this process is not fast enough and is hampering the subsequent selection, prescription and delivery of the medicated feeds. This is the reason why a specific new, simple and efficient strategy (PerformTest) has been designed.

4.2.2 *Vibrio harveyi*

Vibrio harveyi is a Gram-negative, luminous bacterium considered as a normal component of the intestinal microbiota of marine animals. Recently this bacterium has emerged as a serious pathogen that causes significant mortalities in many commercially cultivated marine fish and shellfish species over a wide geographical area.

The clinical signs of infection with these *Vibrio* are various. *V. harveyi* causes cutaneous or gastroenteric infections in most bone fish that frequently hesitate in septicemia (Austin and Zhang, 2006). Skin lesions are often observed which can manifest themselves as erosions or ulcerations, necrosis of the dermis or granulomatous forms, and hemorrhagic effusions at the base of the fins. Eye injuries such as keratitis, corneal opacities and panophthalmitis are described following traumatic injury with secondary irruption of *V. harveyi* as an opportunistic pathogen (Pakingking *et al*., 2018; Minami *et al.,* 2016). Gastrointestinal signs generally manifest as serious enteritis with dilatation of the intestinal lumen due to accumulation of yellowish exudate (Lee *et al.*, 2002). Are described hemorrhagic-necrotic forms affecting the terminal tract of the intestine (Soffientino *et al*., 1999). Furthermore, nervous symptoms have been associated with *V. harveyi* infections, requiring differential diagnosis with VNN and RLO-infections.

The first circulation of strains related to *V. harveyi* in sea bass and sea bream in the Mediterranean area (Greece, Italy, Spain and France) has been reported since 1990 (Pedersen *et al*., 1998). Outbreaks in European sea bass have been reported in Spain since 1999 (Pujalte *et al*., 2003), from 2003 in Turkey (Korun, 2008) and from 2011 in Italy (Florio *et al*., 2012) and cases are still frequently diagnosed in routine practice in farms.

As the early diagnosis of *V. harveyi* infection is crucial to disease surveillance and prevention in cultured marine animals, a fast and accurate method for the detection and identification of this pathogen is required.

The species classified in the “Harveyi clade” according to multilocus sequence analysis (MLSA*)* (Sawabe *et al*., 2013) are *V. harveyi*, *V. alginolyticus*, *V. campbellii*, *V. mytili*, *V. natriegens*, *V. parahaemolyticus*, *V. rotiferianus*, *V. azureus* (Yoshizawa *et al*., 2009) and *V. communis* (Chimetto *et al*., 2011), the latter currently considered junior synonym of *V. owensii* (Urbanczyk *et al.,* 2013). Recently, also other species have been ascribed to the Harveyi clade based on MLSA analysis (*V. sagamiensis*, *V. owensii*, *V. jasicida, V. inhibensis*).

The species associated with the Harveyi clade have phenotypic and genotype traits very similar to each other and in the *V. harveyi* species over time, in single isolated strains, variations of the phenotype and of the genotype (genomic plasticity) due to genetic events such as point mutations (Thompson *et al*., 2004), chromosomal rearrangement, duplication (Zhang *et al.,* 2001), bacteriophage infection (Vidgen *et al.*, 2006) and horizontal gene transfer (Tagomori *et al*., 2002).

Culture, Staining and Biochemical Phenotype

Vibrio harveyi is a Gram negative bacterium, short bacillus, slightly curved, pleomorphic, anaerobic facultative, mobile for single polar flagellum with dimensions 1.0-1.6 × 0.5-0.7 µm (Buller, 2014). Oxidase and catalase positive. *V. harveyi* is a halophilic microorganism and shows growth at NaCl concentrations between 1 - 7% and temperature between 10 - 40° C. Growth is absent at 42° C. Optimal growth is observed at pH 7, salinity of 2% NaCl and temperature of 25 - 28° C. May appear swarming on some media (TSA2% NaCl, Marine Salt Agar, Marine Sea Salt Agar) and not on others (Marine Agar 2216, TCBS). Ferments β-gentiobiose, D-glucuronate, does not ferment arbutin, the hydrolysis of casein is variable. The Voges-Proskauer reaction is negative, urease can be variable; gelatinase and citrate metabolism can take 48-72 hours to show up (Buller, 2014).

V. harveyi show sensitivity to the highest concentration (150 µg) of Vibriostatic 0/129 (2, 4-diamino-6, 7-di-iso-propylpteridine phosphate) but is resistant to that lower (10 µg). Most strains isolated from fish species ferment sucrose and have a yellowish coloration on selective and differential TCBS medium while isolated from Penis shrimp and other invertebrates do not metabolize sucrose and present in TCBS green or blue-greenish coloring. Some strains may exhibit bioluminescence. α and β-hemolysis is observed in blood agar.

Phenotypically *V. harveyi* is highly heterogeneous and therefore extremely difficult to identify using conventional bacteriological tests or kits relying upon biochemical reactions (Vandenberghe *et al*., 2003). For this reason, misidentifications from other *Vibrio* species (mainly with *V. alginolyticus*) using only conventional microbiological tests are frequently detected.

Biochemical tests useful to phenotypically differentiate *V. harveyi* from *V. campbellii* and *V. alginolyticus* (*Vibrio* species with similar biochemical characteristics) are the positivity for ornithine decarboxylase (*V. campbellii* is ODC-), frequently positive urease, negative VP test and no growth at 42 ° C (*V. alginolyticus* is urease negative, VP variable and grows at 42° C) (Buller, 2014).

API system: The use of the miniaturized system API-20E (Biomerieux, France) is valuable for a rapid presumptive identification of the bacterium although the pathogen is not included in the API-20E code index, the system. Buller (2014) report code for *V. harveyi* ATCC 14129: 434612557, *V. harveyi* (*carchariae*): 435412557 and for *V. harveyi* ATCC 35084: 435412557. In other study Pretto (2018) found the most frequent API 20E numeric codes were 4346525 (48%), 4346125 (23%), 4344125 (9%).

Molecular techniques

Several molecular methods have been developed for the specific detection of *V. harveyi* (**Table 7**).

16S rRNA PCR: Oakey *et al* (2003) reported PCR identification of *V. harveyi* based on the 16S rRNA gene. This method requires the phenotypic characterization of swarming on blood agar

and acetoin production (Voges Proskauer test-VP) of PCR-positive strains due to the weak specificity of PCR-positive set for differentiating *V. harveyi* from *V. alginolyticus*. Fukui and Sawabe (2007) suggest an one step *V. harveyi* specific PCR to differentiate *V. harveyi* from related species such as *V. campbelli*, *V. rotiferianus* and *V. alginolyticus* without additional biochemical test.

Phenotypically *V. harveyi* is highly heterogeneous and therefore extremely difficult to identify using conventional bacteriological tests or kits relying upon biochemical The ***toxR*** gene is a virulence gene encodes for regulatory gene of toxin operon and is a useful target for *Vibrio* species identification. This gene is universally distributed in the family Vibrionaceae and it is an effective taxonomic marker for identification of *Vibrio* spp. (Kim *et al*. 1999). Moreover the nucleotide sequences of this gene appears also discriminating within the Harveyi clade (Pascual *et al*., 2010). PCR for detection of *V. harveyi hemolysin* gene (***vhh***), which encodes for a virulence factor involved in pathogenicity to fish and shellfish species, may be useful for species detection or strain differentiation.

Multiplex PCR: several multiplex PCR (mPCR) assay was used for simultaneous detection of more than one *Vibrio* species in a single test (Haldar *et al*., 2010) or for simultaneous detection of several bacterial pathogens (Myoung *et al*. 2014; Ransangan and Lal 2013; Ferreira Pinto *et al*., 2017; Nishiki *et al*., 2018. Kim *et al*. (2019) to identify the *Vibrio* from cage-cultured marine fish, they designed specific primers targeting the RNA polymerase sigma factor (*rpoD*), recombination protein F (*recF*), transcriptional regulator (*toxR*) genes (known as housekeeping genes), and two multiplex PCR sets (PCR set 1 for *V. anguillarum, V. gigan*tis, *V. atlanticus*, *V. harveyi,* and *V. scophthalmi*; and PCR set 2 for *V. lentus, V. splendidus*, *Photoacterium piscicola.*

Real Time PCR (RT-PCR): Pang *et al.* (2019) development a real-time fluorescent RPA (recombinase polymerase amplification) assay for the rapid detection of *V. harveyi* using new primers specifically designed to recognize the *V. harveyi toxR* gene and then hybridized this gene.

AMP assay has been developed for the detection of *V. harveyi* (Cai *et al*., 2010).

Multiplex LAMP: A triplex loop-mediated isothermal amplification (triLAMP) method was developed by Yu *et al*., (2013) to detect three major fish pathogens, i.e., *Vibrio harveyi*, *Vibrio anguillarum* and *Vibrio alginolyticus*. The LAMP primers were designed on the sequences of the *Vhh*P2 gene of *V. harveyi,* the *ToxR* gene of *V. anguillarum*, and the collagenase gene of *V. alginolyticus* . The sensitivity of the triLAMP method was 10^2 to 10^3 times higher than the conventional PCR methods, which was equal to the monoplex LAMP method.

On-chip LAMP: Zhou *et al*. (2014) developed a real-time fluorogenic loop-mediated isothermal amplification assay integrated on a microfluidic disc chip (on-chip LAMP), which was capable of simultaneously detecting 10 pathogenic bacteria in aquatic animals, i.e., *Nocardia seriolae*, *Pseudomonas putida*, *Streptococcus iniae*, *Vibrio alginolyticus*, *V. anguillarum*, *V. fluvialis*, *V. harveyi*, *V. parahaemolyticus*, *V. rotiferianus*, and *V. vulnificus*.

Multilocus sequence analysis (MLSA): Thompson *et al. (*2007) reported on the first standard multilocus sequence analysis (MLSA) scheme for the *V. harveyi* species group based on sequences of genes encoding recombination repair protein (*rec*A), urydilate kinase (*pyrH*), glyceraldehyde 3-phosphate dehydrogenase (*gapA*), an actin-like cytoskeleton protein (*mreB*), a cell division protein (*ftsZ*), DNA gyrase B subunit (*gyrB*), and topoisomerase I (*topA*).

Caño-Gomez *et al*. (2011) also performed a MLSA study focused on the identification of *V. harveyi*-related species by analysis of protein-coding genes (*rpoA*, *pyrH*, *topA*, *ftsZ*, *mreB*). Concatenation of only *topA* and *mreB* gene sequences offered similar resolution to that of full MLSA (5 genes) for identification of V. harveyi-related species. These authors suggested that initial allocation of *V. harveyi*-like isolates into the *V. harveyi* group (by biochemical or 16S rRNA gene analysis) and additional topA−mreB gene analysis offers a reliable identification of these close species, with resolution power comparable to that of a full MLSA analysis.

MALDI-TOF MS: has been described as a fast alternative to sequence analysis and biochemical tests for the identification and classification of bacterial isolates based on their protein composition. Several studies apply MALDI-TOF MS to classify *Vibrio* isolates included *V. harveyi* (Dieckmann *et al.*, 2010; Erler *et al*., 2015).

Table 7. Molecular methods for the identification of Vibrio harveyi

ASSAY	TARGET GENE	PRIMER SEQUENCE (5'-3')	PRODUCT (bp)	SPECIFICITY	REFERENCE
PCR+ cultured method	*16S*	VH-1-AAC GAG TTA TCT GAA CCT TC VH-2-GCA GCT ATT AAC TAC ACT ACC	413	*V. harveyi*	Oakay et al., 2003
PCR	*16S*	VHARF- CCGCATAATACCTACGGGTC VHARR- ACCCGAAGTGCTGGCAAACA	967	*V. harveyi*	Fukui and Sawabe, 2007
PCR	*toxR*	Vh_toxR-F TTCTGAAGCAGCACTCAC Vh_toxR-R TCGACTGGTGAAGACTCA	390	*V. harveyi*	Conejero and Hedreyda, 2003
PCR	*toxR*	toxRF1-GAAGCAGCACTCACCGAT toxRR1-GGTGAAGACTCATCAGCA	382	*V. harveyi*	Pang *et al*., 2006
PCR	*Vhh*	VHF1 ATCATGAATAAAACTATTACGTTACT VhhemoR -GCTTGATAACACTTTGCGGT	308	*V. harveyi*	Conejero and Hedreyda, 2004
Multiplex PCR	*hly*	Vca-hly5- CTATTGGTGGAACGCAC Vca-hly3 -GTATTCTGTCCATACAAAC Vh-hly1F-GAGTTCGGTTTCTTTCAAG Vh-hly1R-TGTAGTTTTTCGCTAATTTC Vp-tlh1GATTTGGCGAACGAGAAC Vp-tlh2 CGTCTCGAACAAGGCG	328 454 695	*V. campbellii* *V. harveyi* *V.parahaemolyticus*	Haldar *et al*., 2010
Multiplex PCR	*toR* *collagenase* *vhhB* *toxR*	MpJRPdF CGGTTATCAAATGATCGCAAC MpJRPdR CTTGCACCCCTTTAACCG MpJRValF CTCTCCCAATTCAGCCCTCTA MpJRValR GACTCTTCACAACAGAACTC MpJRVhF ACGCTTGATGGCTACTGGTGGAG MpJRVhR CTTCGCACCTGCATCGG MpJRVpF CCGTTCCAAAACGAGGCTATC MpJRVpR CGAGTGGTTGCTGTCATGA		*P. damselae* *V. alginolyticus* *V. harveyi* *V. parahaemolyticus*	Ransangan and Lal 2013
Multiplex PCR	*rpoB*	VH-4F GTGATGAAGCTTATCGCGATT VH-7R CGCCTTCTTCAGTTAACGCAGGA PD-2F CAAGACATCATCGATGTGATGCT PD-2R GAAACTTTACCATCTACCACTTTG VI-3FATGCAATCATGCCTCAAGATCTA VI-1R AAATGTACCTTCTTCAGTCAACTT	601 533 434	*V. harveyi* *P. damselae* *V. ichthyoenteri*	Myoung *et al*., 2014
Multiplex PCR	*etfD* *val* *rpoN* *vhh*	EtdF AGCGCAGCTAACGGTAAAGT EtfA_R TGTAACCGTGTTGGCGTAAG ValF CTCTCCCAATTCAGCCCTCTA ValR GACTCTTCACAACAGAACTC rpoN-ang5 GTTCATAGCATCAATGAGGAG rpoN-ang3 GAGCAGACAATATGTTGGATG VhF ACGCTTGATGGCTACTGGTGGAG VhR CTTCGCACCTGCATCGG	426 773 519 606	*E. tarda* *V. alginolyticus* *V. anguillarum* *V. harveyi*	Ferreira Pinto *et al*., 2017
Multiplex PCR	*SufB* *HlyA*	VH298-F-AGCTATTATTCCGCGCCATCTTG VH842-R-AGGACGATCACTTCTACCACCG VA1186-F-CGCATTAACCCGATTGGTTACGC VA1535-R-TCATCACAGCTTGAGGCAGAGAG	545 350	*V. harveyi* *V. anguillarum*	Nishiki *et al*., 2018

	ureC *16S*	PD785-F- CCATTGGTGATCGTGTTATCCACGTA PD1671-R-CTCTGTGGCTGGCTCACAAGTTA CCTGGTAGTCCACGCCGTAA CGAATTAAACCACATGCTCCA	887 168	*P. damselae subsp. damselae*	
Multiplex PCR	*rpoD* *toxR* *recF* *rpoB* *rpoD* *toxR* *rpoD*	Vuni-rpoD- CTTTYGCTTCACCGATAGACAT Vuni-rpoD-F.4 CAAGGCTATCTGACCTACGC Van-rpoD-F.7 CAGACARCAAGAAGAAGACATTCG Vgi-tox-R GGCATGATGAAAGCGATAAGCAGT Vuni-tox-F CCWAARCGCGGTTAYCAAYTKAT Vat-recF-F.2 ATTTGAGAGTTCACTCGCGGGC Vat-recF-R.3 GCTTAGTTCTCGATAGTGTGTTGC VH-4F GTGATGAAGAAGCTTATCGCGATT VH-7R CGCCTTCTTCAGTTAACGCAGGA Vsc-rpoD-F.2 CCAAGTTCAAAACGCCGTTGCA Vle-rpoD-F.3 GAACGGTAATCGTCGCTCAGTCC Vspl-tox-R.1 GTTGTTGCTGGTTCCACTTCAAC Vuni-tox-F CCWAARCGCGGTTAYCAAYTKAT php-rpoD-F.6 AGTACGCACTAGCGAGCGTCTTA Val-rpoD-F AATGAAATGATGCTAGACGTATTCCG Phd-rpoD-F.2 GATGGTGACAGCAGCGACG	1075 125 292 372 601 740 113 218 279 371 519	*Vibrio* spp. *V. anguillarum* *V. gigantis* *V. atlanticus* *V. harveyi* *V. scophthalmi* *V. lentus* *V. splendidus* *P. piscola* *V. alginolyticus* *P. damselae*	Kim *et al.*, 2019
RT-PCR RPA assay	*toxR*	toxRF1-GAAGCAGCACTCACCGAT toxRR1-GGTGAAGACTCATCAGCA F1- CCACTGCTGAGACAAAAGCA R1- GTGATTCTGCAGGGTTGGTT ToxR P1 [TxRED]CAGCCGTCGAACAAGCACCG[BHQ-2] RPA-F1- AGAATCATCGTGTTAGTTGCCCTGCTACTTCCTGT RPA-F2- AATCATCGTGTTAGTTGCCCTGCTACTTCCTGTTG RPA-F3- TGTTAGTTGCCCTGCTACTTCCTGTTGGCGTGT RPA-R1- TGTTTAATTGAGGGTGGTTGATCGGTGTCAT RPA-R2- ATTGTTTAATTGAGGGTGGTTGATCGGTGTC RPA-R3- AGCCAATTGTTTAATTGAGGGTGGTTGATCGG RPA-Probe- CCCTGCAGAATCACAATTTCGTCAAATTGG[FAMdT] G[THF] A[BHQ1-dT] ATCACAACGTGCCAG (C3 Spacer)		*V. harveyi*	Pang *et al.*, 2019
LAMP	*toxR*	ToxR-FIP CTCGTGAAGCTCGTTACGCGATTC-TACTCATGTTGGCAGA (F1c-F2) ToxR-BIP GGAGCAGGGTTTTGAGGTGGACAT-TTTACGTAGAGTGGAAAT (B1c-B2) ToxR-F3 ATTAGGAAGCAACGAAAGC (F3) ToxR-B3 GCGACTTCGTAGAGTCTT (B3) ToxRF GAAGCAGCACTCACCGAT ToxRR GGTGAAGACTCATCAGCA		*V. harveyi*	Cao *et al.*, 2010
Tri LAMP	*vhh* P2(*V. harveyi*)	Vhhp2-F3-CAATTCGAAACAGGCGTG Vhhp2-B3-AGTAAAGCTTGCCACACG Vhhp2-FIP-CGCCACCACCATATCCATCG-GAATTCGGTTAGTCAATGGTGGAACA (*Eco*RI)		*V. harveyi*	Yu *et al.*, 2013

	toxR (*V. anguillarum*) Collagenase (*V. alginolyticus*)	Vhhp2-BIP-GGATGTAAATGAGTTTGGCTTTCCG-GGATCCTTGTCCTATGTTATACGGGTTG (*Bam*HI) ToxR-F3-ATTCGTTAAAACCGTGCC ToxR-B3-TCCCTATTTTTACTTTCGCG ToxR-FIP-TCGTTGGTGGTGTTTTTTTGTGA-TTTTCGAGGTTATCAAATGATCGC ToxR-BIP-GGAACCGTTACTGGCCAGTG-GGATCCTTGGGATGTTGTCTCTTCG (*Bam*HI) ValC-F3-GCGTACTTACCGCAAGTGA ValC-B3-CCCTTAGAGCAAAATCGCCT ValC-FIP-ACACCATTAACCGCATTGCGC-GAATTCCAGTGGCTTACACGTTGGA (*Eco*RI) ValC-BIP-GTGGGCAGTGGAACGAGCAA-TTTTAAGCTTTGGCAAGGTCTGT		*V.anguillarum* *V. alginolyticus*	
on-chip LAMP	*vhhA*	Vha-F3- AACCAATACATCGCTCTGAC Vha-B3- TCATTTCCACGATCTTCGC Vha-FIP- GGCACGCTACGGTTGTAGTT-ttttAACTACAAGCCTGCTAATACC Vha-BIP- GACTACGCGGAAGCCTTGATT ttttTCTGGTAGTGTCATCAACAAC Vha-LF- AGACCAAACTCAAGGGTAAACA Vha-LB- TGCAGGTGCGAAGAACTT		*V. harveyi*	Zhou *et al*., 2014
Whole-Genome Sequence		GenBank accession no. CP009467.2, CP009468.1.		*V. harveyi ATCC 33843*	Wang *et al*., 2015

Serological techniques

Enzyme-linked immunosorbent assays (ELISA) were developed for the rapid detection of *V. harveyi* from penaeid shrimp and water (Robertson *et al*., 1998).

ECL-based novel dot blot assay was developed by Li *et al* (2017) for the rapid and sensitive detection of *V. harveyi*.

4.3 Photobacteriosis by *Photobacterium damselae* subsp. piscicida

Photobacterium damselae ssp. piscicida (Gauthier et al. 1995) is a Gram-negative, nonmotile, bipolar coccobacillus, which was previously known as *Pasteurella piscicida* (Snieszko et al. 1964), belonging to the family *Vibrionaceae*. It is the causative agent of the fish disease photobacteriosis, also known as pasteurellosis or pseudotuberculosis. Fish Photobacteriosis or pasteurellosis (or pseudotuberculosis) is a bacterial septicemia that occurs as an acute septicemic form with massive mortality, especially in young fish, and a chronic form, with the formation of granulomatous lesions in older fish.

Pasteurellosis leads to important economic losses in the marine aquaculture industry both in Japan (yellowtail cultures) and in the Mediterranean area (European sea bass and gilthead sea bream farm) (Andreoni and Magnani, 2014; Vendramin *et al*., 2016). For these reasons a rapid diagnosis is important for the management and control of outbreaks in farmed fish (Andreoni and Magnani, 2014).

Disease diagnosis is usually made using standard microbiological methods based on pathogen culturing and isolation. Biochemical and serological confirmation is also necessary to characterize the bacterium and to discriminate between the two closely related subspecies, *Photobacterium damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* (formerly *Vibrio*

damsela). The latter subspecies has been associated with outbreaks in the gilthead sea bream and European sea bass and has been occasionally associated to human infections as the result of topically acquired infections which could become systemic in immunocompromised people (Haenen *et al.*, 2013).

Infected fish are lethargic, swim slowly near the surface and ultimately sink and rest on the bottom prior to death. Increased ventilation rates and loss of equilibrium may also be observed.

Diseased gilthead seabream in acute forms exhibit no apparent external clinical signs except rare individuals that display hemorrhage around the head and opercular region. Septicemic lesions and even the presence of whitish colonies of bacteria can be seen in spleen and kidney.

In the chronic form small, sometimes few white spots are seen in skin and white "miliary" granuloma-like lesions (pseudotuberculosis) may be seen in the spleen and kidney and are composed by masses of bacterium, epithelial cells and fibroblasts.

Culture, Staining and Biochemical Phenotype

P. damselae subsp. *piscicida* can be isolated from internal organs of diseased fish after 2–4 days incubation at 22°C by using media such as trypticase soy agar (TSA) and blood agar (BA), both supplemented with 1–2% NaCl, or marine agar (MA). The presumptive diagnosis is based on the isolation of a gram-negative, non-motile bipolar rod that is oxidase and catalase positive, fermentative without gas production, sensitive to the vibriostatic agent O/129, fails to grow on thiosulfate citrate bile sucrose (TCBS) agar and has strict salt requirements. The colony is non-hemolytic on blood agar at 48 h post inoculation. However, diagnosis can be hampered by the slow growth of this bacterium in laboratory media, which is easily obscured by other fast-growing bacteria mainly in the cases of the frequently found mixed infections with *Vibrionaceae*. This fact greatly encourages biochemical, serological and genetic characterization studies in order to develop an accurate and specific diagnostic procedure. Otherwise, *P. damselae* subsp. *damselae* is faster growing and 1-2 mm colonies are visible after 24 h of incubation. Biochemical tests may be performed in standard media, however, addition of 1-2% NaCl to the media may enhance growth. For presumptive diagnosis, the bacterium should be an oxidase positive, motile, gram negative rod, a facultative anaerobe that produces acid but no gas from glucose, mannose, and maltose and is positive for arginine dihydrolase, Voges-Proskauer (VP), and urease. The bacterium is sensitive to vibriostatic agent (0/129) and grows on thiosulfate citrate bile sucrose agar (TCBS) producing a green colony.

API system: The homogeneity of *P. damselae* subsp. *piscicida* facilitates the use of the miniaturized system API-20E (Biomerieux, France) for its identification. In agreement with Kent (1982), although the pathogen is not included in the API-20E code index, the system is valuable for a rapid presumptive identification of the bacterium, because all strains have a similar pattern (2005004) (Toranzo *et al.*, 1991) although some strains exhibit aberrant reactions that can lead to misleading results (Thyssen *et al*., 1998). Other codes have been reported by Buller (2004): 001100407, 200400406, 220500407. Instead *P. damselae* subsp. *damselae* displays a code 2015004 (Osorio *et al.,* 2000).

Hence, differentiation of the subspecies *P. damselae* subsp. *damselae* can be achieved when three or more positive results are obtained in the lysine decarboxylase (LDC) production, motility, nitrate reduction to nitrite, gas production from glucose, thiosulfate citrate bile salts-

sucrose (TCBS-1) growth, and urease tests, because all these tests yield negative results for all *P. damselae* subsp. *piscicida* strains.

Molecular techniques

Several molecular methods have been developed for the specific detection of *P. damselae* subsp. *piscicida* (**Table 8**).

16S rRNA PCR: This method can allow the identification of the species *Photobacterium damselae* but cannot differentiate between the two subspecies (Gauthier *et al.,* 1995; Osorio *et al*., 1999). Several authors have reported that 16S rRNA gene does not provide a sufficient phylogenetic resolution at the species level for *Photobacterium* species (Martins *et al.,* 2015). According to previous studies (Osorio *et al*., 1999) the subspecies *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* have 100% similarity between sequences of 16S rRNA gene and only 91% similarity between sequences of toxR gene (Martins *et al*., 2015).

Species-specific PCR and culture on TCBS agar: A simple and rapid method of identification and differentiation between the subspecies of *P. damselae* is done by primers designed to amplify the capsular polysaccharide gene. A pair of primers was selected to amplify a 410-bp fragment of capsular polysaccharide gene (*Cps*) derived from *P. damselae* subsp. *piscicida*. The forward primer, CPSF (5`AGGGGATCCGATTATTACTG3`) corresponding to positions 531–550 of the *P. damselae* subsp. *piscicida* gene for capsular polysaccharide and the reverse primer, CPSR (5`-TCCCATTGAGAAGATTTGAT-3`) corresponding to positions 921–940. Since both subspecies of *P. damselae* are detected using this PCR the subspecies must be differentiated by growth on thiosulfate citrate bile sucrose TCBS agar. *P. damselae* subsp. *damselae* grows on TCBS producing a green colony and *P. damselae* subsp. *piscicida* fails to grow (Rajan *et al.* 2003).

Multiplex PCR: for identification and differentiation between subspecies of *Photobacterium damselae*. A multiplex-PCR approach, employing 2 primer pairs directed to internal regions of the 16S rRNA and u*reC* genes, has been utilized to identify and discriminate between subspecies of *P. damselae* (Osorio *et al.* 2000). *P. damselae* subsp. *damselae*s trains yield 2 amplification products, one of 267 bp and the other of 448 bp, corresponding to internal fragments of the 16S rRNA and ureC genes, respectively. However, *P. damselae* subsp. *piscicida* isolates only show the PCR product of 267 bp (16S rRNA fragment), indicating the absence of the urease gene in its genome (Osorio *et al*. 1999). Amigliani *et al.* (2009) proposed a multiplex PCR with detection limit of 500fg DNA (100 genome equivalent). The nucleotide sequence of partial putative penicillin-binding protein (*Pbp*) 1A from Pdp (GB acc. n. EU164926) was used to design two oligonucleotide primers, designated 76a (5'CCGACTCAACTACAGATCACCCAGTC -3') and 76b (5'-GTGCGGCCTAAATTTCGACGA 3'). The internal amplification control (IC) consisted of the *pDEF* recombinant plasmid previously described by Casabianca *et al.* (2003). A specific set of primers (SDR3 5'CCGGTCTGACCTTTACACTG-3' and SDR4 5'-GGGGTTCTCTGTGAGAAGGT 3') was selected to amplify a 112 bp within the *pDEF* insert. In a subsequent phase of the study, an additional primer set, Ure 5'-Ure 3', previously reported by Osorio *et al*. (2000) was added to the multiplex PCR. The optimized protocol was also pre-validated with spleen, kidney and blood samples from infected and uninfected sea bass, without any culture step, and it can be proposed as a valid alternative to culture standard methods for the rapid and specific diagnosis of photobacteriosis in fish and commercial kit is available (PCR dection Kit by Diatheva).

PCR-RFLP: Zappulli *et al*. (2005) describe a highly sensitive test based on PCR-RFLP analysis that allows culture-independent discrimination between the 2 subspecies. In this study two *P.*

damselae-specific primer pairs were developed: P.dam-1a (CTTAACGCTACGTGGTGACAGTT and AGACGATCGCCTGCAATAAC) and P.dam-5a (CAACCCTGCAACATTTCTACCAAG and GGAGTGCATGCCGAACAAGC) and a restriction analysis (using enzyme Hha I, EcoR V, BsmA I, Bsr I for P.dam 5 and BstU I for P.dam-1) of PCR products was performed. Therefore, the use of this methodology (especially the amplification and restriction of fragment P.dam-1a_F-R) can contribute to an improved efficacy of prevention programs against fish pasteurellosis.

Real-time PCR: Martins *et al*. (2015) developed a real-time PCR for quantification of *P. damselae* in water samples, with a second step based on RT PCR-DGGE (PCR denaturing gradient gel electrophoresis) of *toxR* gene fragments to identify the two subspecies in the same assay. Carraro *et al.* (2017) proposed a single-step, high-sensitivity real-time PCR assay for simultaneous detection and quantification of *P. damselae*. The primers were designed targeting partial sequence of the *bamB* gene and tested for specificity and sensitivity on laboratory-generated samples as well as on experimentally infected seabream tissue samples. Two sets of primers, Ph_PiscA (TGCTGGTGGTGTATTCTGGG and GTCAACTAGACGATCAATTTCAGTT) and Ph_PiscB (TGCTGGTGGTGTATTCTGGG and AACAGGTGTCGCATCAACGT), were designed targeting the region containing the two SNPs. A primer set for the amplification of *Sparus aurata* TRL-9 gene (Sa_TLR9_Forward 5'-GAAATTGT CTGCCAGGTCGC-3' and Sa_TRL9_Reverse 5'-AGGTATACGGGTG AGGCTGT-3') was used as internal control in PCRs. In this study a qPCR assay has been developed for the simultaneous detection and quantification of *P. damselae*. With a theoretical LOQ (theoretical limit of quantification) of nine copies of target DNA in spiked conditions (one copy when pure bacterial DNA is employed), the assay developed in the study of Carraro *et al.* (2017) shows a sensitivity of at least one or two orders of magnitude higher than the assays proposed by Amagliani *et al*.(2009) (i.e., 100 molecules of pure bacterial DNA) and by Martins *et al*.(2015) (i.e., 1 x 10^3 molecules of pure bacterial DNA), respectively. The only previous assay with a LOD (limit of detection) lower than 10 copies was the one proposed by Zappulli *et al.* (2005); however, its sensitivity has only been tested on pure bacterial DNA and an additional RFLP protocol on amplified DNA is needed to discriminate between *P. piscicida* and *P. damselae* subspecies. A Real time PCR Commercial kit is available: *Photobacterium damselae* subsp. *piscicida* Primerdesign™genesig® Kit.

Fingerprinting methods for typing: Mancuso *et al*. (2007) evaluated the effectiveness of three different molecular techniques REP-PCR (repetitive extragenic palindromic PCR), ERIC-PCR (enterobacterial repetitive intergenic consensus sequence PCR) and RAPD-PCR (random amplified polymorphic DNA) for rapid typing of *P. damselae* subsp. *piscicida*. The results obtained showed that RAPD and ERIC-PCR were more discriminative and can be used in epidemiological studies of photobacteriosis infections.

Whole-Genome Sequence: In recent years several whole genome sequences have been carried out and are available in the GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

MALDI-TOF: within the PerformFish project, Pečur Kazazić *et al*. (2019) applied MALDI-TOF technology for a rapid and accurate identification of *Photobacterium damselae* subsp. *piscicida* isolated from European seabass and gilthead seabream.

Table 8. Molecular Methods for the identification of Photobacterium damselae subsp. Piscicida

ASSAY	TARGET GENE	PRIMER SEQUENCE (5'-3')	PRODUCT (bp)	SPECIFICITY	REFERENCE
PCR	*16S*	Car1 GCTTGAAGAGATTCGAGT Car2 CACCTCGCGGTCTTGCTG	267	*P. damselae* species	Osorio *et al.* 1999
PCR+ cultured method	*Cps*	CPSF AGGGGATCCGATTATTACTG CPSR TCCCATTGAGAAGATTTGAT	410	*P. damselae subspecies**	Rajan *et al.*, 2003
Multiplex PCR	*ureC* *16S*	Ure 5' TCCGGAATAGGTAAAGCGGG Ure 3' CTTGAATATCCATCTCATCTGC Car 1 GCTTGAAGAGATTCGAGT Car2 CACCTCGCGGTCTTGCTG	448 267	*P. damselae* subspecies	Osorio *et al.*, 2000
Multiplex PCR	*Pbp-1* IC *ureC*	76aCCGACTCAACTACAGATCACCCAGTC 76b GTGCGGCCTAAATTTCGACGA SDR3 CCGGTCTGACCTTTACACTG SDR4 GGGGTTCTCTGTGAGAAGGT Ure 5' TCCGGAATAGGTAAAGCGGG Ure 3' CTTGAATATCCATCTCATCTGC	297 112 448	*P. damselae* subspecies	Amagliani *et al.*, 2009 Commercial kit available: PCR dection Kit by Diatheva
PCR-RFLP	-	P.dam1aF CTTAACGCTACGTGGTGACAGTT P.dam1aR AGACGATCGCCTGCAATAAC P.dam5aF CAACCCTGCAACATTTCTACCAAG P.dam-5a R GGAGTGCATGCCGAACAAGC	*BstU* I *Hha* I, *EcoR* V, *BsmA* I, *Bsr* I	*P. damselae* subspecies (restriction analysis)	Zappulli *et al.*, 2005
RT-PCR and **Nested PCR-DGGE**	*toxR*	ToxRn1-F GGCTATTRCAKCAACSGAACA ToxRn1-R TTTGGYGTTACRACTTGCACCCC ToxRn2-(GC)-FCAGCAACGGAACACGCAGAAG ToxRn2-R TGCACCCCTTTAACCGAAAAGA	323 300	*P. damselae* species *P. damselae* subspecies	Martins *et al.,* 2015
RT-PCR	*bamB* *TRL-9* (IC)	Ph_Pisc A-F TGCTGGTGGTGTATTCTGGG Ph_Pisc A-R GTCAACTAGACGATCAATTTCAGTT Ph_Pisc.B-F TGCTGGTGGTGTATTCTGGG Ph_Pisc.B-R AACAGGTGTCGCATCAACGT Sa_TLR9-F5 GAAATTGT CTGCCAGGTCGC Sa_TRL9-R AGGTATACGGGTG AGGCTGT	130 148	*P. damselae* subsp. *piscicida*	Carraro *et al.,* 2017
RT-PCR	*trpB*			*P. damselae* subsp. *piscicida*	Commercial kit available: *Photobacterium damselae* subsp. *piscicida* (Primerdesign™genesig® Kit)
ERIC-PCR		ERIC 1 ATG TAA GCT CCT GGG GAT TCA C ERIC 2 AAG TAA GTG ACT GGG GTG AGC G		typing of *Ph.damselae* ssp. *piscicida*	Mancuso *et al.*, 2007 Commercial kit available w/o primers (Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech)
REP-PCR		REP ID NNN RCG YCG NCA TCM GGC REP 2D RCG YCT TAT CMG GCC TAC		typing of *Ph.damselae* ssp. *piscicida*	Mancuso *et al.*, 2007 Commercial kit available w/o primers (Ready-To-Go™ PCR

					beads (Amersham Pharmacia Biotech)
RAPD-PCR		P5 AACGCGCAAC P6 CCCGTCAGCA		typing of *Ph.damselae* ssp. *piscicida*	Mancuso *et al.*, 2007 Commercial kit available w/o primers (Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech)
Whole-Genome Sequence		GenBank accession no. GCA_000300355.3 AP018045 to AP018048 BDMQ01000001 to BDMQ01000007			Osorio *et al.*, 2015 Teru *et al.*, 2017 Aoki et al., 2017

Serological techniques

From a serological standpoint, *P. damselae* subsp. *piscicida* is also a highly homogeneous group, which makes it impossible to establish serotypes.

Latex agglutination-based assay (BIONOR Mono-Pp-kit). Agglutination tests based on preculturing of sample. The test reagents consist of monodisperse particles coated with specific antibodies against fish pathogenic bacteria. he detection limit of the kit is 10^{10} bacteria ml^{-1} (Romalde *et al*., 1995a).

ELISA-based (Bionor AQUARAPID-Pp test). A manual system based on Enzyme Immuno Assay principle. Available in 40 tests per kit.The kit showed a sensitivity cut-off between a positive and a negative sample of 10^6 bacteria/0.5g tissue (Romalde *et al.,* 1995b).

Magnetic particle enzyme immunoassay (Bionor AQUAEIA-Pp test). Semiautomated system based on Magnetic Particle Enzyme Immuno Assay principle. Available in 50 tests per kit. Detection limit 10^4 cells (Romalde *et al*., 1999; Lores *et al*., 1998). The kit can detect both clinical and subclinical cases of pasteurellosis and it was able to detect the pathogen in fish samples when primary isolation culture did not. Furthermore, the ability to detect the pathogen in sexual products in a non-destructive analysis method permits monitoring populations and selection of disease-free broodstock as an additional preventive measure to avoid disease outbreaks. Both test systems (AQUARAPID and AQUAEIA) are designed for diagnostic testing for fish pathogens at laboratories and at fish farms and the results are obtained within 1 hour with little hands-on time.

Immunohistochemistry: monoclonal anti-*Photobacterium damselae* subsp. *piscicida* monoclonal antibody (Product no; P02, Aquatic Diagnostics Ltd., Scotland) are also available.

4.4 Sparicotylosis by *Sparicotyle chrysophrii*

Polyopisthocotyleans are pathogenic monogenean trematodes worldwide distributed and threatening fish of great commercial importance. Amongst them, *Sparicotyle* (syn. *Microcotyle*) *chrysophrii* (Van Beneden et Hesse, 1863) is one of the most limiting factors for gilthead seabream (*Sparus aurata* L.) production, producing mortality outbreaks in farmed fish (Faisal and Iman, 1990; Sanz, 1992; Alvarez-Pellitero, 2004). The main pathological effects of *S. chrysophrii* in gilthead sea bream are the respiratory dysfunction due to the association of mechanical lesions and obstruction of water flow between gill filaments by the massive body of the flukes, and the progressive anemia induced by the hematophagous attitude of the parasite

and by the hemorrhages caused by the disruption of the epithelial and vascular structures (Sitjà-Bobadilla and Alvarez-Pellitero, 2009). Lower food intake is reported in gilthead seabream with high monogenean burdens (Sitjà-Bobadilla et al., 2006), so that the possible contribution of anorexia to the anemic condition of fish in heavy infections cannot be excluded (Sitjà-Bobadilla and Alvarez-Pellitero, 2009).

Field Diagnosis

Clinical signs and behavioural changes

Disease signs commonly reported by fish farmers and fish pathologists are lethargy, emaciation and severe anemia dependent on parasite density (Sitjà-Bobadilla and Alvarez-Pellitero, 2009). Pronounced pathology can be observed even at low infection density (eight parasites per gill arch) (Mahmoud et al., 2014). Lesions caused by the parasite can easily become infected by aquatic bacteria and gill co-infections with *Tenacibaculum* are seen quite often.

Detection

Since *Sparicotyle chrysophrii* has big dimensions, the presence of the parasite could be easily detected by two methods based on wet mounts (Sitjà-Bobadilla and Alvarez-Pellitero, 2009). The first one is based on fresh (F) diagnosis, by which fish are slightly anesthetized with MS-222 (Sigma, Saint Louis, MO) (100 mg/l) and gill scrapings taken from the external gill arch are examined using light microscope. A second diagnostic method by the stereomicroscope (S) could be applied: fish are euthanized and all their gills arches excised and examined under the stereomicroscope. In both cases, the number and type of stages of the monogenean could be recorded (Sitjà-Bobadilla and Alvarez-Pellitero, 2009).

The outermost arch is reported as the most parasitized (Oliver, 1984; Rigos et al., 2016) and also in the field the two external arches are usually checked as easily dissected (with the second one often more infected) or all the four arches on one side are examined to count the total number of parasites in the fish. A further approach setup by veterinarians working in the field is to count the number of parasites and divide the total by the weight of the fish; if the index is above a certain threshold both clinical signs and mortality could start.

Morphological identification

Traditional morphological approach according Oliver (1968) and Radujkovic and Euzet (1989) leads to species identification just starting from microscopical examination.

The main morphological features within the class are marked differences in the attachment apparatus of the different species. The main characters of the species *S. chrysophrii* is a symmetrical, triangular and leaf-shaped opisthaptor with more than 100 clamps (55 pairs in adult specimens) situated on its edge (Caffara et al., 1998) and each composed of a peduncle and two opposable hinged jaws (anterior and posterior) connected to the tegument and supported by a median sclerite.

Parasites collected from gills of larger fish have been reported as significantly larger than those collected from smaller fish and the largest clamp was wider in the parasites from the largest hosts (Villar-Torres et al., 2014). It has been hypothesized also a rather marked difference on size between parasites collected from farmed (infected by larger parasites) and wild fish (dr. Ivona Mladineo, data not published).

Further morphological features are related to the shape of the eggs, with typical prolonged polar filaments, and aspect of reproductive organs, in particular the genital atrium in which the shape and the number of the spines have taxonomic relevance.

Scanning electron microscopy could be utilized for the surface topography: Fixation in cold (4°C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, dehydration through a graded acetone series (30%, 50%, 75%, 90% and 100%), critical point dried and sputter coated with gold/palladium and observation by scanning microscope (Antonelli et al., 2010; Jun, 2015).

Molecular identification

Polymerase Chain Reaction and sequencing (Jun, 2015)

DNA extraction by Commercial Kit following the manufacturer's instruction. Amplification of the 28S rDNA by PCR using C1/D2 primer pair: C1: 5'-ACC CGC TGA ATT TAA GCA T-3', D2: 5'-TGG TCC GTG TTT CAA GAC-3'. The amplification was carried out as previously reported (Chisholm et al., 2001). The amplified PCR product was sequenced using the BigDyeTM terminator cycle sequencing kit (Macrogen Genomic Division). The sequence obtained in this study was aligned with that of the *S. chrysophrii* 28S ribosomal RNA gene (AF311719).

Phylogenetic analysis (Mladineo et al., 2009): Fixation in absolute alcohol and sample storing at 4 °C. For phylogenetic analysis, genomic DNA was isolated using the QIAGEN DNeasy Blood and Tissue Kit (Qiagen). Quantity and purity of isolated DNA and amplified fragments afterwards was checked by bio-photometer (Eppendorf). A mitochondrial DNA locus, COI (cytochrome oxidase I) was amplified by PCR using 0.8 µM of each of the following primers that amplify ~370bp of *S. chrysophrii* COI. (Mladineo et al., 2009):

Forward primer 5' GTG CTA ATA CTA CCA GC 3' (sparicoF)

Reverse primer 5' GCT ACA CGA CCA TCT ATC 3' (sparicoR)

Development of RAD markers to distinguish wild from farmed *Sparicotyle* populations (Trumbić et al., 2017)

A genotyping-by-sequencing approach has been applied, through a double digest Restriction-site Associated DNA sequencing (ddRAD-Seq), to generate a genome-wide SNP marker dataset with the long term aim of investigating the degree of differentiation among individuals collected from farmed and wild hosts. One ddRAD library for *S. chrysophrii* was constructed (96 and 138 samples in total), producing over 16 and 21 million reads with 26, 566 unique RAD loci identified. Considerable variability in number of reads, polymorphic loci and SNPs identified was observed between individuals for both species. SNPs genotyped in at least 50% of the individuals were identified (507 in *S. chrysophrii*) and used to compare the structure between samples currently available. The method was implemented in the context of ParaFishControl project.

4.5 Enteric parasitic diseases of gilthead sea bream due to *Enteromyxum leei*, *Enterospora nucleophila* and *Cryptosporidium molnari*

4.5.1 Enteromyxum leei

Enteromyxum leei Diamant, Lom, Dyková, 1994 is a microscopic myxozoan parasite (Cnidaria, Myxozoa, Myxosporea) infecting the intestinal tract of several fish species, particularly

pathogenic for the Sparidae, causing a negative economic impact on fish farming in the Mediterranean Sea. In particular, Enteromyxosis has caused the abandonment of the farming of sharpsnout sea bream (*Diplodus puntazzo*), particularly susceptible species, in several Mediterranean countries. In gilthead bream it induces severe enteritis followed by anorexia, emaciation up to cachexia and death of susceptible fish, and is the cause of significant growth retardation and mortality in intensive farming systems.

The life cycle of myxosporeans generally involves two alternating hosts, fish and annelids but, for *E. leei* direct spontaneous fish-to-fish transmission has been demonstrated in various marine fish (Sitjà-Bobadilla et al., 2007; Sitjà-Bobadilla & Palenzuela, 2012). *E. leei* developmental stages (trophozoites) and myxospores are excreted into the water with faeces or from dead fish. Released trophozoites are infective to other fish, and thus responsible for the horizontal transmission. Although it is probable that this myxozoan also has an indirect life cycle involving an invertebrate host in the wild, fish-to-fish transmission by oral, cohabitation, contaminated water and anal routes has been experimentally confirmed. This unique mode of horizontal transmission favors the spread of enteromyxosis in cultured fish stocks.

Enteromyxosis cannot be diagnosed by clinical signs as these are non-specific. Below the already existing diagnostic techniques to detect *E. leei*, together with the new ones developed during the ParaFishControl EU project (H2020), are reported.

For the detection of *E. leei* non-lethal and lethal sampling are available. The first one is carried out by squeezing the anus and collecting a little drop of intestinal content by coverslip followed by microscopical observation (10X-40X). This method is very useful for a rapid screening especially for large fish avoiding euthanasia. Intestinal contents can be collected also by probing the rectum with a cotton swab for molecular analyses. This procedure has been validated against a gold standard (histological observation of the whole intestinal tract) showing high sensitivity (0.96) and specificity (Sitjà-Bobadilla & Palenzuela, 2012; Picard-Sánchez et al., 2020). Concerning the lethal sampling methods, depending from the downstream analyses, intestinal mucosa could be collected in different ways. For fresh smear, after the isolation of the whole gut and longitudinal opening, gentle scrape the mucosa with a coverslip and observe under light microscope (10×-40×). This method is useful also to understand the distribution of the parasite along the whole intestine by dividing it in anterior, medium and posterior tract. Confirmatory diagnosis consists in the detection of spores or developmental stages in either fresh or smears stained with May-Grünwald Giemsa or Giemsa (Sitjà-Bobadilla & Palenzuela, 2012).

For downstream molecular analyses the posterior rectal ampoule could be sampled, representing the site which has the maximum predictive value for detection of the parasite in gilthead sea bream. The sample is then preserved in 80% ethanol before DNA extraction.

For **histology** the tissue specimen is preserved in 10% neutral buffered formalin, embedded in paraffin or Technovit resin. The sections (1–3 µm) could be stained with Periodic acid-Schiff (PAS), Giemsa, Toluidine blue or lectins (Sitjà-Bobadilla & Palenzuela, 2012). Infection intensity is evaluated in the histological sections following Estensoro et al. (2017) whom evaluated the infection intensity in Giemsa stained section of each intestinal segment semiquantitatively following a conventional scale from 1+ to 6+, with the following ranges: 1+ = 1-5; 2+ = 6-10; 3+ = 11-25; 4+ = 26-50; 5+ = 51-100; 6+ > 100 parasite stages per microscope field observation at 120×. *Enteromyxum leei* stages were classified as spores, sporoblasts and proliferative stages, the latter corresponding to stages one to three described in Alvarez-Pellitero et al. (2008). A fish

is considered positive for infection when the parasite is found at least in one intestinal segment. Examination of histological sections of intestine is considered the standard procedure to detect *E. leei*, unfortunately, when the parasite is in latent location or with low intensity the infection may be missed.

From the same samples prepared for histology, ***in situ* hybridization (ISH)** has been developed by Cuadrado et al. (2007) to detect *E. leei* developmental stages. Briefly, two oligonucleotide probes, 5'-GAATATCGGTGACGCCAATC-3' and 5'-CCGGAGAAGCCAACGTAT-3', were designed to specifically hybridise *E. leei* rDNA and rRNA according to original *E. leei* rDNA gene sequence data (Palenzuela et al., 2002). These probes were labelled with a single 3' digoxigenin (DIG) molecule by means of a modified dideoxyuridine-triphosphate (DIGddUTP) using a commercial kit (Roche Diagnostics, Barcelona, Spain). Labelled probes were mixed in equimolar amounts, and the cocktail was used in the ISH protocol as described previously for *Ceratomyxa shasta* (Palenzuela and Bartholomew, 2002), with minor modifications. Briefly, 5-µm-thick sections were taken on superfrost-plus slides. Deparaffinized sections were digested with 15 µg ml−1 proteinase K (15 min at 37°C), and they were allowed to hybridize with the probes overnight at 37°C. Unbound probes were washed off by double successive stringency washes at 37°C in 2×, 1× and 0.25× saline sodium citrate (SSC) buffer (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Immunological detection was carried out using an AP-conjugated anti-DIG Fab fragment and nitroblue tetrazolium/5-bromo-4-chloro-indolyl-phosphatase (NBT/BCIP) substrate (Roche Diagnostics), after the procedures recommended by the provider. The slides were counterstained with light green and mounted in permanent medium (DPX).

Several **molecular methods** have been developed to identify infected fish, to study on the life cycle and for phylogenetical purpose. Palenzuela et al. (2002) amplified by PCR the 18S rDNA of *E. leei* and other myxozoans with the primer pair MM18Sf (ctggttgattctgccagtggtc) and MM18Sr (cggtactagcgacgggcg) allowing a product of 1589 bp. Golomazou et al. (2014) developed a sybr green real-time PCR with species-specific primers (EL1F: ACCAAGTCTTCGGGCTCTG and EL2R: TCTGTCATTCTGTCAATCCTATCG) targeting the 18S rDNA of *E. leei.* qPCR was used allowing earlier diagnosis and identification of all developmental stages of the parasite throughout its life cycle, significantly before any outbreak occurred, thus combining parasitosis' prevalence and intensity. Recently Shin *et al*. (2018) developed species-specific multiplex PCR amplifying *E. leei* and *Parvicapsula anisocaudata* 18S rDNA (ELNMF CGGTGACGCCAATCCGTG (*E. leei* forward), PANMF AGGAACGTTACATAGCCGGCA 694 (*P. anisocaudata* forward) and MyNMR GACGGTATCTGATCGTCTTCGA (*E. leei/P. anisocaudata* common reverse) 192 bp and 502 bp respectively) from olive flounder *Paralichthys olivaceus* in Korea.

During the EU project ParaFishControl several new techniques have been developed, as indicated below.

Estensoro et al. (2017) carried out a research on *E. leei* to assess intestinal cell turnover and parasite proliferation, by administering the cell proliferative marker BrdU that is incorporated into synthetizing nuclear DNA during the S phase of the cell cycle and can be later immunohistochemically detected (Walsh & Eckert, 2014). As Gratzner (1982) described a monoclonal antibody to BrdU, this technique has proven to be very useful in many research fields to assess cell proliferation in normal tissues and tumours and to trace cells during development and differentiation (Goodlad, 2017). This technique has been applied previously in teleost fish to evaluate the effects of gastrointestinal distress (Dezfuli et al., 2016; Schall et al., 2015; Takahashi et al., 2006; Yoshida et al., 2016), but had not been previously used for

sparids or for the study of host–parasite interactions involving myxozoans. Altogether, BrdU immunolabelling and *pcna* gene expression showed the rapid proliferative response of the fish intestines upon a myxozoan infection and how this response is effectively triggered even before the parasite reaches or establishes in the site.

Piazzon *et al*. (2018) carried out a study with the aim to gain insights into fish T cell responses in the gilthead sea bream-*E. leei* infection model using a PCR-array with 30 signature molecules for different leukocyte responses in head kidney, spleen, anterior and posterior intestine. The PCR-array results suggest that *E. leei* induced migration of T cells from head kidney to intestines where TH1, CTL and TH17 profiles were activated and kept in balance by the upregulation of regulatory cytokines. These results were partially validated using cross-reacting antibodies and BrdU immunostaining to monitor proliferation. Zap70 immunostaining supported the increased number of T cells in the anterior intestine detected by gene expression, but double staining with BrdU did not show active proliferation of this cell type at a local level, supporting the migration from lymphohaematopoietic tissues to the site of infection. Global analyses of the expression profiles revealed a clear separation between infected and exposed, but non-infected fish, more evident in the target organ. Exposed, non-infected animals showed an intermediate phenotype closer to the control fish.

4.5.2 *Enterospora nucleophila*

Enterospora nucleophila is a microsporidian parasite infecting the teleost fish gilthead sea bream (*Sparus aurata*). It develops primarily within the nuclei of rodlet cells and enterocytes, at the intestinal epithelium (Palenzuela *et al*., 2014). It can also be found within the cytoplasm of other cell types, including phagocytes, at subepithelial layers. It is the causative agent of gilthead sea bream emaciative microsporidiosis, a chronic condition manifesting as a severe growth retardation up to stunted growth of infected fish, which can be accompanied by low-level but sustained trickling mortality (0.1-0.3% daily, up to 1% at peaks per sea cage) (Palenzuela *et al*., 2014). Affected fish normally appear lethargic and cachectic, with other nonspecific signs like discoloration and occasional scale loss (Palenzuela *et al*., 2014). The clinical condition seems to appear in gilthead sea bream during their first winter in sea cages. As a result of the arrested growth, infected animals can average half the weight of the unaffected ones.

The disease was first noticed in the early 2000´s. However, the difficulties in the diagnosis of the parasite probably delayed acknowledgement of its presence and impact. Indeed, the parasite and its association with gilthead sea bream emaciative microsporidiosis were not described until recently, but retrospective studies identified it in samples taken in 1993 (Palenzuela *et al*., 2014).

The main clinical signs are only noticed in severe infections and can be largely masked by other infectious diseases of gilthead sea bream, so approaches to understand the true impact of the disease can only be afforded after the development of appropriate diagnostic methods to conduct specific epidemiological and risk-assessment studies. Besides the mortality, the main economic impact of the parasite is related to the segregation of sizes caused by the infection within affected sea cages, as it results in inefficient feeding and serious biomass and quality losses at the harvest.

Only the development within gilthead sea bream is known (Palenzuela *et al*., 2014) and it is currently unknown if its life cycle could involve other hosts, as described for other close relatives

of the family Enterocytozoonidae that infect crustaceans (e.g. *Enterospora canceri* or *Enterocytozoon hepatopenaei*), some of which present heteroxenous cycles alternating crustacean and fish hosts (e.g., *Desmozoon lepeophtheri*) (Nylund *et al*., 2010).

Presumptive diagnosis can be made based on clinical signs and histopathological examination of the intestinal epithelium. The most common observation in heavy infections is the presence of numerous hypertrophied cell nuclei and a remarkable hypercellularity (Palenzuela *et al*., 2014). When present, tiny microsporidian spores (1.67 x 1.05 µm) can be identified. Like in other microsporidioses, the detection of spores can be facilitated with Calcofluor-white M2R or Luna stains (Petersen *et al*., 2010). More reliable confirmatory diagnosis of *E. nucleophila* is possible with molecular-based methods, such as RT-PCR tests (Hossam *et al*., 2017) and *in situ* hybridization (Hossam et al., 2019), developed during the EU project ParaFishControl.

4.5.3 *Cryptosporidium molnari*

Alvarez-Pellitero and Sitjà-Bobadilla (2002) described for the first time *C. molnari* in gilthead sea bream *(Sparus aurata* L.) and the European sea bass (*Dicentrarchus labrax* L.) farmed in Spain causing, at high infection intensity, abdominal swelling, whitish faeces and ascites. The parasite is found mainly in the stomach and seldom in the intestinal epithelium and fingerlings and juveniles are the main age classes infected. The diagnosis based on microscopic observation of Ziehl-Neelsen stained smear is difficult due to the small dimension of the oocysts (4.72 x 4.47 µm). For this reason, the molecular analysis (PCR on 18S rDNA, COWP, *gp*60 and qPCR), already applied for terrestrial animals, should be considered as gold standard.

5 PerformFISH activities focused to improve and/or setup diagnostic methods

To improve the diagnostic techniques useful for a rapid and reliable detection of the main pathogens of European sea bass and gilthead sea bream a diversified approach based on the different expertises (bacteriologists, virologists, biotechnologists, parasitologists) of partners involved in task 3.2 was used, aimed at improving already available methods or developing new techniques that may be of use to industry. A two-level approah was developed: a first, more generalistic approach based on Technology Readiness Level (TRL) concept for each identified need for diagnostics and a much more focused approach on particular needs for improvement for each diagnostic.

For Betanodavirus, the current diagnostic techniques and mainly qPCR have achieved a high level of readiness (TRL 7-8). In fact, many laboratories can offer sensitive qPCR detection techniques based on standarized protocols. The recent ring-test organised by IZSV in the context of MedAID and PerformFish also demonstrated a relevant level of harmonisation in the results obtained but at the same time allowed to detect some problems. Howewer, the recent changes due to the emergence of new reassortant types lead to the necessity for new techniques and particularly the simultaneous detection of different strains. With this purpose, a multiplex RT-PCR aiming at the simultaneous detection of different Betanodavirus strains, including emerging

reassortants, has been developed till TRL level 3 and is ready for further validation in laboratory and field environments.

Concerning *Vibrio anguillarum*, the variety of techniques available for routine diagnostic is quite wide, as it can be noticed in the current techniques section and therefore, the TRL is also very high (7-8) for most of the techniques but requires improvements in specific areas, particularly for molecular characterization of the strains of these species using molecular nucleic acid analysis (PCR-related techniques) and also techniques for the identification of the biomolecular profile of these strains and also strains of *Photobacterium damselae* subsp. *piscicida* (MALDI TOF). For particular uses, such as continuous microbiological water quality assessment in some areas in Mediterranean fish farming (broodstock, hatchery, live feed production). In this case, a probe-based biosensor has been developped, achieving TRL levels of 2-3.

Related to bacterial pathogens, antibiotic sensitivity identification was still an outstanding issue, as the methodology and interpretation in many laboratories does not take into account the specific particularities of aquatic bacterial pathogens. Relevant metrics such as MIC still require a major consensus on the way they are obtained to develop more sophisticated future approaches such as clinical breakpoints and epidemiologial cut-off values in the farms. At the same time, the industry requires faster methods to obtain good data on sensitivity and resistance of the different strains in order to take fast and efficient decisions for antimicrobial therapeutics (see also PerformFISH deliverable 3.4). The new method developped by UNIBO has achieved a TRL level 3 and is ready to be expanded to TRL4.

No developments in diagnostic methods for Sparicotyle were considered necessary due to the particular characteristics of the parasite.

Finally, concerning intestinal parasitic infections in gilthead seabream, it should be highligted that *Enterospora* and *Enteromyxum* are already well covered by the H2020 project ParaFishControl and particularly diagnostics have been already delivered. With this previous background and with diagnostics strongly covered by ParaFishControl, PerformFish approach on diagnostics was focused on the development of a complementary differential diagnostic method to *Enterospora*, as another parasite, *Cryptosporidium*, another gastrointestinal parasite widely present in gilthead seabream farms in the Mediterranean was not covered in ParaFishControl.

Therefore, the new developments on diagnostic techniques in PerformFISH are:

1. a new technique for the simultaneous detection of different Betanodavirus strains, including reassortants, has been set up with a multiplex PCR approach, relatively rapid and cost-effective in comparison with standard virological/molecular assays.
2. an improvement of the molecular characterization and identification of *Vibrio anguillarum* and preliminary setup of a molecular probe-based biosensor for its detection in the field for its possible application in critical production sites such as hatcheries.
3. MALDI-TOF studies for identification of *V. anguillarum* and *Photobacterium damselae* subsp. *piscicida* to produce data useful for diagnostic labs.
4. a field method to test the antibiotic sensitivity of bacterial strains isolated during disease outbreak has been designed and preliminarily developed (Perform-TEST), although the restrictions due to Covid-19 outbreak forced to stop the activities before finalization.

5. a multiplex PCR to detect simultaneously different criptic enteric parasites, such as *Enterospora* and *Cryptosporidium*, is under development, yet research halted due to Covid-19 outbreak.

5.1 Multiplex RT-PCR for Betanodavirus

Betanodaviruses are small, non-enveloped, single-stranded positive-sense RNA viruses and their genome consist of two segments named RNA1 and RNA2 which encode for the RNA-depend RNA polymerase and the coat protein, respectively (Thiery et al., 2012). Based on a partial nucleotide sequence of the coat protein gene, betanodaviruses have been clustered into four genotypes, currently accepted by the International Committee on Taxonomy of Viruses (ICTV) as official species of this genus: Striped jack nervous necrosis virus (SJNNV), Tiger puffer nervous necrosis virus (TGNNV), Barfin flounder nervous necrosis virus (BFNNV) and Redspotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997; Thiéry et al., 2012). Furthermore, two new reassortant strains originated from the reassortment of SJNNV and RGNNV genotype and named SJNNV/RGNNV (containing the RNA1 segment deriving from the SJNNV genotype and the RNA2 segment from the RGNNV genotype) and RGNNV/SJNNV (containing the RNA1 segment deriving from the RGNNV genotype and RNA2 segment from the SJNNV genotype) were reported in Southern Europe (Toffolo et al., 2007; Olveira et al., 2009).

Historically, the RGNNV strains were the most impacting the Mediterranean aquaculture causing severe mortality outbreaks in European seabass (*Dicentrarchus labrax*). More recently the RGNNV/SJNNV reassortant strain expanded betanodavirus host range causing massive mortality outbreaks in gilthead seabream larvae (*Sparus aurata*) (Toffan et al., 2017; Volpe et al., 2020).

Several diagnostic techniques are available for NNV detection (electron microscopy, cell culture isolation, immunodetection and molecular methods) but according to the World Organization for Animal Health (OIE), the 'Gold Standard' method to detect VER is the isolation of viral agents in cell culture, followed by immunological or molecular identification (OIE, 2019). Furthermore, given the wide co-circulation of different betanodaviruses in the same geographic region and considering their different features in pathogenicity host and temperature tropism, laboratories are expected not only to detect, but also to genotype isolated/detected viruses (Toffan et al., 2018; Zrnčić et al., 2020). In this regard, sequencing of both viral genome segments (RNA1 and RNA2) is, at the moment, the method of choice to genotype viral strains.

However, both cell culture isolation and genome sequencing are time-consuming and resource-intensive techniques and consequently, are not optimal for the rapid detection and identification of NNV from field cases and routine surveillance programs (Doan et al., 2017). Multiplex PCR assays have gained popularity because of their convenience in terms of cost and time (Giridharan et al. 2005).

Some PCR-based reactions have been developed in order to discriminate between RGNNV and SJNNV genotypes; however, these assays remain limited to the independent detection of RNA1 or RNA2, making them unable to distinguish between parental RGNNV and SJNNV genotypes from their reassortant strains (RGNNV/SJNNV and SJNNV/RGNNV) using a single reaction (Valle et al., 2000; Valle et al., 2005; Lopez-Jimena et al., 2010; Panzarin et al., 2010; Baud et al., 2015; Toubanaki et al., 2015; Toubanaki and Karagouni, 2017; Kim et al., 2018).

The purpose of this study was to develop a one-step multiplex RT-PCR assay as rapid and cheap diagnostic technique to detect the presence of NNV and to discriminate between RGNNV, SJNNV genotypes from the RGNNV/SJNNV and SJNNV/RGNNV reassortant strains and to identify possible co-infections.

The simultaneous detection of several viral targets with a multiplex PCR approach is relatively rapid and cost effective compared to standard virological/molecular assays. The mRT-PCR assay developed was conducted in a mix reaction volume equivalent to that of a singleplex; in this way, the cost and the time of execution of the test for the simultaneous detection and identification of NNV and its variants circulating in Southern Europe are equivalent to those necessary to test only one genotype leading to a considerable saving of time and costs.

Materials and methods

Primers design

In order to design primer pairs, two alignments were generated, one for the RNA1 and another one for RNA2 viral genome segments. Both alignments included RGNNV and SJNNV genotypes and the reassortant strains (RGNNV/SJNNV and SJNNV/RGNNV). Sequences were obtained from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and aligned with Clustal W implemented in the BioEdit software (http://www.mbio.ncsu.edu/ BioEdit/bioedit.html). Several primers in their forward and reverse forms targeting RGNNV-RNA1, RGNNV-RNA2, SJNNV-RNA1 and SJNNV-RNA2 were designed and combined together and with other 7 primers already described in the literature (Nishizawa et al., 1994; Toffolo et al., 2007).

Optimal primers were selected according to their position within each genotype's RNA sequence, melting temperature (Tm) and generated amplicon size. Primer pairs were initially tested in different PCR conditions (singleplex, duplex and triplex) and finally under RT-PCR conditions (one-step multiplex RT-PCR).

The screening, the optimization of primers and the thermal cycle setup were conducted using already characterised NNV strains: It/351/Sb (RGNNV) isolated from *D. labrax* (Ciulli et al., 2006); 416-Dec17 (RGNNV/SJNNV) isolated from *S. aurata* larvae (Volpe et al., 2020). The strains 389/I96 (SJNNV/RGNNV) and 484.2.2009 (SJNNV) were kindly provided by Dr Anna Toffan of IZSVe and were isolated from *D. labrax* and *S. senegalensis* respectively (Panzarin et al., 2012; Vendramin et al., 2014).

The viral strains were cultivated on SSN-1 cell line monolayer using 25 cm^2 culture flasks (Frerichs et al. 1996) to produce virus for viral titration and for RNA extraction. Flasks were incubated at 20°C± 1°C (SJNNV and SJNNV/RGNNV) and at 25 ± 1°C (RGNNV and RGNNV/SJNNV) until cytopathic effects were observed.

RNA from each NNV strain was extracted from viral supernatants using NucleoSpin®RNA (Macherey-Nagel GmbH & Co., Germany) according to manufacturer's instructions and stored at -80°C until use.

Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized from the previously extracted RNA from each reference strain (It/351/Sb, VIR416/18, 389/l96 and 484.2.2009) using the Applied Biosystems™

High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, US) according to the manufacturer's instructions. The cDNA obtained was used in the PCR assays or stored at -20°C.

Reaction setup and optimization

First, new primer pairs were individually tested under PCR conditions (singleplex) by using a temperature gradient (58°C, 60°C and 62°C). The PCR reaction mixture contained 10x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM for each primer, 1.25 U of Taq DNA polymerase, nuclease-free water and 1µl cDNA in a final volume of of 25µl for each PCR reaction (Invitrogen™ Taq Polymerase, Recombinant; Invitrogen, USA). The thermal cycling conditions were 95°C for 3 min, followed by 40 amplification cycles of 95°C for 30s, 58°C, 60°C or 62°C for 30s and 72°C for 30s. A final extension was performed at 72°C for 10 min. To avoid any cross-contamination, negative controls were run along with all reactions. PCR products obtained from these reactions were visualized through gel electrophoresis.

The optimal primer pairs resulted from single PCR assays were tested under multiplex PCR conditions (duplex and triplex PCR) by using the same mixture used for the single PCR assay except for the primers concentrations that were different for each primer sets. The thermal cycle conditions were the same used in the single PCR test screening. A total of 3 duplex PCR reactions, targeting RGNNV-RNA1 and SJNNV-RNA2, were performed to test primers combinations. Optimal primer pairs that shown well defined and specific band product in the duplex PCR assays were used to test a third primer combination targeting RGNNV-RNA2. Two triplex PCR reactions were performed adding a third primer pair selected on the basis of its amplicon and its similarity in Tm regarding with the other primer combination used.

The primer pairs candidates resulted from the prior PCR trials were tested with a one-step multiplex RT-PCR by using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA). To avoid any cross-contamination, negative controls were run along with all reactions. The amplified products were visualized through gel electrophoresis.

Evaluation of the Multiplex RT-PCR sensitivity

In order to determine the Limit of Detection (LOD) of the developed one-step multiplex PCR method, the viral titre for each reference strain was calculated. Titrations of the viruses were performed according to the end-point dilution method. Briefly, ten-fold serial dilutions (10^{-2} to 10^{-9}) of viral solutions were inoculated onto 24h-old SSN-1 cell cultures in 96-well plates and incubated at 20 ± 1°C (SJNNV and SJNNV/RGNNV) and 25 ± 1 °C (RGNNV and RGNNV/SJNNV). The viral adsorption period was of 1h for each strain. Daily readings were carried out up to one-week post-inoculation. The titre was expressed as $TCID_{50}$ 0.1 ml^{-1} and calculated using the Spearman-Karber method. Limits of detection for the one-step multiplex RT-PCR were conducted individually for each reference strain using extracted RNA from at least five viral dilutions.

Results

Primer design and PCR assays

Fifty-two pairs of primers targeting RGNNV-RNA1, RGNNV-RNA2, SJNNV-RNA1 and SJNNV-RNA2 were designed and combined together with other 7 primer pairs already described in the literature (Nishizawa et al., 1994; Toffolo et al., 2007). In order to proceed with the PCR-based

assays thirteen primers pairs combinations were selected according to their position within each genotype's RNA sequence, Tm and amplicon size (**Table 9**).

Table 9. ***Tested primer combinations for Betanovirus multiplex RT-PCR. For each primer target, name and sequence are reported. For each combination amplicon position and size are reported.***

Target	Combination number	Primers	Tm (°C)	Sequences (5'→3')	Reference	Position	Amplicon (bp)
RG_RNA1	1	RG_Fw1_RNA1	58.8	GACTCAGATCCAGCGGGAA	This study	247-414	169
		RG_Rev6_RNA1	60.0	TCCAACCTCACGGGGTGAT	Toffolo et al., 2007		
	2	RG_Fw1_RNA1	58.4	GACTCAGATCCAGCGGGAA	This study	247-655	410
		VNNV8	50.7	CAGCAACACGGTAGTG	Toffolo et al., 2007		
	3	RG_Fw3_RNA1	58.0	CACCAAGCTCGGTATTGCG	This study	798-1150	354
		VNNV6	58.4	ACCGGCGAACAGTATCTGAC	Toffolo et al., 2007		
	4	RG_Fw5_RNA1	50.6	GCGCAACAACATTGC	This study	972-1150	180
		VNNV6	58.4	ACCGGCGAACAGTATCTGAC	Toffolo et al., 2007		
RG_RNA2	5	RG-SPCF2_RNA2	50.6	TGACACAAGGTTCCCTGT	This study	695-1041	348
		R3	58.4	CGAGTCAACACGGGTGAAGA	Nishizawa et al., 1994		
	6	RG_SPCF1b_RNA2	54.5	CAATCGTCGGCGTAGTAATC	This study	107-711	606
		RG_SPCFRev2_RNA2	54.1	ACAGGGAACCTTGTGTCA			
	7	RG_SPCF1b_RNA2	54.5	CAATCGTCGGCGTAGTAATC		107-761	656
		RG_SPCFRev3b_RNA2	57.7	AGGAGGATGGACTTGAAGTC			
	8	RG_SPCF1_RNA2	54.6	TAACAATCGTCGGCGTAGTAA		110-1041	933
		R3	58.4	CGAGTCAACACGGGTGAAGA	Nishizawa et al., 1994		
SJ_RNA1	9	SJ_SPCF1_RNA1	53.5	CACGAGTATCCAGAAGCAG	This study	247-655	410
		VNNV8	50.7	CAGCAACACGGTAGTG	Toffolo et al., 2007		
	10	SJ_SPCF1_RNA1	53.5	CACGAGTATCCAGAAGCAG	This study	247-800	555
		SJ_SPCFRev2_RNA1	53.6	AGATAGAGGTAGCCACACAAA			
	11	SJ_SPCF1_RNA1	53.5	TTTGTGTGGCTACCTCTATCT		779-1291	514
		SJ_SPCFRev6_RNA1	62.6	AGGGGTCGATGTCGTACGTGTC			
SJ_RNA2	12	SJ_F2_RNA2	58.8	ATTACTACCCAGGCGCCAC	This study	691 - 1041	352
		R3	58.4	CGAGTCAACACGGGTGAAGA	Nishizawa et al., 1994		
	13	SJ_F7_RNA2	57.1	TGTCACTGACAAACCGTTGC	This study	804 - 1041	239
		R3	58.4	CGAGTCAACACGGGTGAAGA	Nishizawa et al., 1994		

Primer pairs were tested individually with different Ta gradient (58°C, 60°C and 62°C) and 60°C was chosen as best Ta. In fact, for some primer pairs non-specific bands were observed when primers were tested at 58°C and 62°C. Furthermore, the reaction conducted at Ta of 62°C showed positive fainted bands for some primer combinations (combination 1).

Primers pairs that showed non-specific bands were rejected and were not tested under multiplex PCR conditions. Consequently, nine (9) primer combinations were tested in duplex and

triplex PCR to determine the primer pair's behaviour under multiplex conditions testing different PCR parameters (primer and $MgCl_2$ concentrations).

Duplex and Triplex PCR

A total of three (3) duplex PCR reactions, targeting RGNNV-RNA1 and SJNNV-RNA2, were performed to test primers combinations. Duplex 1 was used to test combination between primer pairs 1 and 12; Duplex 2 was used to test combinations 4 and 12; Duplex 3 was used to test combinations 2 and 13.

The Duplex PCR assays showed that both Duplex 1 and 2 produced well defined and intense amplicon band of the expected size (169bp for primer pair 1, 180 bp for primer pair 4 and 352bp for primer pair 12) and also did not produce any non-specific bands. Consequently, these primer pairs were selected to test a third primer combination in the triplex PCR assays. On the contrary, Duplex 3 was rejected because the reaction did not amplify all the targeted amplicons and also a non-specific band was observed in the RGNNV sample (It/351/Sb) at approximately 239bp.

The first Triplex amplification assay produced a non-specific band at approximately 352bp in the RGNNV sample (It/351/Sb) and a fainted band at 180bp when using 1.5mM $MgCl_2$. Consequently, and in order to increase the primers' specificity, the same reaction was tested by using different $MgCl_2$ concentrations (2.0 mM and 2.5 mM).

Triplex 1 was performed by using 2.5mM in $MgCl_2$ concentration and produced well defined and intense bands of the expected size (180 bp for primer pair 4, 606bp for primer pair 6 and 352 for primer pair 12) while non-specific bands were absent. Following up on these results, it was decided to initially test Triplex 2 with 2.5mM $MgCl_2$. Triplex 2 amplified all the targeted amplicons, however, due to the low intensity of the amplified bands in the SJNNV/RGNNV sample (389/196) in comparison with the other samples (RGNNV, RGNNV/SJNNV and SJNNV), it was decided to increase the $MgCl_2$ concentration up to 3.5mM.

One-step multiplex RT-PCR assay

A one-step multiplex RT-PCR targeting RGNNV-RNA1, RGNNV-RNA2 and SJNNV-RNA2 was setup to detect and identify simultaneously the presence of RGNNV, SJNNV or one of their reassortant strains (RGNNV/SGNNV, SJNNV/RGNNV). The optimized reaction consisted of 15 µl of reaction mix containing 3 µl RNA, 2X Reaction Mix SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, USA), 0.45µM RGNNV-RNA1 primer, 0.3µM RGNNV-RNA2 primer, 0.2µM SJNNV-RNA2 primer and 0.3 µl Superscript III Platinum Taq enzyme mix. The thermal cycling conditions were 45°C for 30 min, 94°C for 2 min, followed by 40 amplification cycles of 95°C for 30s, 60°C for 30s and 30s at 72°C. A final extension was performed at 72°C for 10 min.

No non-specific products were amplified and the amplicons produced clear and intense bands (**Figure 2**), rendering this reaction optimal for the identification of RGNNV and the reassortant RGNNV/SJNNV strain and to distinguish them from SJNNV and the reassortant SJNNV/RGNNV strains.

Determination of the Limit of detection (LOD)

The viral titre ($TCID_{50}$ ml^{-1}) was calculated for each reference strain (RGNNV, RGNNV/SJNNV, SJNNV and SJNNV/RGNNV) in order to determine the LOD of the method. The infectious titres of the produced viral solutions were $10^{7.4}$, $10^{5.8}$, $10^{6.3}$ and $10^{4.2}$ $TCID_{50}$ 0.1 ml^{-1} for RGNN, SJNNV, RGNNV-SJNNV and SJNNV/RGNNV, respectively (

Figure 3). The analysis of tenfold dilution of titrated viral solutions evidenced a similar sensitivity for three out of four target viruses (**Table 10**).

The highest sensitivity was to the detection of the RGNNV/SJNNV strain. The mRT-PCR assay was able to detect the viral RNA extracted from the fifth tenfold dilution of a solution with a titre $10^{6.3}$ TCID50 0.1 ml^{-1} corresponding to an inferred titre of $10^{1.3}$ TCID50 0.1 ml^{-1}. For RGNNV, SJNNV and SJNNV/RGNNV, the mRT-PCR was able to detect the viruses into dilutions with inferred titres of $10^{2.4}$, $101^{.8}$ and $10^{2.2}$ TCID50 0.1 ml^{-1}, respectively.

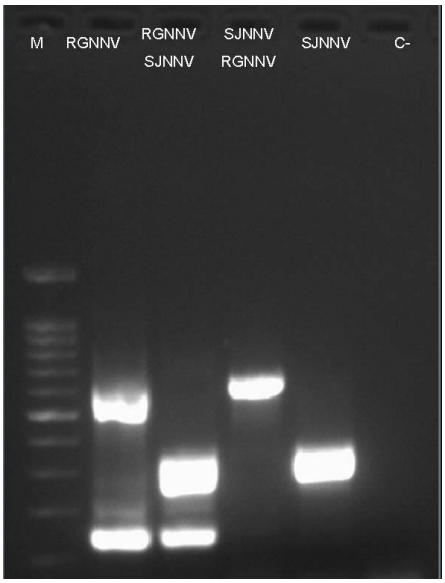

Figure 2. One-step multiplex RT-PCR targeting RGNNV-RNA1 (169 bp), RGNNV-RNA2 (655 bp) and SJNNV-RNA2 (351 bp). M: 100 bp molecular marker (Invitrogen). C-: negative control.

Table 10. ***Limit of detection of mRT-PCR was determined on tenfold dilution of viral solutions***

Dilution factor	RGNNV It/351/Sb		SJNNV 484.2.2009		RGNNV/SJNNV Sa-416-Dec17		SJNNV/RGNNV 389/I96	
	mRTPCR	Virus titre	mRTPCR	Virus titre	mRTPCR	Virus titre	mRTPCR	Virus titre
10^{-0}	+	$10^{7.4}$	+	$10^{5.8}$	+	$10^{6.3}$	+	$10^{4.2}$
10^{-1}	+	$10^{6.4}$	+	$10^{4.8}$	+	$10^{5.3}$	+	$10^{3.2}$
10^{-2}	+	$10^{5.4}$	+	$10^{3.8}$	+	$10^{4.3}$	+	$10^{2.2}$
10^{-3}	+	$10^{4.4}$	+	$10^{2.8}$	+	$10^{3.3}$	-	$10^{1.2}$
10^{-4}	+	$10^{3.4}$	+	$10^{1.8}$	+	$10^{2.3}$	-	$10^{0.2}$
10^{-5}	+	$10^{2.4}$	-	$10^{0.8}$	+	$10^{1.3}$	n.d	n.d.
10^{-6}	-	$10^{1.4}$	-	$10^{0.08}$	-	$10^{0.3}$	n.d	n.d.
10^{-7}	-	$10^{0.4}$	n.d	n.d.	-	$10^{0.03}$	n.d	n.d.
10^{-8}	-	$10^{0.04}$	n.d	n.d.	-	$10^{0.003}$	n.d	n.d.

Discussion and conclusion

In this study a one-step multiplex RT-PCR targeting RGNNV-RNA1, RGNNV-RNA2, SJNNV-RNA1 and SJNNV-RNA2 was successfully developed. The method was able to detect the presence of NNV but also to identify RGNNV genotype and reassortant strain RGNNV/SJNNV and to differentiate them from the SJNNV genotype and the reassortant strain SJNNV/RGNNV in a single PCR reaction. The absences of non-specific products being amplified in the reaction, makes this a highly specific reaction. The method showed a quite high sensitivity in accordance with the detection limit reported for other mRT-PCR assays (Yoshinaka and Yoshimizu, 1998; Pinheiro et al., 2016) and it is suitable to be applied to confirm VNN during mortality field outbreaks in European sea bass and gilthead sea bream. Both two-step (PCR) and one-step (RT-PCR) methods have been tested. The one-step method provides a more rapid and simple method, reducing the risk of cross-contaminations.

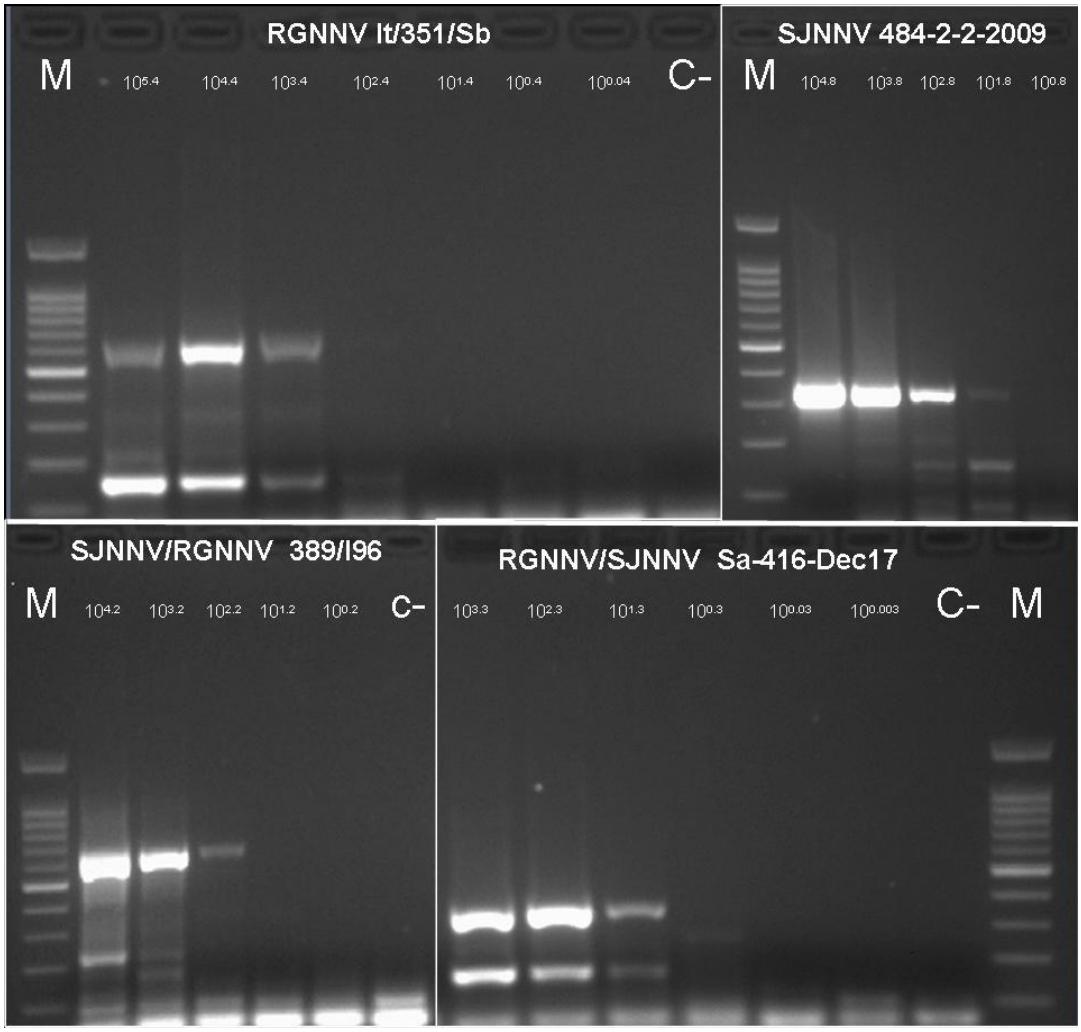

Figure 3. ***Limit of detections established for the reference strains testing ten-fold serial dilutions of viral titred solutions. M: 100 bp molecular marker (Invitrogen). C-: negative control.***

The simultaneous detection of several viral targets with a multiplex PCR approach is relatively rapid and cost effective compared with the standard virological/molecular assays. The mRT-PCR assay developed was conducted in a mix reaction volume equivalent to that of a singleplex; in this way, the cost and the time of execution of the test for the simultaneous detection and identification of NNV and its variants circulating in Southern Europe are equivalent to those necessary to test only one genotype leading to a considerable saving of time and costs.

In conclusion, the results obtained in this study demonstrate that the developed multiplex RT-PCR method was able to efficiently detect and identify the viral RNA in infected cell culture supernatants of each reference strain showing its usefulness as rapid identification technique. A selection of field samples already characterized was ongoing in the last months before the research activity was stopped due to covid-19 pandemia. Selected and new field samples will be tested as soon as the research activity resumes; this activity will be carried out together with WP6 activities that already include the integration of developed diagnostic methods in operational environments.

5.2 Improvement of characterization and identification of *Vibrio anguillarum* and biosensor

The rapid and correct identification of *Vibrio anguillarum* in the initial phase of an outbreak is of fundamental importance for taking appropriate and effective control measures. Traditional methods based on culturing used for detecting this bacterial pathogen, although necessary for conducting antibiotic sensitivity tests, require various days to conclude identification at species level. Among the number of techniques that have been set up in the past and described in section 4.2, molecular methods can meet the requirements of rapid and specific detection since in 24 hours it is possible to obtain results. Tsukamoto *et al*. (1993) proposed a PCR with primers annealing 16S rDNA, Thompson *et al*. (2004) the *recA* gene, and the *gyrB* gene to identify *V. anguillarum* using a nested PCR. Furthermore, nanotechnologies represent an important tool to achieve the expected goals of rapidity and specificity needed in microbiological analyses for pathogen detection. Recent advances in nanotechnologies render them useful for the development of new devices and protocols for the direct detection of *V. anguillarum* in fish.

With this aim, the work focused on the design of new primers to detect specifically *V. anguillarum* by qPCR techniques and on the design of a specific ssDNA probe to develop a bioassay, based on the utilization of an electrochemical biosensor, a portable device which has the advantage of allowing the sensitive and specific analyses of samples in field, avoiding the need to bring samples to the laboratory.

Materials and methods

Bacteria strains

Bacterial strains listed in

Table *11* were recovered for the experiments. The first step consisted in the revitalization of the bacteria from -80°C. Briefly, the cells were streaked onto specific media depending on the bacteria species:

- Tryptone Soya Agar (TSA, Oxoid, Milan, Italy) supplemented with 2.5% NaCl at 30°C for 48 h for *Vibrio anguillarum, V. ordalii, V. parahaemolyticus, V. harveyi, Photobacterium damselae* subsp. *piscicida*;
- Brain Heart Infusion (BHI, Oxoid, Milan, Italy) at 37°C for 24 h for *Escherichia coli, Salmonella enterica, Staphylococcus aureus*;
- BHI at 30°C for 48 h for *Aereomonas hydrophila;*
  de Man Rogosa Sharpe (MRS, Oxoid, Milan, Italy) at 30°C for 48-72 h in microaerophilic conditions for lactic acid bacteria.

DNA Extraction

Pure colonies were used for DNA extraction by a mechanical breakage of cells with glass beads, followed by breaking buffer (2% Triton, 1% SDS, 100 mM NaCl, 10 mM tris-HCl, 1 mM EDTA), TE (10 mM Tris, 1 mM EDTA, pH 8, and phenol/chloroform/isoamyl alcohol solutions (SigmaAldrich, Milan, Italy. The supernatant was collected and precipitated with absolute ethanol at 20°C and washed with 70% ethanol. After drying, the DNA was re-suspended in sterile distilled water, treated with RNase, and stored at -20°C. DNA integrity was evaluated by running 5 µl of the extracted DNAs in a 1.5 % agarose gel at 120 V for 45 min, stained with ethidium bromide (0.5 µg/ml; Sigma-Aldrich, Milan, Italy), and visualized under UV light; the image was acquired with bioimaging system GeneGenius (SynGene, Cambridge, England). **Figure 4** shows the results about the integrity of the extracted DNAs.

The samples extracted showed a good concentration of DNA a part for *Lactobacillus curvatus* DSMZ 20019 and *Photobacterium damselae* subsp*. piscicida* B 07/8 DI4 that were of 91.9 and 118.5 ng/µl. The ratio A260/280 was good and ranged from 1.68 to 2.13 (

Table *11*).

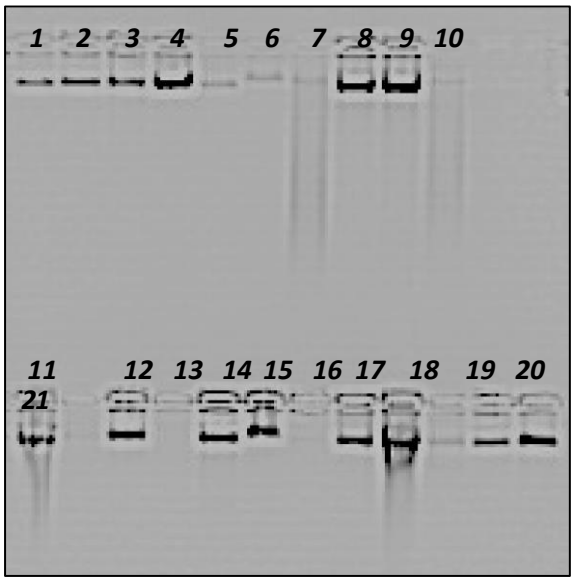

Figure 4.*Integrity of the DNA extracted from bacteria listed in Table 11.*

Line 1: V. anguillarum 117/2013 2 DIMEVET*; Line 2: V. anguillarum 117/2013* DIMEVET*; Line 3: V. anguillarum 199/2016 A* DIMEVET*; Line 4: V. anguillarum 199/2016 A* DIMEVET*; Line 5: V. anguillarum 130/2015* DIMEVET*; Line 6: V. anguillarum 130/2015* DIMEVET*; Line 7: V. anguillarum 189/2013* DIMEVET*; Line 8: V. anguillarum 189/2013* DIMEVET*; Line 9: V. anguillarum 188/2015* DIMEVET*; Line 10: V. anguillarum 188/2015* DIMEVET*; Line 11: Potobacterium damselae subsp. piscicida B07/8 DI4A*; *Line 12: St. aureus* DI4A; *Line 13:/; Line 14: V. prahaemolyticus 10027* DSMZ*; Line 15: V. ordalii 19621* DSMZ*; Line 16:/; Line 17: Aeromonas hydrophila 30187* DSMZ; *Line 18:*

Bacillus cereus 2301 DSMZ; *line 19: Lb. brevis* 20054 DSMZ*; Line 20: Salmonella enterica 9386* DSMZ*; Line 21: Escherichia coli 3* DISTAM.

Table 11. ***Bacteria strains used, their source and DNA concentrations and quality***

Bacteria	Source	NA ng/µl	A260/A280 nm
Vibrio anguillarum	251/2016 A DIMEVET*	924.9	2.04
Vibrio anguillarum	117/2013 DIMEVET	590	2.05
Vibrio anguillarum	199/2016 DIMEVET	3,029.9	2.06
Vibrio anguillarum	130/2015 DIMEVET	4,628.3	2.11
Vibrio anguillarum	189/2013 DIMEVET	1,002.6	1.98
Vibrio anguillarum	188/2015 DIMEVET	3,545.2	2.02
Vibrio anguillarum	4/2015 B DIMEVET	2,310.9	2.13
Vibrio anguillarum	21597 DSMZ°	2,916.8	2.13
Vibrio anguillarum	32/TT DI4A **	700.1	1.85
Vibrio ordalii	19621 DSMZ	103.30	1.47
Vibrio parahaemolyticus	10027 DSMZ	597.9	1.83
Photobacterium damselae subsp. ***piscicida***	B 07/8 DI4A	118.5	1.81
Staphylococcus aureus	DI4A	475.8	1.78
Lactobacillus curvatus	20019 DSMZ	91.9	1.68
Aeromonas hydrophila	30187 DSMZ	1,074.8	2.11
Bacillus cereus	2301 DSMZ	976.4	2.07
Lactobacillus brevis	20054 DSMZ	738.2	1.89
Salmonella enterica	9145 DSMZ	1,705.1	1.98
Escherichia coli	3 DISTAM§	641.4	1.79

DIMEVET Dipartimento di Scienze Mediche e Veterinarie, Università di Bologna (Italy);*
DISTAM§ Department of Science and Food Technology and Microbiology (Milan, Italy);
DSMZ° Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweigh, Germany);
*DI4A** Dipartimento di Scienze e Tecnologie Alimentari dell'Università degli Studi di Udine (Udine, Italy).*

5.2.1 Tests on GroEL gene

Primers, PCR and qPCR

The *GroEL* gene, which encodes the chaperonin GroEL, was selected as a target for the design of the primers to use in the specific detection of *V. anguillarum*. *GroEL* sequences from *Vibrio* and non-*Vibrio* enteric species were retrieved from GenBank using the software provided by NCBI

(https://www.ncbi.nlm.nih.gov). Sequences were aligned with the software MultAlin (Multiple sequence alignment with hierarchical clustering, Corpet, 1988) to evaluate traits with high variability that were compared with sequences of bacteria used as negative controls. The characteristics of the designed primers were tested using Oligo Analyzer 3.1 IDT (https://eu.idtdna.com/calc/analyzer) and an *in-silico* PCR was performed with Amplifix1.7.0 (Jullien, 2013) in order to evaluate primer dimer formation, to confirm primers specificity, and to predict the amplicon size.

The primers named VibrioFW and VibrioRV (**Table 12**) were expected to produce an amplicon of 193 bp, as calculated by the software *in silico* amplification using Amplifix 1.7.0 on *Vibrio anguillarum* strain JWA3 (Accession No KX528017.1). Only the target species *Vibrio anguillarum* among the bacteria listed in

Table *11* produced amplification with the *in-silico* tests, indicating that the new primer pair as suitable for specific detection of *Vibrio anguillarum* by PCR analysis.

Table 12. *Primers designed for Vibrio anguillarum*

Primers	Tm (°C)	%GC	Length (bp)
VibrioFW	56	47	23
VibrioRV	58	57	21

PCR with primers VibrioFW and VibrioRV

To evaluate specificity and sensitivity of the newly designed primers, VibrioFW-VibrioRV were used in PCR using as target 1 µl DNA of bacteria listed in

Table *11* after standardization at 100 ng/µl. Annealing temperature and $MgCl_2$ concentration were optimized to improve PCR specificity and reaction yield. To assess sensitivity, decimal dilutions (from 100 ng/µl to 1 fg/µl) of the DNA of *Vibrio anguillarum* DSMZ 21597 were used in PCR. The reaction mixture contained 5 µl AmpliTaq® Buffer 10x (Applied Biosystems, Foster City, CA, USA), 1 µl MgCl2 1.5 mM (Applied Biosystems), 1 µl PCR Nucleotide Mix 10 mM each (Applied Biosystems), 1µl of each primer (VibrioFW and VibrioRV at 10 µM), 0.25 µl AmpliTaq® DNA Polymerase 5 units/µl (Applied Biosystems).

In each assay, a blank control called NTC (no-template control) was included, in which the template DNA was replaced with an equal volume of nuclease-free water. The cycling protocol consisted in an initial denaturation step at 95°C for 5 min, 30 cycles of denaturation at 95°C for 15 sec, annealing at 54°C for 15 sec, and extension at 72°C for 15 sec, followed by a final elongation at 72°C for 5 min. PCR products were resolved in 1.5 % agarose gel at 120 V for 40 min, stained with ethidium bromide (0.5 µg/ml). The image was acquired with bio-imaging system GeneGenius (SynGene, Cambridge, England).

Vibrio-FW and Vibrio-RV, tested in end-point PCR showed specificity for *Vibrio anguillarum* after optimization of the amplification protocol, the annealing temperature and $MgCl_2$ concentration in the reaction mixture. The annealing temperature of 54°C was tested with the $MgCl_2$

concentrations of 1.5 and 2 mM on DNA of *V. anguillarum* DSMZ 21597, *V. anguillarum* 32TT D4A, and *V. anguillarum* 251/2016 A (**Figure 5**).

The concentration of 2mM $MgCl_2$ produced a good yield and was selected for verifying specificity on the bacteria listed in Table 11. As reported in **Figure 6**, only *V. anguillarum* strains produced the expected amplicon of 193 bp. DNA from strains used as negative controls were not amplified.

Vibrio-FW and Vibrio-RV were tested for sensitivity varying the DNA template concentrations (*V. anguillarum* DSMZ 21597) from 100 ng to 1 fg. The detection limit obtained was of 1 pg as shown in **Figure 7**.

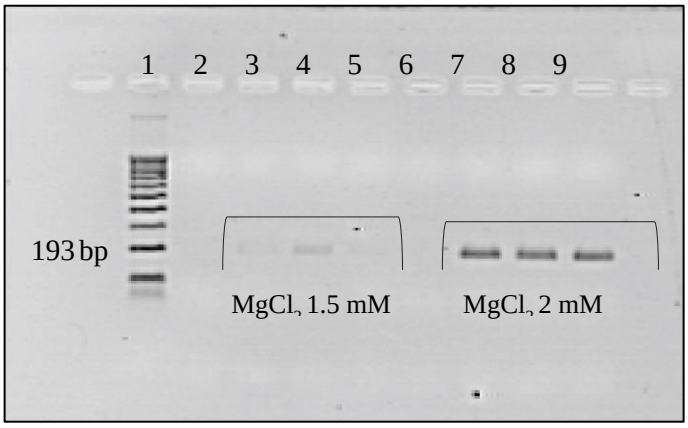

Figure 5. Agarose gel electrophoresis of V. anguillarum PCR products obtained using VibrioFW-VibrioRV primers. Ta 54°C, $MgCl_2$ 1.5 mM and 2 mM. Line 1: 100bp DNA ladder (Promega); Line 2: negative control; Line 3: V. anguillarum 21597 DSMZ; Line 4: V. anguillarum 32/TT DI4A; Line 5: V. anguillarum 251/2016 A DIMEVET; Line 6: negative control; Line 7: V. anguillarum 21597 DSMZ; Line 8: V. anguillarum 32/TT DI4A DI4A; Line 9: V. anguillarum 251/2016 A DIMEVET.

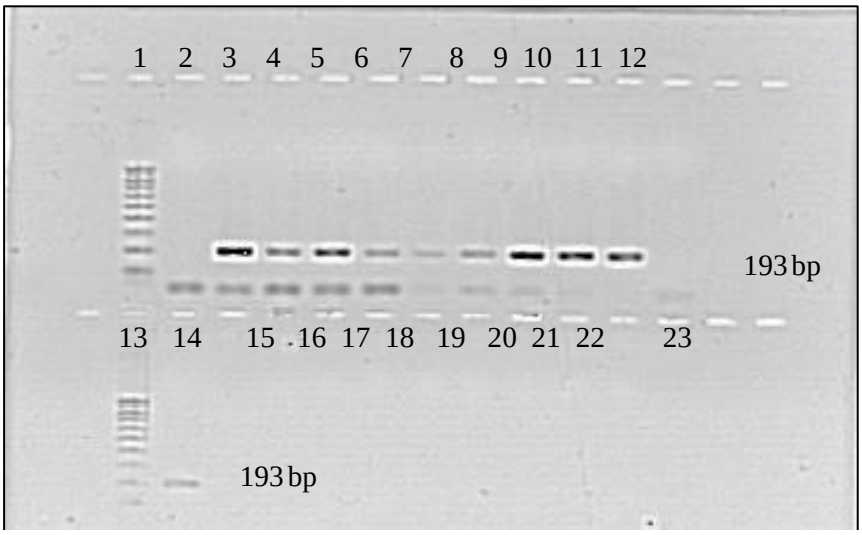

Figure 6. Agarose gel electrophoresis of V. anguillarum PCR products obtained using VibrioFW- VibrioRV. Ta 54°C, $MgCl_2$ 2 mM. Linea 1: 100bp DNA ladder (Promega); Linea 2: Negative Control; Linea 3: V. anguillarum 251/2016 A DIMEVET; Line 4: V. anguillarum 117/2013 DIMEVET; Line 5: V. anguillarum 199/2016 A DIMEVET; Line 6: V. anguillarum 130/2015 DIMEVET; Line 7: V. anguillarum 189/2013 DIMEVET; Line 8: V. anguillarum 189/2013 DIMEVET; Line 9: V. anguillarum 188/2015 DIMEVET; Line 10: V. anguillarum 4/2015 B DIMEVET; Line 11: V. anguillarum 21597 DSMZ; Line 12: Ph. dmaselae subsp. piscicida B07/8 DI4A; Line 13: 100bp

DNA ladder (Promega); Line 14: V. anguillarum 32/TT DI4A; Line 15: St. aureus DI4A; Line 16: V. parahaemolyticus 10027 DSMZ; Line 17: A. hydrophila 30187 DSMZ; Line 18: B. cereus 2301 DSMZ; Line 19: Lb. brevis 20054 DSMZ; Line 20: Salmonella enterica 9145 DSMZ; Line 21: E. coli 3 DISTAM; Line 22: V. ordalii 19621 DSMZ.

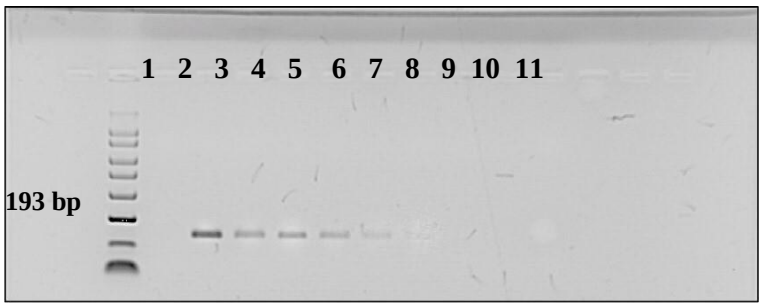

Figure 7. PCR sensitivity of VibrioFW-VibrioRV using decimal dilutions of DNA of V. anguillarum 21597 *DSMZ*. Ta 54°C, $MgCl_2$ 2 mM. Line 1: 100 bp DNA ladder (Promega); Line 2: NTC; Line 3: 100 ng/µl DNA; Line 4: 10 ng/µl DNA; Line 5: 1 ng/µL DNA; Line 6: 100 pg/µL DNA; Line 7: 10 pg/µl DNA; Line 8: 1 pg/µl DNA; Line 9: 100 fg/µl DNA; Line 10: 10 fg/µl DNA; Line 11, 1 fg /µl DNA.

qPCR with primers VibrioFW and VibrioRV

VibrioFW and VibrioRV were tested in a protocol of qPCR to give quantitative results. A calibration curve was built using decimal dilutions of the DNA of *V. anguillarum* DSMZ 21597, from 100 ng/µl to 1 fg/µl. The threshold limit setting was performed in automatic mode. SsoFastTM EvaGreen® kit (Bio-rad, Irvine, CA, USA) was employed, according to the manufacturer's instructions. The reaction mixture contained the following reagents: 10 µl SsoFastTM EvaGreen® Supermix 2x, 1 µl of each primer at 10 µM. The final volume of 20 µl consisted in 13 µl of reaction mixture, 7 µl of nuclease-free water and 1 µl of template DNA at various concentrations. In each assay, a blank control called NTC was included. Samples were amplified in the Rotor Gene Q (Venlo, Limburg, Netherlands) and the thermal program applied consisted of 98°C hot start activation for 2 min, 40 cycles of 98°C denaturation for 5 sec and 60°C annealing/extension for 20 sec. A melting curve was constructed rising the temperature from 60 to 95 °C (+0.5°C/5 sec) to assess if the intercalating dye has produced single, specific products. **Table 13** shows the Ct values obtained for *Vibrio anguillarum* DSMZ 21597 at various concentrations. The detection limit was 100 fg, in fact dilutions lower than 100 fg didn't produce a detectable signal. The NTC confirms that there were no contamination.

The standard curve equation obtained can be used to calculate the concentration [C] of unknown DNA samples based on their CT value [C= 10^(-0.323*CT + 5.850) and R^2= 0.9902]. A slope of -3.094 corresponds to efficiency of 110.5%.

At the end of the amplification cycle melting curves analysis was performed in order to confirm that the fluorescence data obtained correspond to specific amplicons. The melting curves obtained confirmed that the measured fluorescence belonged to the amplicons of the target gene.

Table 13. Ct values, and DNA concentrations of serial dilution of Vibrio anguillarum *21597 DSMZ* used for the calibration curve

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
1		Bianco	NTC				
2		V.anguillarum 100 ng	Standard	12,38		100,000000	70,958527
3		V.anguillarum 10 ng	Standard	14,99		10,000000	10,106309
4		V.anguillarum 1 ng	Standard	18,26		1,000000	,892529
5		V.anguillarum 100 pg	Standard	20,84		,100000	,130550
6		V.anguilVlarum 10 pg	Standard	23,00		,010000	,026207
7		V.anguillarum 1 pg	Standard	27,74		,001000	,000766
8		V.anguillarum 100 fg	Standard	31,18		,000100	,000060
9		V.anguillarum 10 fg	Standard			,000010	
10		V.anguillarum 1 fg	Standard			,000001	

Detection of V. anguillarum from fish tissue after experimental infection

To evaluate the efficiency of the protocols used to detect/quantify *V. anguillarum* directly from fish samples a qPCR test was conducted using DNA extracted from European sea bass liver samples inoculated with three different concentrations of *V. anguillarum* DSMZ 21597 (10^2, 10^3, 10^4 cells/g).

Three fresh European sea bass were purchased from local supermarket and processed the same day of the analysis. The livers were extracted with the help of sterile scalpels, divided into 8 aliquots of 2 g each and stored in a Petri dish on ice up to the analysis.

Bacterial suspensions of *V. anguillarum* were prepared by diluting the pure culture in saline solution up to an initial value of optical density (OD_{600}) of 0.489 corresponding to 2.45 x 10^8 cells/ml. Subsequently, ten-fold dilutions were performed in saline-peptone water (8 g/l NaCl, 1 g/l bacteriological peptone; Oxoid) to reach cell densities of approximately 10^5, 10^4 and 10^3 cells/ml, which served as inocula. The suspensions were analysed by spreading 200 µl of each dilution onto TSA plates with 2.5% NaC to verify the cell/ml concentrations. 200 µl of each dilution was also used to inoculate the 2 g of liver (in double) and left to incubate 30 min at room temperature before the analyses. Non-inoculated liver sample was used as negative control. Inoculated liver samples were homogenized in 5 ml saline-peptone water, serially diluted and used for colony count on TSA plates. One ml of homogenate was used for DNA extraction as previously described for reference bacteria strains. One µl of DNA from each series was used as a template for qPCR with VibrioFW and VibrioRW primers.

No signals of amplification were detected for the samples at the three different inocula (10^2, 10^3 and 10^4 cell/g) of *V. anguillarum.* These results indicate that the amount of DNA used in qPCR was lower than the LOD of the test, 100 fg/µl. Although the amount of DNA estimated by using

the spectrophotometer was in the range of 40.8 ng/µl to 270.6 ng/ µl, the amount of DNA of *V. anguillarum* is unknown. Moreover, the liver contains high percentage of lipids that can affect the efficiency of the extraction thus a new extraction method has to be optimized to improve the sensitivity (low LOD) in field samples.

DNA probe designed and utilization on GroEL gene

GroEL gene was also selected as a target for the design of a DNA probe for the rapid and direct detection of *V. anguillarum.* Sequences were retrieved from GenBank. After alignments with MultAlin software (Corpet, 1988) the regions showing variability were detected and used for probe design, using the software OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer). The probe VIBRIO-DIG (Sigma-Aldrich Milan, Italy), tailed with digoxigenin at 5' end, was used in dot blot to tests its specificity before the biosensor development. A ssDNA sequence, complementary to the probe, was used as positive control in dot blot tests (**Table 14**).

Dot blot

Dot blot was applied in order to verify the specificity of the probe. The protocol was optimized by testing hybridization temperatures of 45, 43, and 41°C and concentrations of the saline-sodium citrate (SSC) buffer (used for the washing steps) of 1X, 05X and 0.1X.

The probe was tested on the sequence complementary to the probe (positive control) at 100, 10, 1 ng/µl, 100, 10, 1 pg/ µl, 100, 10 and 1 fg/µl. The specificity of the probe was tested on the DNAs extracted from the strains listed in Table 1 after standardization at 100 ng/µl.

Table 14. Characteristics of the VIBRIO probe

VIBRIO-DIG probe			5'-GAGATGGCGTTATCACTGTTGAGAAGGTCAAGCAC-3'	
Sequence Complementary to VIBRIO-DIG probe			5'-GTGCTTGACCTTCTTCAACAGTGATAACGCCATCTC-3'	
36 bp	GC (%) 47	Tm 65 (°C)	Self-dimer min ΔG (kcal/mole) -5.47	Hairpin min ΔG (kcal/mole) - 0,49

** According to software guide the ΔG conditions are not problematic (OligoAnalyzer 3.1, http://eu.idtdna.com/calc/analyzer).*

After DNA denaturation at 95°C for 10 min, 1 µl of each DNA sample was spotted onto a positively charged nylon membrane (Zeta-Probe GT; Bio-Rad, Irvine, CA, USA) and cross-linked by exposure to UV light (254 nm) for 10 min. The membrane was first pre-hybridized in Dig Easy Hyb buffer (Roche Diagnostics, Mannheim, Germany) at the selected hybridization temperature and after hybridized overnight at the same temperature after addition of the VIBRIO-DIG probe at final concentration of 100 ng/µl.

The non-hybridized probe was removed by serial washing steps: twice with 2X SSC (Promega) with 0.1% (w/v) SDS at room temperature (RT) for 5 min shaking, twice with 1X SSC with 0.1% (w/v) SDS at RT for 15 min shaking, once with 1X washing buffer (Roche Diagnostics) at RT for 10 min shaking.

The membrane was then incubated with fresh prepared 1X blocking solution (Roche Diagnostics) at RT for 30 min shaking and subsequently with antibody solution AntiDIG-AP diluted in

blocking solution (Roche Diagnostics) at RT for 30 min shaking. The membrane was washed again twice with 1X washing buffer for 15 min shaking and neutralized with 1X detection buffer (Roche Diagnostic) for 5 min. For the detection of the probe-target hybrid, the membrane was incubated with the detection color solution (NBT/BCIP stock solution diluted in 1X detection buffer, 100 µl for 5 ml; Roche Diagnostics) in the dark and without shaking. After 50 min, the membrane was rinsed with sterile water to stop the reaction and visualized.

The sensitivity of VIBRIO-DIG probe at the concentration of 100 ng/µl was 10 pg (**Figure 8**), spot 2B) using the complementary sequence as a positive target. After testing SSC buffer at 1X, the best results were obtained using the SSC buffer 0.5X, at the temperature of 45°C selected for hybridization, as visible in spots reported in Figure 8. No visible spots for concentrations of 1 pg (2C), 100 fg (3A), 10 fg (3B) and 1 fg (3C).

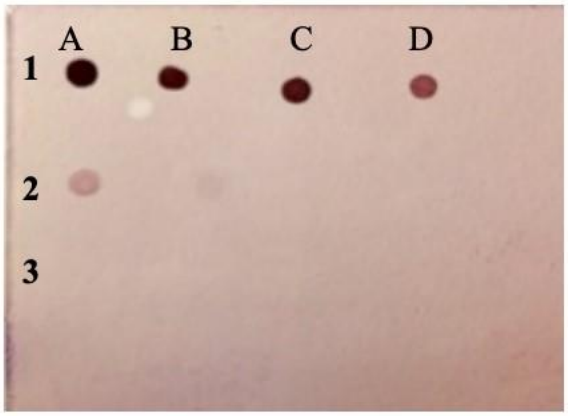

Figure 8. Sensitivity of VIBRIO-DIG probe at 100 ng/µl, hybridization temperature 45°C, using as target 1 µl of the positive control (sequence complementary to the probe) at the concentrations of 100 ng (1A), 50 ng (1B), 10 ng (1C), 1 ng (1D), 100 pg (2A), 10 pg (2B), 1 pg (2C), 100 fg (3A), 10 fg (3B) and 1 fg (3C).

After the optimization of the hybridization conditions using the sequence complementary to the VIBRIO-DIG probe, the probe at 100 ng/µl was tested at 45°C with SSC 0.5X on various strains of *V. anguillarum* from international collections, isolated from fish, to ensure the capability of the probe to hybridize different strains *of V. anguillarum*. dsDNA extracted and standardized at 100 ng/µl were spotted (1 µl) onto the nylon membrane. The hybridization temperature of 45°C was too stringent, in fact, none of the *V. anguillarum* strains tested produced the expected blue spot (**Figure 9**), thus it was lowered first to 43°C, and later to 41°C. The SSC buffer was tested at 0.5 X and 0.1X to ensure specificity.

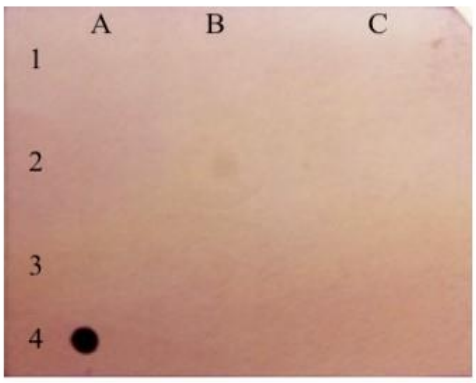

Figure 9. Results of specificity of VIBRIO-DIG probe at 100 ng/ µl using dsDNA of different strains of Vibrio anguillarum at 100ng/ µl. Hybridization T of 45°C, SSC 0.5X. Spot 1A: V. anguillarum 251/2016 A *DIMEVET*; 1B: V. anguillarum 117/2013 *DIMEVET*; 1C: V. anguillarum 199/2016 *DIMEVET*; 2A: V. anguillarum 130/2015 *DIMEVET*; 2B: V. anguillarum 189/2013 *DIMEVET*; 2C: V. anguillarum 188/2015 *DIMEVET*; 3A: V. anguillarum 4/2015 B *DIMEVET*; 3B: V. anguillarum 21597 *DSMZ*; 3C: V. anguillarum 32/TT *DI4A*; 4A: positive control; 4B: E. coli *3 DISTAM* (negative control).

As showed in Figure 10a, a spot was visible for *V. parahemolyticus* DSMZ 10027 (2C) and a faint spot for A*eromonas hydrophila* DSMZ 30187 (2D), both negative samples. The use of SSC solution at 0.1 X improved specificity, in fact, only *Photobacterium damselae* subsp*. piscicida* B07/8 produced a positive spot (**Figure 10b**, 3B).

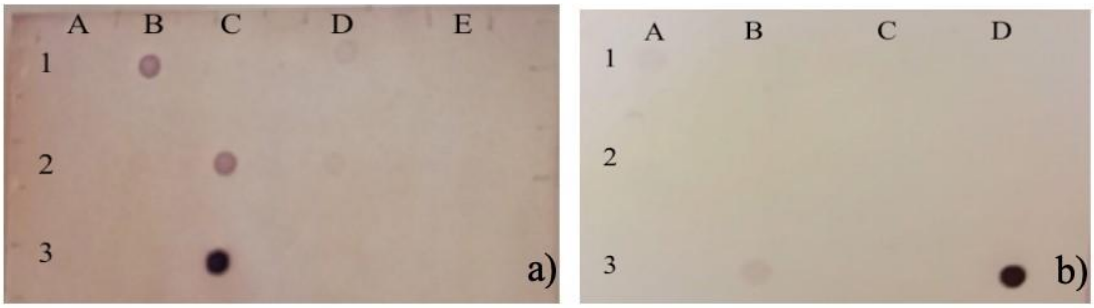

Figure 10. Dot blot specificity with VIBRIO-DIG probe at 100ng/ µl with positive and negative controls at 100ng/ µl at 41°C. a) SSC 0.5 X. Spots 1A: V. anguillarum 251/2016 A *DIMEVET* ; 1B: V. anguillarum 188/2015 *DIMEVET*; 1C: V. anguillarum 4/2015 B *DIMEVET*; 1D: V. anguillarum 21597 *DSMZ*; 1E: V. anguillarum 32/TT *DI4A*; 2A: Photobacterium damsaelae subsp. piscicida B07/8 *DI4A* ; 2B: Staphylococcus aureus *DI4A*; 2C: V. parahemolyticus 10027 *DSMZ*; 2D: Aeromonas hydrophila 30187 *DSMZ*; 2E: Bacillus cereus 2301 *DSMZ;* 3A: Lactobacillus brevis 20054 *DSMZ;* 3B: V. ordalii *19621 DSMZ*; 3C: positive control (sequence complementary to the probe). b) SSC 0. 1 X. Spots 1A: V. anguillarum 21597 *DSMZ*; 3B: Photobacterium damselae subsp. piscicida B07/8 *DI4A*; 3D: positive control (sequence complementary to the probe).

Nevertheless, the persistent positive signal of a negative control (*Photobacterium damselae* subsp*. piscicida* B07/8) after repeated tests, resulted in rejecting the probe designed on the *GroEL* gene for the development of a biosensors due to low specificity.

5.2.2 Tests on *tonB2* gene

To improve the sensitivity of primers and ensure specificity for *V anguillarum* an accurate bibliographical study was performed and the gene *tonB2*, was selected as target for the design of specific primers and a specific probe for *V. anguillarum.* The gene *tonB2* is essential to the transport of the siderophore anguibactin, required by an active iron uptake mechanism, associated with bacterial virulence, and it's present in *V. anguillarum* but absent in *V. ordalii*, a species very close to *V. anguillarum*. Sequences of the genome of microorganisms presenting this gene were retrieved from GenBank: *V. anguillarum, A. hydrophila* and *V. parahaemolyticus* (Chromosome 1 and 2). The sequences were aligned using the MultAlin software (Corpet, 1988), with the aim of identifying the regions of low similarity to be used for the design of the primers.

Design of new primers specific for V. anguillarum

The primers VaFw and VaRw were manually designed and tested *in silico* with the software AmplifX 1.7.0 on the genomic sequences of bacteria listed in Table 15, retrieved from GenBank. AmplifX 1.7.0 gives some characteristics of the primers: the melting temperature (Tm), the percentage of guanine and cytosine of the primer, the 3' end stability, degeneracy statistics with poliX and self-dimers (**Table 15**).

Table 15. Parameters of the primers VaFw and VaRw

	length (bp)	Tm (°C)	GC %	3' end stability	self dimer	amplicon (bp)
VaFw	19	56.5	47	2	10	159
VaRw	19	54.5	52	1	12	

Another tool used to verify the quality of the designed primers was OligoAnalyzer (https://eu.idtdna.com/calc/analyzer). The last step was a check using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) that provides the comparison between nucleotide primers or amplicons and the sequences present in nucleotide databases on-line.

PCR with primers VaFw and VaRw

The primers VaFw and VaRw, newly designed was tested by PCR as follows: the reaction mixture contained the following reagents: 5 µl AmpliTaq® Buffer 10X (Applied Biosystems, Foster City, CA, USA), 1 µl $MgCl_2$ 1.5 and 2 mM (Applied Biosystems), 1 µl PCR Nucleotide (dNTP's) Mix 10 mM (Applied Biosystems), 1µl of each primer (VaFw and VaRw at 10 µM), 0.25 µl AmpliTaq® DNA Polymerase 5 units/µl (Applied Biosystems), 1µl of DNA. A blank control called NTC was also included. The final volume of 50 µl was reached by adding nuclease-free water (Sigma-Aldrich). Cycling protocol: 95°C for 5 min, 30 cycles of 95°C for 15 sec, annealing (were used 52.5, 53 and 54 °C) for 15 sec, and extension at 72°C for 15 sec, followed by a final elongation at 72°C for 5 min.

PCR results

Considering the melting temperature of the primers VaFw and VaRw, an initial annealing temperature of 52.5°C and a concentration $MgCl_2$ of 1.5 mM were tested.
These conditions produced amplicons for both *V. anguillarum* and *V. ordalii* leading to the decision to increase the temperature at 53°C and the concentration of $MgCl_2$ to 2 mM to make the reaction more specific. These conditions produced an amplicon for *V. ordalii*, negative control, although *tonB2* gene is not present in this species. The annealing temperature was increased at 54°C with $MgCl_2$ concentration of 2 mM. The results obtained showed the amplification of both *V. anguillarum* and *V. ordalii*, indicating that the primers were not specific for *V. anguillarum.*

Design of a specific probe on tonB2 gene

The gene *tonB2*, present in *V. anguillarum*, and absent in *V. ordalii* was selected as target for the design of a specific DNA probe able to distinguish selectively *V. anguillarum*. Sequences of the genome of microorganisms possessing this gene and pathogenic for fish, such as *V. anguillarum, Photobacterium damselae* subsp. *piscicida* and *V. parahaemolyticus*, were retrieved from GenBank. *tonB2* sequences were aligned using the MultAlin software (Corpet, 1988), to identify the regions of low similarity for manually design the probe.
The selected probe was tested *in silico* with AmplifX 1.5.4 software on the whole genomic DNA of different microorganisms with and without *tonB2* gene, and with BLAST (https://www.ncbi.nlm.nih.gov) for alignment. Once verified *in silico*, the probe tailed with

digoxigenin at 5'-end was subjected to experimental tests with dot blot for specificity and sensitivity. A ssDNA sequence, complementary to the sequence of the probe, was used as a positive control for both probe sensitivity and specificity test. Two ssDNA short sequences of *V. ordalii* and *V. harveyi* were used as negative controls for the tests aimed to optimize conditions. The selected probe, of 33 bp, 42 %GC, T_m 62°C, was named VANG-DIG probe.

Dot blot

Dot blot was performed according to standard protocol (Cecchini *et al*., 2012) by testing various hybridization temperatures. The probe was tested for sensitivity on the sequence complementary to the probe (positive control) at 100, 10, 1 ng/µl, 100, 10, 1 pg/ µl, 100, 10 and 1 fg/µl.

The sensitivity of VANG-DIG obtained at 47°C was of 10 ng/ µl using DNA extracted from *V. anguillarum* DSMZ 21597. To improve the sensitivity, the hybridization temperature was lowered until 42°C which allows to obtain an increased sensitivity of 1 ng/µl (**Figure 11**).

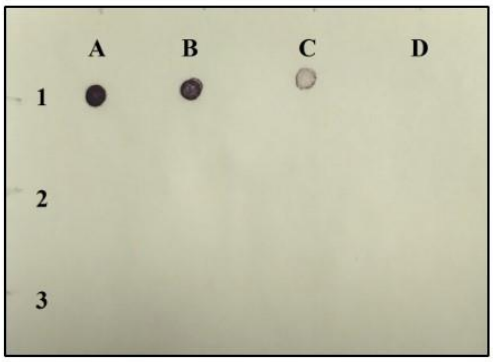

Figure 11.: Sensitivity of VANG-DIG probe at 100 ng/µl, at 47°C hybridization temperature. Spots of the sequence complementary to the probe, 1A: 100 ng/µl; 1B: 50 ng/µl; 1C: 10 ng/µl; 1D: 1 ng/µl; 2A: 100 pg/µl; 2B: 10 pg/µl; 2C: 10 pg/µl; 3A: 100 fg/µl; 3B: 10 fg/µl; 3C: 1 fg/µl.

After sensitivity, the VANG-DIG probe was tested for specificity on the DNAs extracted from the strains listed in Table 11 after standardization at 100 ng/µl. The test was carried out at 42°C with DNAs of *V. anguillarum* DSMZ 21597; *V. anguillarum* 189/13; *A. hydrophila*. DSMZ 30184; *V. parahemolyticus* DSM 10027; *V. ordalii* DSMZ 19621; *E. coli* DIAL; *Photobacterium damselae* subsp*. piscicida* DSMZ 22834.

These conditions produced one blue spot only for the positive control at 50 ng/µl. With the aim of optimizing the protocol, two different brands of nylon membranes (Bio-Rad and Sigma) were used with the same reagents at the same conditions, but no variations in the results were obtained. The hybridization temperature was lowered to 40°C with the aim to allow the hybridization of the DNAs of *V. anguillarum* samples at concentrations of 50 ng, 100 ng and 200 ng/µl. The results showed no differences from the previous ones, in fact only the positive control (sequence complementary to the probe) showed the blue spot.

To verify the hybridization temperature, and the quality of DNA for hybridization, an experiment was conducted at 35°C in parallel in two membranes at the same conditions, using DNAs extracted with: i) glass beads and phenol/chloroform/isoamyl alcohol and ii) Wizard® kit. In one membrane DNAs were spotted as previously described, in the second membrane DNAs were spotted after a treatment with formamide to avoid the formation of secondary structures (Dedish *et al*., 2001). As reported in **Figure 12**, the positive blue spots were visible for the

samples extracted and purified with the Wizard® kit in both membranes. The treatment with formamide produced no effects.

The conditions used allowed the hybridization of *A. hydrophila*, a negative control indicating that the 35°C used were not optimal for specificity. To make conditions specific, the hybridization temperature was increased at 40°C and the SSC was used at 0.1X with 0.1% SDS. These conditions were specific, in fact, the only blue spot corresponded to *V. anguillarum* DNA. The optimized conditions were tested on the sequence complementary to the probe at various serial dilution. The sensitivity increased at 10 pg/µl (**Figure 13**).

The optimized conditions were then used to test DNA extracted both by glass beads and phenol/chloroform/isoamyl alcohol, and by the kit (GenElute™) from a sample of about 25 mg of kidney isolated from a European sea bass and tested positive for *V. anguillarum* by PCR (**Table 16**). However, the dot blot result was negative as no blue spots were visible, maybe due to a concentration of *V. anguillarum* DNA below 10 pg/µl.

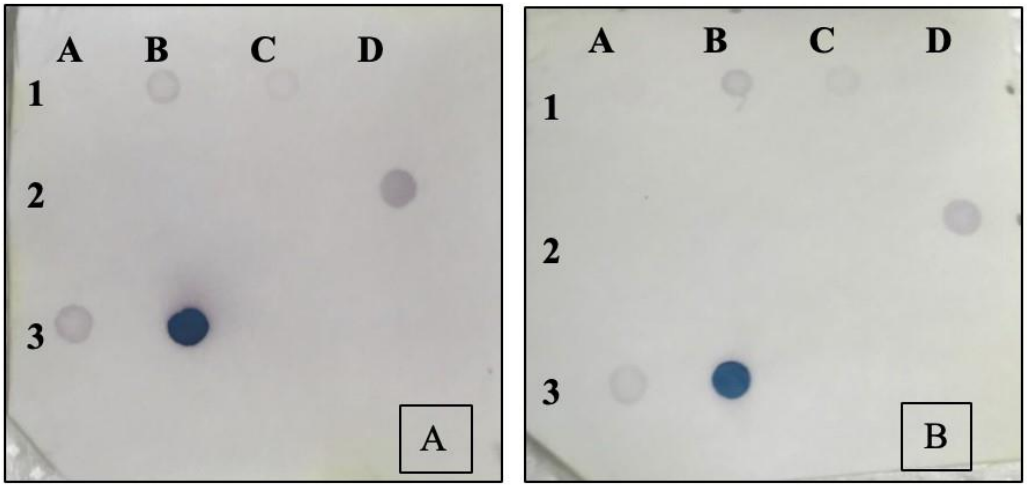

Figure 12. Sensitivity of VANG-DIG probe at the concentration of 100 ng/ µl, hybridization temperature 35°C, DNA at 100 ng/ µl. A) samples without formamide treatment; B) samples treated with formamide. 1A: V. anguillarum 189/2013 DIMEVET; 1B: V. anguillarum 251/2016 A DIMEVET; 1C: V. anguillarum 130/2015 DIMEVET; 2A: V. anguillarum 117/2013 DIMEVET; 2B: V. anguillarum 199/2016 DIMEVET; 2D: V. anguillarum 21597 DSMZ; 3A: Aereomonas hydrophila 30187 DSMZ; 3B: positive control at 50 ng/µL. 1A-1B-1C-2D-3A samples extracted with kit Wizard®; 2A-2B-2C samples extracted with phenol/chloroform.

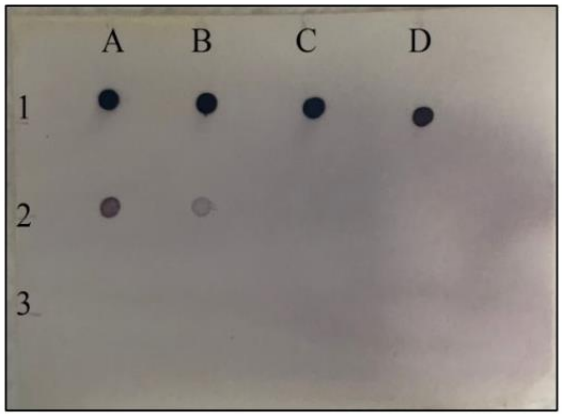

Figure 13.Sensitivity of VANG-DIG probe at 100 ng/µl, hybridization temperature 40°C. Positive control (sequence complementary to the probe) at: 1A: 100 ng/µl; 1B: 50 ng/µl; 1C: 10 ng/µl; 1D: 1 ng/µl; 2A: 100 pg/µl; 2B: 10 pg/µl; 2C: 10 pg/µl; 3A: 100 fg/µl; 3B: 10 fg/µl; 3C: 1 fg/µl.

Table 16. DNA extracted from kidney of sea bass

Sample	extraction method	ng/µl	A260/A280
kidney	kit (GenElute™)	31.70	2.03
kidney	kit (GenElute™)	28.30	2.11
kidney	glass beads- phenol/chloroform/isoamyl alcohol	2882.80	1.85
kidney	glass beads- phenol/chloroform/isoamyl alcohol	1245.50	1.76

5.2.3 Electrochemical biosensor

Screen-printed gold electrodes (SPAuEs) were selected for the development of a DNA based biosensor (**Figure 14**). The sensor is composed by three electrodes: the gold working electrode (WE), round shaped where reaction take places; the gold counter electrode (CE), which balances the overpotentials on the WE; and the Ag reference electrode (RE), placed around the WE, which has a fixed known potential in order to determine a potential difference between RE this and the WE.

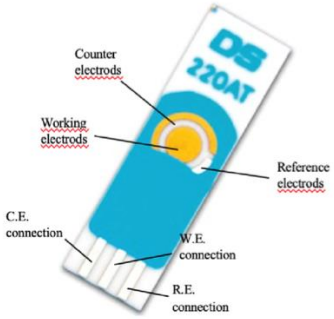

Figure 14. Gold screen printed electrode

Before utilization SPAuEs were cleaned by immersion in a specific electrochemical cell containing 8 ml solution of H_2SO_4 0.5 M (96% sulfuric acid, Carlo Erba), and subjected to 10 voltammetry cycles from 0 to +1.3 V. SPAuEs were then rinsed twice with 500 µl of bi-distilled H_2O on both sides and dried in a laminar flow cabinet.

After cleaning, SPAuEs were functionalized with the specific probe (VANG) selected in the *tonB2* gene of *V. anguillarum*, after modification with a thiol group at the 5'-end. The modified probe was called capture probe (HS-VANG) designed on *tonB2* gene of *V. anguillarum* (33 bp). To allow the immobilization of the probe to the gold surface, the thiol group of the HS-VANG was deprotected to make the thiol group reactive, according to manufacturer's instructions. Briefly,

addition of 10 mM TCEP shaking for 60 sec at RT; precipitation by addition of 3M NaAc, addition of ethanol and shake gently, then incubate 20 min at -20°C. After spin for 5 min at 13.000 rpm, discard the supernatant before drying at RT. SPAuEs were incubated with 20 µl of 10 ng/µl HS-VANG probe in PBS 1X, overnight at room temperature, and then rinsed with 500 µl deionized water.

Blocking

The resulting functionalized SPAuEs surfaces were blocked with 20 µl of MCE (C_2H_6OS 2mercaptoethanol) 10 mM in PBS 1X, pH 7.4 for 30 min at RT to saturate the surface used for the detection and to allow the captured probe to assume an organized position. The surface was then rinsed with 500 µl PBS 1X (5 times), dried under a sterile laminar flow cabinet and used for detection.

Once prepared, the chip was subjected to cyclic voltammetry analysis to evaluate the cyclic voltammetry (CV) value that will be used as reference. Afterwards, the sequence complementary to the probe was used as positive control. It was diluted at concentrations of 1000, 100, 10, 1 pg/µl in PBS 1X, kept at 95°C for 10 minutes and immediately put on ice before being used for hybridization. 20 µl of the positive control were deposed on the functionalized surface of the working electrode (WE) of the screen printed and left to incubate for 2 h at RT to hybridize. After incubation, the electrodes were washed 5 time with 500 µl of PBS 1X in order to remove the not hybridize positive control. The electrodes were then air-dried under a sterile laminar flow cabinet.

Voltammetry measurements

The measurement of the CVs was made by immersing the SPAuE into 8 ml of 10 mM $K_4[Fe(CN)_6]$-$3H_2O$ in PBS 1X and waiting 5 min before the measurement (**Figure 15**). CV measurements were conducted between - 0.2 V and + 0.6 V (against the Ag/AgCl reference electrode) at 100 mV/s, before (blank) and after hybridization. After, a voltammetry analysis will be performed to evaluate the CV value. If a difference between the values of CV measured before and after the hybridization is detected it can be recognized as a confirmation of the hybridization between the probe and the target. If no difference were detected it means that there was no hybridization between the probe and the target.

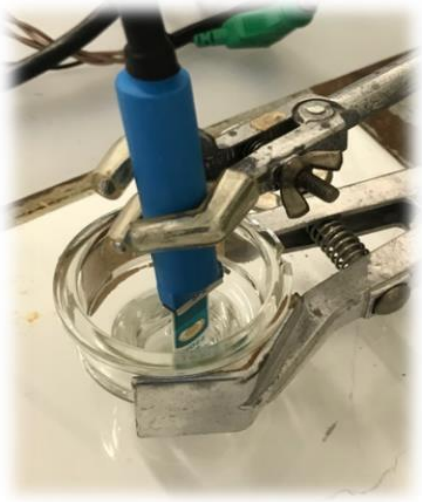

Figure 15. Electrochemical detection. Immersion of the SPAuE in $K_4[Fe(CN)_6]$-$3H_2O$ in PBS 1X.

Preliminary test

Tests were conducted in 10 mM potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$) in PBS 1X. The first test was performed to evaluate the signal produced by the SPAuE without and with the addition of blocking (MCE, 10 mM).

During the cyclic voltammetry measurements, the potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$) molecule undergoes a reversible redox process generating an anodic peak during the anodic scan (Ipa) and a corresponding cathodic peak during the reverse scan (Ipc). The difference between anodic peak current reached after DNA hybridization (Ipa) and anodic peak current reached after the funzionalization with DNA-probe (Ipa-blank), was measured according to the equation: ΔIpa = Ipa – Ipa-blank. Data reported in **Table 17** confirms that the blocking with MCE does not interfere with probe functionalization.

Table 17. Values of anodic peak current of SPAuEs after functionalization and after blocking

	Sample	Ipa	Standard Deviation
no blocking	1- HS-VANG	0.000161	3.61E-06
	2- HS-VANG	0.000161	6.16E-06
with blocking	1- HS-VANG -MCE	0.000179	2.69E-06
	2- HS-VANG -MCE	0.000182	3.38E-06

Hybridization test with positive control

Functionalized and blocked SPAuEs were hybridized at 40°C with 20 µl of the complementary denatured probe (95°C for 10 min) and measured according to the protocol. The data obtained and reported in **Table 18**, identifying a signal variation, following hybridization, due to the presence of the sequence complementary to the probe.

The signals of the SPAuEs after hybridization with the positive control were different depending on the presence or absence of blocking, as showed in **Figure 16**. Serial decimal dilutions of the positive control were tested. The data obtained showed a decrease in the difference between the currents at the decrease of the concentration of the positive control used for hybridization, except for the 1 ng/µl. The analysis conducted using voltammetry seems promising, as first results were able to distinguish different concentrations of the target (**Table 19**). Nevertheless, some parameters need optimization.

Table 18. Data of the measurement of anodic peak current for SPAuEs functionalized, blocked and hybridized with the positive control (PC)

	Sample	Ipa	Standard Deviation
no blocking	1- HS-VANG - PC	0.000163	5.20E-06
	2- HS-VANG - PC	0.000158	4.34E-06
with blocking	1- HS-VANG -MCE- PC	0.000168	3.99E-06
	2- HS-VANG -MCE- PC	0.000170	3.84E-06

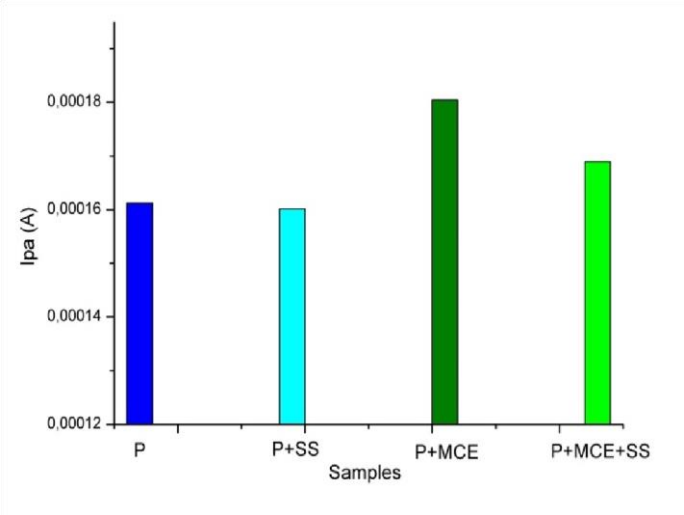

Figure 16. Comparison between screen-printed functionalized and hybridized with and without MCE. P: SPAuE functionalized; P+SS: SPAuE functionalized and hybridized with positive control; P+MCE: SPAuE functionalized and blocked with MCE; P+MCE+SS: SPAuE functionalized, blocked and hybridized with positive control.

Table 19. Measurement of anodic peak currents of various dilutions of the positive control. ΔIpa: (Ipa samples – Ipa blank)

Sample	Ipa	Standard Deviation	Δ Ipa (Ipa samples – Ipa blank)
blank	0.000182	-	-
10 ng/µl	0.000166	6.09E-06	-1.58E-05
1 ng/µl	0.000166	7.76E-06	-1.59E-05
100 pg/µl	0.000169	6.63E-06	-1.33E-05
10 pg/µl	0.000167	8.32E-06	-1.49E-05
1 pg/µl	0.000177	7.00E-06	-5.23E-06

New functionalization-blocking strategies

With the aim to improve the response of the bioassay, the blocking agent was changed from mercaptoethanol (MCE) to 6-Mercapto-1-hexanol (MCH) and measurements were performed by covering with 80 µl of potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6]$-$3H_2O$) (SigmaAldrich) 10 mM in PBS 1X the WE, instead of immersing the SPAuE in 8 ml 10 mM $K_4[Fe(CN)_6]3H_2O$ in PBS 1X. Functionalization and blocking were performed in one step, and two steps before hybridization and testing.

Functionalization-blocking in one step

A solution of 6 µl of MCH $5x10^{-7}$ mM in PBS 1X and 6 µl of 10 ng/µl HS-VANG probe in PBS 1X (ssDNA corresponding to $9.63x10^{-4}$ mM) were drop casted on the WE surface and incubated overnight at 25°C, rinsed twice with 500 µl of bi-distilled H_2O and dried.

Functionalization-blocking in two steps

The solution (12 µl) containing the specific HS-VANG capture probe at 10 ng/µl ($9.63X10^{-4}$ mM) diluted in PBS 1X was deposed on the gold surface of the working electrode and incubated over night at 25°C. After functionalization the electrode surface was rinsed twice with 500 µl of bi-distilled H_2O, dried under laminar flow cabinet and then blocked for 1h at 25°C using 12 µl of MCH (Sigma-Aldrich) in PBS 1X to obtain a final concentration of 1 mM. The SPAuE was then rinsed twice with 500 µl of bi-distilled H_2O, and dried.

Hybridization

Hybridization step allows the binding of the target DNAs to the HS-VANG probe previously immobilized on the working electrode (functionalization step). The sequence complementary to the probe, used as positive control, was diluted at different concentrations (1,000, 100, 10, 1 pg/µl) in PBS 1X, denatured at 95°C for 10 min and used for the hybridization step. 12 µl of the lower concentration were spotted on the WE surface and incubated for 1 h at 40°C, rinsed twice with 500 µl of bi-distilled H_2O and dried.

Detection

The first measure was performed with the functionalized and blocked screen-printed without the addition of the target (blank), then measurements were made after hybridization starting from the lowest (1 pg/µl) to the highest (1,000 pg/µl) concentration of the sequence complementary to the probe (positive control).

After hybridization, the electrode was connected to the potentiostat, and the SPAuE was covered with 80 µl of potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]$-$3H_2O$) (Sigma-Aldrich) 10 mM in PBS 1X. The oxidation of ferrocyanide to ferricyanide produced a signal detected by the instrument. CV measurement and DPV measurement were performed in sequence. Electrochemical measurements were performed with a computer-controlled µ-AutoLab type II potentiostat run by NOVA 2.1.2 software (EcoChemie, Utrecht, The Netherlands). The CVs were performed from -0.2V to +0.6V at 0.1 V/s while DPVs were performed from -0.2V to +0.4V at 0.01 V/s. After CV and DPV measurements, the electrode was washed twice with 500 µl of bi-distilled H_2O, dried under laminar flow cabinet, and subjected to the addition of a new concentration of the sequence complementary to the probe.

CV and DPV measurements were repeated to evaluate the current intensity after each hybridization step (Ipa sample). An Ipa sample current lower than the Ipa blank current confirms the binding between the DNA target and the HS-VANG probe.

Results for functionalization and blocking in one step

As reported in **Figure 17**, CVs and DPVs current peaks did not decrease proportionally with the concentration of the positive control used. CV and DPV curves recorded seems overlapped. This result may be ascribed to competition between MCH blocking solution and the thiolated DNA probe during the immobilization step on the Au. Consequently, it seems that the probe attached to the gold surface is in a lower amount than the MCH. On the basis of these results functionalization and blocking were performed in two separate steps, immobilization of the HS-VANG capture probe followed by blocking with MCH.

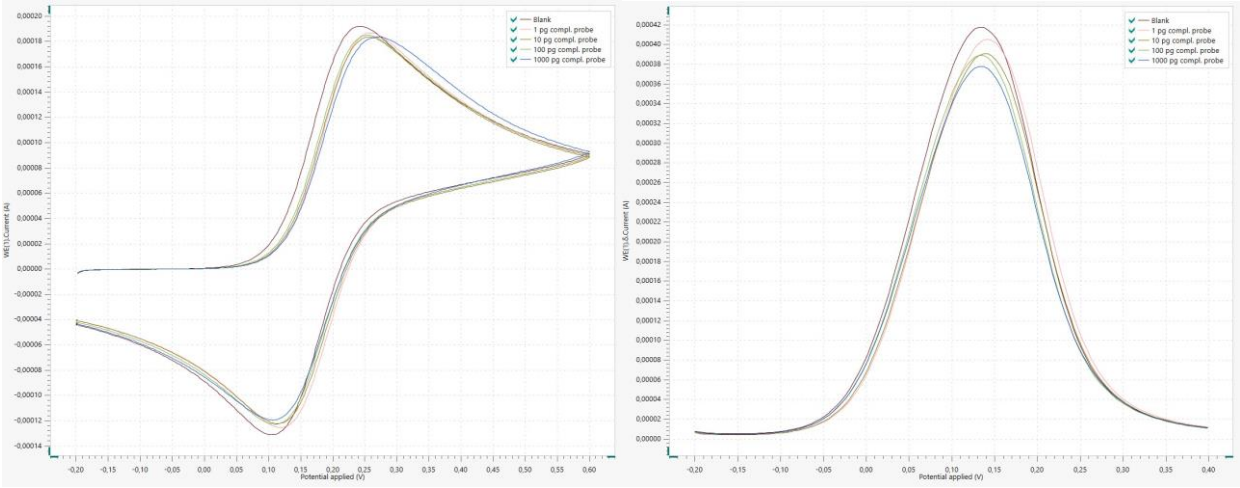

Figure 17. CVs (a) and DPVs (b) of ferrocyanide $[Fe(CN)_6]^{4-}$ coupling functionalization and blocking in one step. Black, SPAuE measure after blocking; Pink, SPAuE measure after hybridization with 1 pg/µl of sequence complementary to the probe; Brown, SPAuE measure after hybridization with 10 pg/µl of sequence complementary to the probe; Green, SPAuE measure after hybridization with 100 pg/µl of sequence complementary to the probe; Blue, SPAuE measure after hybridization with 1,000 pg/µl of sequence complementary to the probe.

Results of functionalization and blocking in two steps

Figure 18 shows the results obtained performing functionalization and blocking in two separate steps. The CV and DPV values obtained plotting ΔIpa (µA) against the logarithmic concentration of the positive control expressed in pg/µl, showed an appreciable decrease at increasing concentrations of the positive control (sequence complementary to the probe). The ΔIpa represents the difference between anodic peak current recorded by CV and DPV after hybridization with the HS-VANG complementary sequence (Ipa sample) and the anodic peak current recorded after SPAuE functionalization and blocking (Ipa blank).

As expected, the addition of the HS-VANG complementary probe produced a current decrease due to its steric hindrance which reduced the charge exchange between the ferrocyanide and the gold surface in relation to the increased concentration of the target added. Both CV and DPV measurements, showed good R^2 values of R^2 = 0,9896 and R^2 = 0,9967 respectively**.** The

sensitivity of DPV measurements were about five times higher than the sensitivity of CV measurements (**Figure 19**).

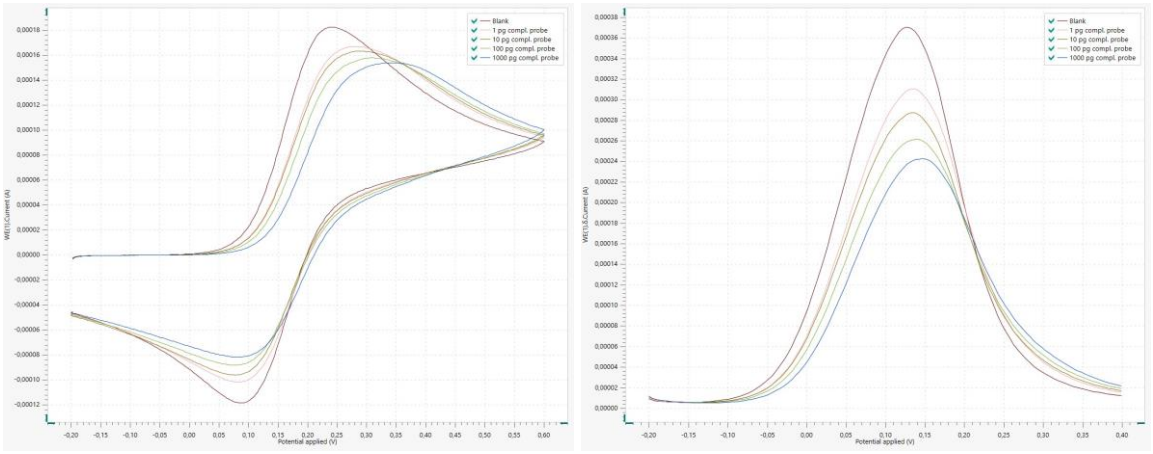

Figure 18. CVs (a) and DPVs (b) of ferrocyanide $[Fe(CN)_6]^{4-}$ using different concentrations of the sequence complementary to the probe. Black, SPAuE measure after blocking; Pink, SPAuE measure after hybridization with 1 pg/µl of sequence complementary to the probe; Brown, SPAuE measure after hybridization with 10 pg/µl of sequence complementary to the probe; Green, SPAuE measure after hybridization with 100 pg/µl of sequence complementary to the probe; Blue, SPAuE measure after hybridization with 1,000 pg/µl of sequence complementary to the probe.

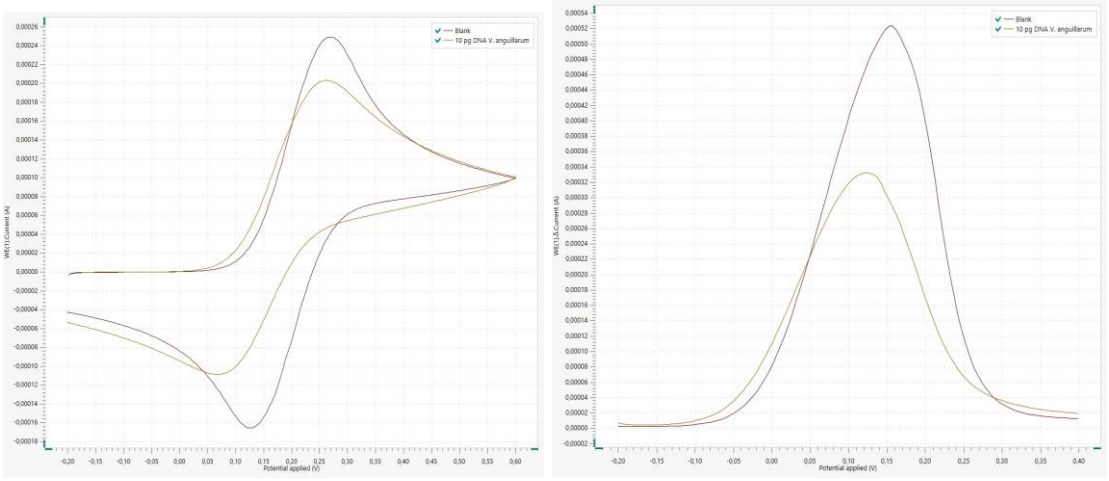

Figure 19. CV (a) and DPV (b) calibration line. On the x axis the logarithmic concentration of the HS-VANG complementary sequence (Log[ssDNA]) expressed pg/µl. On the y axis the ΔIpa (according to the equation: ΔIpa = Ipa sample – Ipa blank) expressed in µA.

Utilization of DNA extracted from V. anguillarum

To confirm the results, the same conditions were used for a preliminary test using a concentration of 10 pg/µl of DNA of V. anguillarum DSMZ 21597. DNA after dilution in PBS 1X was denatured at 95°C for 10 min and used for hybridization to the HS-VANG probe previously functionalized on the WE surface at 10 ng/ µl. 12 µl were spotted on the WE surface of the SPAuE, incubated for 1 h at 40°C, rinsed twice with 500 µl of bi-distilled H_2O, and dried under a hood. Detection was performed as described previously.

As expected, the anodic curve showed a decrease of the current after hybridization (**Figure 20**). This result confirmed the effectiveness of the screen-printed Au electrodes modified performing in two separate steps functionalization and blocking, allowing the development of an appropriate protocol to create a calibration curve with *V. anguillarum* DNA at serial dilutions to create an assay for the detection of the pathogen in field samples.

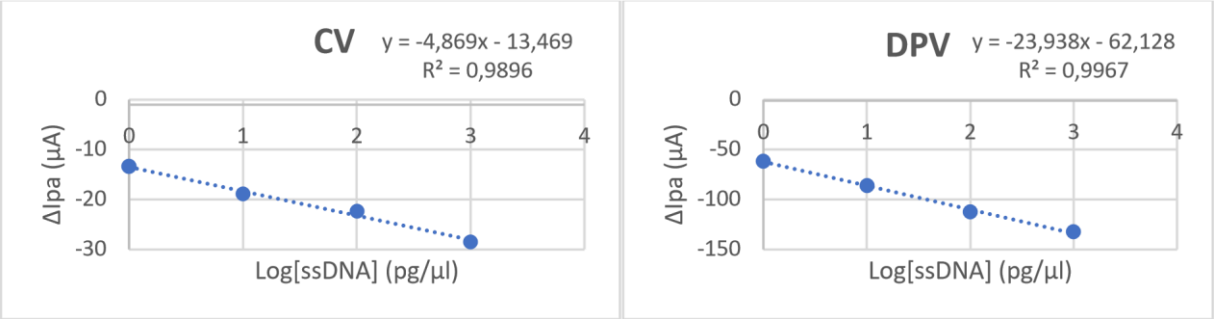

Figure 20. **CVs (a) and DPVs (b) measures after hybridization with 10 pg/µl of V. anguillarum 21597 *DSMZ* DNA. Two separate steps for immobilization and blocking were used.** Black: SPAuE measure before hybridization; Brown: SPAuE measure after hybridization with 10 pg/µl of genomic DNA.

Utilization of SSC solution for washing

To verify the utilization of water for washing steps after immobilization, a test was conducted by substituting water with the buffer SSC. The measurements were performed following the procedure previously described in two separate steps, the functionalization and the blocking. The SSC 2X solution was prepared from SSC 20X diluted in bi-distilled H_2O and sterilized at 121 °C for 15 min. SPAuE was rinsed twice with 500 µl SSC 2X solution, then twice with 500 µl of bi-distilled H_2O, and dried. This procedure was repeated before every measure, for every different concentration of the positive control used.

As can be observed in **Figure 21**, the results of both CV and DPV were not expected; in fact, inverse behaviors were obtained. CV and DPV current peaks showed an increase at increasing DNA target concentrations. SSC buffer used showed a strong washing out action, revealed by the increased value of the current peaks, which may suggest that DNA capture probe molecules and MCH molecules were progressively displayed from the gold surface. The results confirmed that water is the best solution for washing steps.

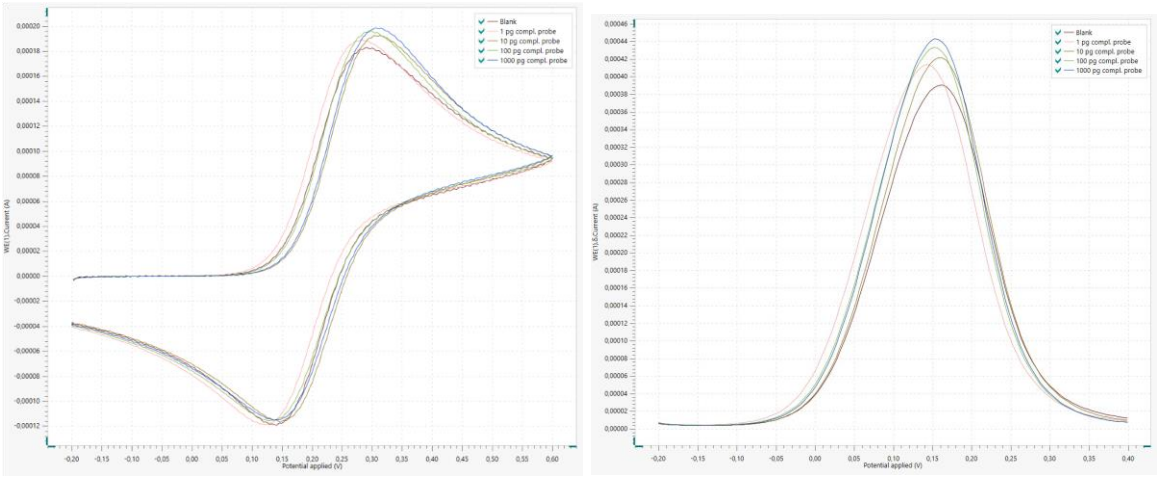

Figure 21. CVs (a) and DPVs (b) of ferrocyanide $[Fe(CN)_6]^{4-}$ using SSC 2X as washing reagent. Black, SPAuE measure after blocking; Pink, SPAuE measure after hybridization with 1 pg/µl of sequence complementary to the probe; Brown, SPAuE measure after hybridization with 10 pg/µl of sequence complementary to the probe; Green, SPAuE measure after hybridization with 100 pg/µl of sequence complementary to the probe; Blue, SPAuE measure after hybridization with 1,000 pg/µl of sequence complementary to the probe.

Discussion

Vibriosis caused by *Vibrio anguillarum* and other *Vibrio* spp. is one of the most severe and world spread marine fish disease, which result in great economic losses. Therefore, the availability of rapid and species-specific diagnostic methods for the early diagnosis and accurate identification of this species are needed. The morphological and biochemical identification of *V. anguillarum* is both time-consuming and inaccurate, leading often to misidentification. PCR, *in situ* hybridization and other molecular diagnostic tools, due to their rapidity and efficacy, have rapidly become available for the diagnosis of pathogenic fish bacteria. PCR methods for detection of *V. anguillarum* were developed based on 16S rRNA, *recA*, haemolysin, *empA, rpoN, amiB,* and *rpoS* genes. However, none of these targets was suitable to discriminate between closely related strain as *V. ordalii* due to the high level of sequence similarity (Kita-Tsukamoto *et al*., 1993; Rodkhum *et al*., 2006; Xiao *et al*., 2009). A more recent study has demonstrated the validity of the use of the *GroEL* gene, coding for a 60 –kDa heat shock protein, as a PCR target for specific detection of *V. anguillarum* (Kim *et al*., 2010). Therefore, *GroEL* sequences of our target bacteria and similar species were analysed and compared to find a region with a high number of differences between *V. anguillarum* and the other related species. These regions were used to design specific primers and a probe that were after used to optimize molecular protocols. **The designed primers VibrioFW and VibrioRV were specific for *V. anguillarum* and obtained a sensitivity of 1 pg/µL in classic endpoint PCR and 100 fg/µl in qPCR.** The results obtained for the test carried out with the artificial inoculum of fish liver suggest that the extraction step was not enough efficient to produce a DNA amount to give a clear signal.

The primers designed on *tonB2* gene were not able to distinguish between the two species, they may require a second step, such as a restriction on the amplicon to distinguish *V. anguillarum* from *V. ordalii.*

The probe VIBRIO-DIG due to the lack in specificity cannot be used with the purpose to detect *V. anguillarum* in field samples. For this reason, a DNA probe was identified in a different suitable gene, the *tonB2* gene. This probe (HS-VANG) was specific when tested by dot blot using not complementary sequences, and genomic DNAs from various bacteria, positive and negative controls. **The HS-VANG was used to develop a biosensor assay**. Future trends could evaluate the response of the biosensor with more genomic DNA concentrations, and other *Vibrio* species.

Conclusions

The results obtained with our work confirmed the possibility to use a sensor for the purpose of reducing the time of specific analyses necessary for the detection of fish pathogens. Screen-printed gold electrodes were used by using voltammetry and DPV. DPV showed higher sensitivity than CV.

5.3 MALDI-TOF MS for *V. anguillarum* and *Photobacterium damselae* subsp. *piscicida* identification

Introduction

Although some of the tests listed in section 4 are straightforward and highly reproducible in identification of *Ph. damselae* subsp. *piscicida* and *Vibrio (Listonella) anguillarum*, the complete identification frequently is time consuming, some methods require specialized training, technical skills and support, often are labour-intensive, while some require enrichment of target organism and costly reagents (Topić Popović et al., 2017). Nevertheless, *Ph. damselae* subsp. *piscicida* and *Vibrio (Listonella) anguillarum* outbreaks in fish require fast and reliable identification in order to move to the next step of prescribing treatment and control measures. To that effect, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is the most promising method for bacterial identification. It is a rapid and accurate proteomic method, detecting ribosomal protein fractions of bacteria to be used for classification of the organisms (Lay, 2001). Further identification is based on the detection of mass signals from proteins specific at genus, species or subspecies levels (Benagli et al., 2012). MALDI-TOF MS analyses whole bacterial cells within minutes after cultivation, with high throughput and low running costs (Topić Popović et al., 2017).

However, the identification requires a pure and intact colony, while the accuracy of the method may be impacted by culture media, incubation time of the bacterium, and sample preparation (Demirev et al., 1999; Saffert et al., 2011; Veloo et al., 2014). Therefore, the aim of this work was to investigate the sensitivity and reproducibility of MALDI-TOF MS for accurate identification of *Ph. damselae* subsp. *piscicida* and *Vibrio (Listonella) anguillarum*. This was tested by analysing clinical isolates of *Ph. damselae* subsp. *piscicida* grown on two different media, cultured at three different incubation times, and applied on the target plate by the direct sample spotting, the on-target extraction and by the full extraction method. *V. anguillarum*

isolates were grown on standard media with and without the addition of NaCl, cultured at three incubation temperatures, and at three incubation periods.

Materials and methods

Bacterial strains

The cultures used in this study were the isolates of *Ph. damselae* subsp. *piscicida* from various fish species farmed in the Mediterranean. A total of 26 *Ph. damselae* subsp. *piscicida* strains were used. Before MALDI-TOF MS analyses, all isolates were cultured at 22°C on TSA and Blood Agar, BA (Certifikat doo, Croatia) enriched with 1.5% NaCl.

Also, a total of 27 *V. anguillarum* strains were used, and cultured on tryptone soy agar (TSA) (Oxoid Ltd, England UK). The medium formula contained pancreatic digest of casein (15g/L), enzymatic digest of soya bean (5 g/L), agar (15g/L), and sodium chloride (5g/L). The non-supplemented medium thus inherently contained 0.5 % NaCl. Strains were also cultured on TSA medium supplemented with 1.5 % NaCl.

MALDI-TOF MS

The application of the isolates on the plate was performed by the direct sample spotting, the on-target extraction and by the full extraction method, after 24, 48, and 72 hours of incubation on each growth medium (TSA and BA). All the procedures were performed in triplicate for each individual strain.

Direct sample spotting: a single bacterial colony from each tested strain was smeared onto a 96-spot polished steel target plate (Bruker Daltonik, Bremen, Germany). They were allowed to air dry at room temperature (RT), after which 1 µL of MALDI matrix was added to each bacterial colony (saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile and 2.5% trifluoroacetic acid.

On-target extraction: a single bacterial colony from each tested strain was smeared onto a 96-spot steel target plate. Subsequently 1 µL of 70 % formic acid (Kemika, Croatia) was added to each bacterial colony. After drying, 1 µL of MALDI matrix was added to each spot.

Full extraction: a loopful of a bacterial colony from each tested strain was suspended in 300 µL of LC-MS-grade water (Fisher Chemical, St. Louis, MO) and immediately vortexed. Further, 900 µL of 100 % ethanol (Kemika, Croatia) was added to the suspension, vortexed and centrifuged at 16 000 *g* for 2 minutes. The supernatant was discarded and the pellet recentrifuged. After discarding the supernatant, the pellet was dried at RT and resuspended in 20 µL of 70 % formic acid. The suspension was mixed by pipetting and 20 µL of acetonitrile was added, mixed and centrifuged at 16 000 *g* for 2 min. Before overlying with 1 µL of MALDI matrix, 1 µL of supernatant was added to each plate spot and allowed to dry.

MALDI-TOF MS was performed with a Bruker Biotyper (Bruker Daltonik, Bremen, Germany) system. The microflex LT mass spectrometer was calibrated with a bacterial test standard (Bruker Daltonik). Spectra were acquired in the positive linear mode between 2 to 20 kDa of mass range using FlexControl software in automatic mode. Bruker Biotyper 3.0 software (Bruker Daltonik) were used to analyse the spectra. Identification criteria were following: a log score of 2.300 to 3.000 indicated highly probable species level identification, a score of 2.000 to 2.299 indicated probable species identification, a score 1.700 to 1.999 indicated probable

identification to the genus level, while a score of < 1.700 was considered unreliable. In order to minimize random effects, data obtained with replicate measurements were added to the calculation.

Results

5.3.1 *Photobacterium damselae* subsp. *piscicida*

Three sample preparation protocols were tested for preparation of bacterial samples. The application of the *Ph. damselae* subsp. *piscicida* isolates on the plate was performed by the direct sample spotting, the on-target extraction and by the full extraction method. Samples prepared by the on-target extraction were overall better identified than by the other two protocols. **The on-target extraction gave high log scores indicating highly probable species level identification, in both culture media, in all incubation times and in all replicates, when compared with the direct sample spotting, and to the full extraction method.** Of 1,404 individual measurements in total, the on-target extraction gave 85.47 % and 87.18 % highly probable species level identifications for samples cultivated on TSA and BA, respectively. The direct spotting resulted in 100 % identification to the genus level in all cases, while the full extraction method identified to the species level 97.86 % and 97.01 % of strains cultivated on TSA and BA respectively, all other factors considered.

There was a difference regarding the culture media and successful acquisition of mass spectra. Identification of samples grown on BA outperformed identification on TSA for 0.64% for the direct sample spotting and the on-target extraction methods, as the BA mass spectra had the greater number of signals and the higher signal-to-noise ratio. However, when performing the full extraction, samples grown on BA had 13.68 % of unreliable identifications and 11.11 % of no-identifications, compared with 5.13 % unreliables and 1.71 % no-identifications for samples grown on TSA. TSA medium enabled correct identification of *Ph. damselae* subsp. *piscicida* samples against the Biotyper database as highly probable species level identification in 49.57 % compared with 41.45 % for BA when performing the full sample extraction.

The *Ph. damselae* subsp. *piscicida* strains were incubated for 24, 48, and 72 h on each growth medium (**Table 20**). Samples incubated for 24 h gave better results over other incubation times as the number of successful identifications to the species level was higher, as well as the mass spectral quality sufficient for automatic acquisition. Reliable species identification (log scores of 2.300 to 3.000 and 2.000 to 2.299) after 24 hours were 61.54 %, 84.61 %, and 53.85 % for samples grown on TSA and isolated by the direct sample spotting, the on-target extraction and by the full extraction method, respectively. However, when compared with identification results of samples grown for 48 and 72 h and isolated by the on-target extraction, the results did not change significantly (83.33 % and 87.18 % respectively). Reliable species identification (log scores of 2.000 to 3.000) after 24 hours were 76.92 %, 96.15 %, and 30.77 % for samples grown on BA and isolated by the direct sample spotting, the on-target extraction and by the full extraction method, respectively. Again, when identified by the on-target extraction, the results did not change significantly for 48 h (93.58 %), but declined for isolates grown for 72 h (71.79 %).

All measurements were performed in triplicate for each individual strain. The identical identification result for all three measurements was for: TSA medium and the direct sample spotting 9.40 %, the on-target extraction 20.94 %, by the full extraction method 12.82 %; for BA

medium and the direct sample spotting 15.38 %, the on-target extraction 23.93 %, and by the full extraction method 3.74 %. The on-target extraction, therefore, for both media gave the most reliable results in terms of identical identification of replicates. The reliable identification with the highest score (log scores of 2.000 to 3.000) from the first measurement amounted to 100 % only for the on-target extraction from BA (24 h), and 92.31 % (48 h), whereas the on-target extraction from TSA amounted to 84.61 % (24 h), 76.92 % (48 h), and 84.61 % (72 h). If the first measurement was not reliable, the number of subsequent measurements needed for probable to highly probable species level identification was the least for the direct method after 24 h of incubation on both media.

5.3.2 *Vibrio* (*Listonella*) *anguillarum*

Overall, MALDI-TOF MS identified 15.22 % of isolates accurately only to the genus level and 73.85 % to the species level, when observing results irrespective of the NaCl supplementation of the medium and culture conditions. However, when comparing species identification for different culture conditions, the results varied significantly by media type ($p<0.001$), incubating temperature ($p<0.001$), and time of measurement ($p<0.001$).

Identification of strains grown on NaCl-supplemented TSA outperformed identification on non-supplemented TSA. The best scores were retrieved for *V. anguillarum* strains grown on NaCl-supplemented TSA at 22°C and incubated for 48h, followed by incubation at 37°C at 48h. The samples grown on non-supplemented TSA gave the best readings when incubated at 22°C for 72h, followed by incubation at 15°C for 72h. The results obtained for isolates grown at 37°C for 7 days on both media have to be interpreted with caution since they included a significant percent of unreliable scores.

The incubation temperature affected the MALDI-TOF MS species identification results significantly ($p<0.001$), and incubation at 37°C yielded the most unreliable results. Unreliable identifications and no-identifications were growing with the incubation duration at 37°C, on both media, amounting to 88.89 % for 7d incubation on supplemented TSA, and 92.60 % for 7d incubation on non-supplemented TSA.

The correct identification of *V. anguillarum* strains was diminishing with the prolongation of the incubation time, on both media and all temperatures. The 7d incubations enabled correct identifications of less than 30 % of overall specimens in various culture conditions, while only for supplemented TSA at 15°C it amounted to 88.89 %. The logistic regression modelling showed no significant association of strains ($p=0.4$) and the final fixed effects model included all variables of culture conditions as independent main effects since no interactions of variables showed a significant improvement of the model. According to K-fold cross validation, the final model had an average accuracy of 93.3% and by the receiver operating characteristics the area under the curve of 0.964.

The NaCl-supplemented TSA enabled the correct identification of *V. anguillarum* against the Biotyper database as highly probable species level identification in 2.1 % of cases, when observing all culture conditions with that medium. When observing the 22°C incubation at 48h, it amounted to 11.11 %.

There was a difference between the NaCl supplementation of the culture media and successful acquisition of mass spectra. The mass spectra of bacteria grown on the supplemented media

had the greater number of signals and the higher signal-to-noise ratio. Most of the ions were appearing reproducibly across all the conditions tested, i.e. media, temperature, and incubation duration. The reproducible appearance of these ions at the specific *m/z* ratios were used for comparison of identification results, and the mass spectral quality sufficient for automatic acquisition was on the highest level for specimens grown at 15°C for 72h (**Figure 22**).

Table 20. Identification results of different sample preparations of *Ph. damselae* subsp. *piscicida* strains cultured on Tryptic soy agar (TSA) supplemented with 1.5% NaCl (as log score). The samples were applied on the target plate by the direct sample spotting (A), the on-target extraction (B) and by the full extraction method (C). Results presented are the average of three measurements.

Strain ID	24 h incubation			48 h incubation			72 h incubation		
	A	B	C	A	B	C	A	B	C
177/04	2.009	2.088	2.067	1.947	2.046	2.165	2.001	2.035	2.048
235/04	2.040	2.044	2.047	1.905	1.965	2.109	2.003	2.032	1.905
299/C/04	2.048	1.999	1.953	1.951	2.084	2.038	1.999	2.062	1.888
319/04	2.020	2.072	2.045	1.931	2.017	2.051	2.035	1.994	2.006
79/05	1.993	1.989	2.073	1.866	2.051	2.086	1.948	1.896	1.758
114/05	1.974	2.042	2.040	2.024	2.047	2.071	1.959	2.045	1.947
189/A/05	2.016	2.028	2.071	1.889	2.097	2.050	1.979	2.025	1.954
189/C/05	2.054	1.956	2.063	1.944	2.041	1.967	1.993	2.071	2.065
256/05	1.994	2.019	2.030	2.008	2.066	2.021	2.035	1.955	1.823
314/05	2.029	1.978	2.011	2.026	2.110	2.112	2.006	2.068	1.952
328/05	2.003	2.124	2.093	2.039	1.993	2.029	2.051	2.028	2.120
82/06	2.008	2.058	2.037	2.085	2.088	1.923	2.006	2.093	1.787
262/08	1.850	2.030	1.933	2.005	1.948	2.116	1.914	1.996	1.351
243/10	2.078	2.086	1.938	2.019	2.028	2.018	2.004	2.011	2.037
97/14	2.017	2.032	1.814	2.003	2.054	2.065	1.973	2.067	1.599
325/C/14	2.032	2.065	1.939	2.101	2.105	1.570	2.045	2.061	2.063
352/B/14	1.925	2.092	2.079	1.738	2.052	1.686	1.948	2.054	1.911
395/14	1.836	2.058	1.912	1.864	2.070	1.807	1.995	2.044	1.851
396/14	1.882	2.046	1.850	1.957	1.995	1.910	2.045	2.031	2.072
399/14	2.016	2.040	1.931	1.863	2.085	1.643	1.983	2.045	1.960
277/A/15	2.015	2.076	1.757	1.979	2.037	2.003	1.934	2.077	1.950

278/16	1.898	1.998	1.975	1.925	2.043	2.019	1.914	2.046	2.029
305/15	1.815	2.085	1.931	1.829	2.041	1.919	1.994	2.049	1.856
335/15	2.041	2.033	1.859	1.898	2.123	1.921	1.969	2.011	2.074
340/16	2.015	2.133	1.809	1.955	2.031	1.952	2.039	2.046	2.039
342/A/16	2.043	2.153	1.918	1.915	2.062	2.071	1.951	2.034	1.998
% strains identified to genus/species with highly probable/probable level identification:									
genus	100	100	100	100	100	88.46	100	100	92,31
species	65.38	80.76	46.15	34.61	84.61	61.54	42.31	84.61	38.46

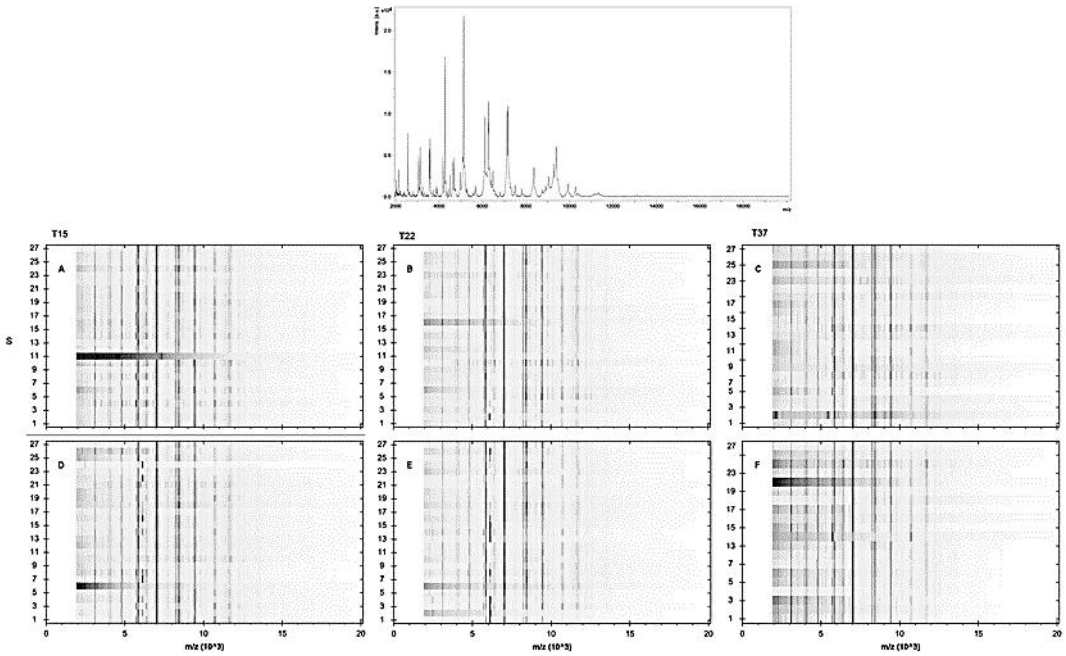

Figure 22. Gelview of signatures obtained from analysis of 27 strains of *Vibrio anguillarum* grown on different media, characterized by mass/charge ratio peak (m/z) and frequency of appearance in respect of the supplementation of TSA medium with sodium chloride.

The results of this work were included in the following publications:

KAZAZIĆ S, TOPIĆ POPOVIĆ N*, STRUNJAK-PEROVIĆ I, FLORIO D, FIORAVANTI M, BABIĆ S, ČOŽ-RAKOVAC R (2019): Fish photobacteriosis – the importance of rapid and accurate identification of *Photobacterium damselae* subsp. *piscicida*. Journal of Fish Diseases, 42, 8, 1201-1209. https://doi.org/10.1111/jfd.13022

KAZAZIĆ S, TOPIĆ POPOVIĆ N*, STRUNJAK-PEROVIĆ I, BABIĆ S, FLORIO D, FIORAVANTI M, BOJANIĆ K, ČOŽ-RAKOVAC R (2019): Matrix-assisted laser desorption/ionization time of flight mass spectrometry identification of *Vibrio* (*Listonella*) *anguillarum* isolated from sea bass and sea bream. PLoS ONE 14(11): e0225343. https://doi.org/10.1371/journal.pone.0225343

5.4 Development of a field antibiotic sensitivity test - "Perform-TEST"

5.4.1 Introduction

In order to reduce the amount of time between the first clinical signs of a bacterial disease and the diagnosis (including the results of the antibiotic sensitivity test) a new method aimed to test the antibiotic susceptibility according to the breakpoint criteria, together with the cultural exam has been designed for field use, to give fast and accurate indications on the authorized antimicrobial treatment of choice. This method has been designed to fulfil the following criteria: accuracy, rapidity, simple use, inexpensive and fit to be performed by a field technician.

5.4.2 Description of the procedure

Sampling of at least five symptomatic fish (suspected of bacterial infection) from each cage or tank.

Necropsy of the fish sampled in a suitable way to access the anterior kidney. This activity must be carried out in asepsis to avoid contamination of the sample.

Inoculation of the anterior kidney on different culture media: Trypticase Soy Agar (TSA) +1.5%NaCl/ Marine agar plate (MA), Thiosulphate Citrate Bile Salt Sucrose agar (TCBS) and possibly a Blood-agar plate (if an infection by *Photobacterium damselae* is suspected). In addition, streaking the same organ into Perform-PLATES (see section 5.4.3) containing Mueller-Hinton II and specific antibiotic (e.g. oxytetracycline, etc.). The inoculation on TSA+1.5%NaCl/Marine Agar, TCBS and Blood Agar plates and Perform-PLATES should be performed with disposable sterile loop (1µl).

Incubation in aerobic condition for 24-48 hours (longer time for slow-growing species such as *Photobacterium damselae*) (**Figure 23**) at 25°C±1.

After the incubation, if the bacteria growth on the plates (TSA+1.5%NaCl, TCBS and Blood agar) it will be possible to observe in Perform-PLATES:

- Bacterial growth: bacteria are resistant to the tested antibiotic.
- Absence of bacterial growth: bacteria are susceptible to the tested antibiotic.

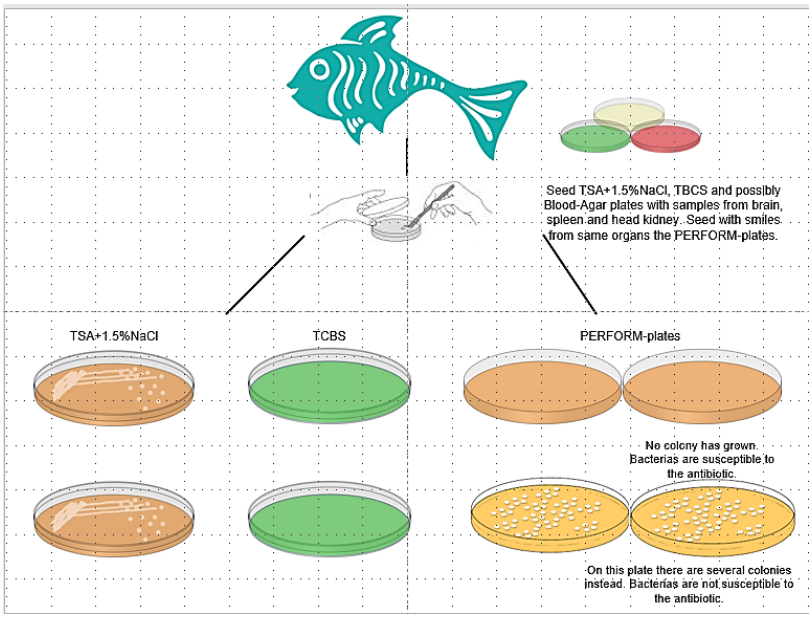

Figure 23.: Schematic workflow of the execution and interpretation of Perform-TEST.

5.4.3 Preparation of Perform-PLATES (Figure 24)

Step 1 (prepared in the lab or by industry):

Preparation of the media containing Müller-Hinton II.

Preparation of the different antibiotics supplement in a concentration close to the breakpoint value defined for each molecule.

Step 2 (in the farm):

Dissolve the media by boiling (100°C).

Let the media cool at 50°C.

Reconstitute antibiotics supplement and add it aseptically to the media.

Plate the media into sterile, empty Petri dishes (thickness of 4mm).

When the plates are dry, store it at 2-8 °C for no more than 3-5 days. The Petri plates must be placed into plastic bags to avoid drying out.

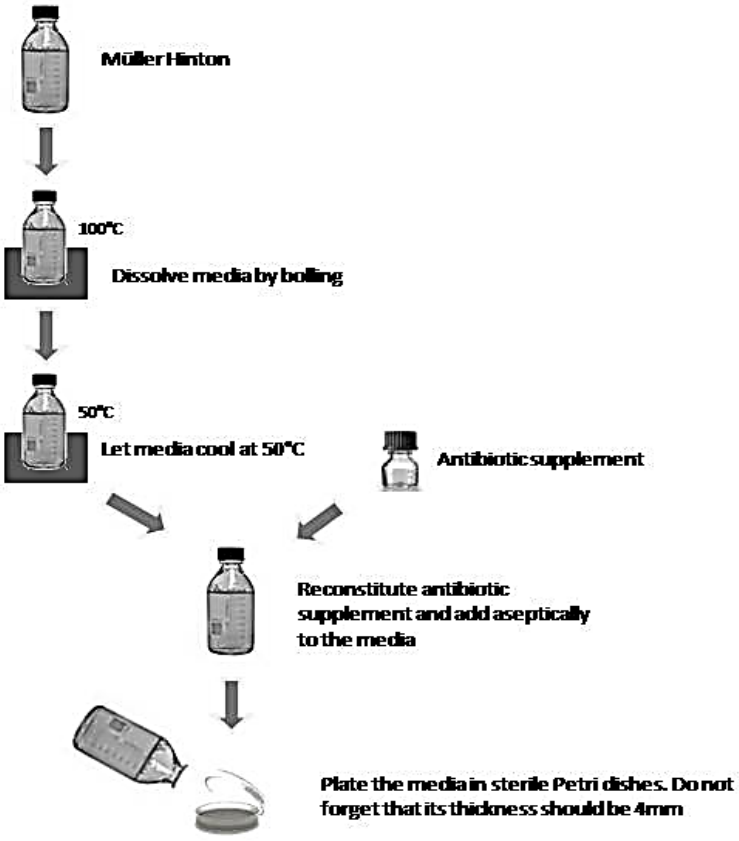

Figure 24. Preparation of Perform-PLATES

5.4.4 Critical issues and possible solutions

Critical Issues	*Possible Solutions*
Reproducibility	Section 5.4.5
Definition of breakpoint values for each molecule	Section 5.4.6
Shelf life of plates. Antibiotics, in particular tetracyclines and penicillin, are extremely sensitive to light and have a short shelf life.	The shelf life of the plates can be tested by sowing plates prepared the same day, every day for 7 days with strains known to be sensitive or resistant to the antibiotics.
Operator. Both preparation and sowing of the media should be carried out by a trained operator to not compromise the results.	Training course for both field operators and other labs.
Sterility. It is a critical point in both preparation and sowing of the plates.	To maintain the sterility, the media is prepared in liquid form, and the antibiotics added in a second time as it may represent an important critical point.
Plates. The volume of the media should be standardized.	
Medium. Müller-Hinton might not be suitable for some bacteria with special requirements (filamentous bacteria)	Use of alternative media (FMM, other) if sensitive bacterial species are suspected

5.4.5 Assessment of the reproducibility of Perform-TEST during the inoculation of the sample

A critical issue that emerged during the development of Perform-TEST was the reproducibility during the inoculation of the sample, established as the minimum number of morphologically identical bacterial colonies found in individual plates starting from the same matrix (anterior kidney). For this purpose, five fish (1 fish = 1 sample unit) with symptoms from the same outbreak, were examined as follow.

Fish samples were collected during vibriosis outbreaks (epidemiologically independent) and examined within 12 hours. From each subject, the anterior kidney was inoculated initially on a TCBS plate and subsequently on six TSA + 1.5% NaCl plates using 1µl sterile loop for each plate. All plates were incubated at 25 ± 1 °C for 24-48 hours.

The following criteria were then defined:

- The number of positive samples had to be not less than 10. A single sample (consisting of 5 fish) was included in the reproducibility test provided that at least three subjects were positive for bacteriological culture; when a number of colonies referable to *Vibrio* spp. were isolated on the first plate of TCBS and in an amount not less than 20 colonies.

- The number of positive sample units for each individual sample had to be not less than 3; The single sample unit was considered positive when colonies referable to Vibrio spp. were counted on all the six TSA plates and in an amount not less than 20 units.
- It was established that the reproducibility test is considered positive if at least eight of the ten samples examined were found to comply with both of the requirements described above.

5.4.6 Definition of breakpoint values for different antibiotics against European sea bass and gilthead sea bream bacterial pathogens

In order to obtain the breakpoints for different antibiotics against sea bass and sea bream bacterial pathogens we decided to use the epidemiological cut-off values. In general, interpretive criteria can be of two types, clinical breakpoints or epidemiological cut-off values. The aims of the first one is to categorize an isolate based on the probable outcome of the administration of an antimicrobial agent to a host infected by that bacterium. These breakpoints categorize isolates as either sensitive (S), intermediate (I) or resistant (R).

The categorization of an isolate as S with respect to an agent implies that an administration of that agent, using a standard dose regime, could be expected to have a positive therapeutic outcome for a specified host infected by that isolate.

A categorization as R would imply that it was improbable that such a therapy would have any beneficial outcome. The categorization of an isolate as I is used to indicate an uncertainty as to the probable clinical outcome or, in some cases, that the infection might be controllable if a higher dose was administered.

Setting clinical breakpoints is a complex process involving the consideration of a variety of data: *in-vitro* susceptibility of the bacterium, the pharmacokinetics and pharmacodynamics (PK/PD) of the agent in the host and the historical records of the clinical outcomes of previous treatments of the host. Both the nature of the host and dose regime used in the administration of the agent will influence the PK/PD and the resultant clinical outcomes. Consequently, clinical breakpoints will be dose and host specific. Their validity is always limited to situations where the therapeutic administration of a specified, standard dose of an agent is given to a specified host. What is certain is that it will not be possible to generate globally applicable clinical breakpoints as a basis for interpretation of susceptibility test data for bacteria isolated from aquatic animals.

The achievement of clinical breakpoints relating to specific therapies of specific hosts in a specific clinical and environmental context may be possible, but application of these breakpoints will always be of local and not general validity.

The Clinical and Laboratory Standard Institute (CLSI) presented a very limited number of clinical breakpoints relevant on the therapy of aquatic animals. The CLSI guideline M45-A3 (CLSI, 2016) and CLSI document M100-S27 (CLSI, 2017) provides some clinical breakpoints applicable to data for *Aeromonas* spp., *Streptococcus* spp. and *Vibrio* spp. but they could only have a valid application when the aim of the study is to investigate susceptibility in terms of therapies of human patients. They cannot and should not be used to predict the outcomes of therapies of aquatic animals. Only VET03/04-S2 document (CLSI, 2014a) gives breakpoints relevant to the therapy of Aeromonas salmonicida infections. The limited data supporting these breakpoints were from studies on salmon in freshwater at low temperatures.

For these reasons in the Aquatic Animal Health Code (https://www.oie.int/standard-setting/aquatic-code/) published by the World Organisation for Animal Health (OIE), in order to monitor and promote surveillance programs of antibiotic susceptibilities of bacteria isolated from aquatic animals recommends that where possible, the primary susceptibility data collected in such programs should be interpreted by the application of internationally agreed epidemiological cut-off values.

Epidemiological cut-off values (ECVs for CLSI or ECOFFs for EUCAST) are, in common with clinical breakpoints, agent-specific, protocol-specific and species-specific interpretive criteria. Their aim is, however, different. The aim of the application of an ECV is to facilitate the categorizing of an isolate as a fully susceptible member of its species or as manifesting a reduced susceptibility (as wild type, WT or non-wild type, NWT). The only data needed to set these cut-off values are those concerning in-vitro susceptibility measurements in contrast to the possibly insurmountable difficulties in obtaining the data required to set harmonized clinical breakpoints.

Currently very few ECVs have been established by CLSI for bacteria isolated from aquatic animals. The supplement VET03-VET04-S2 (CLSI, 2014a) presents ECVs that can be applied to A. salmonicida, A. hydrophila, F. columnare and F. psychrophilum.

In order to obtain epidemiological cut-off values we started with MIC assay of select strains present in our collection: 52 Vibrio spp. (27 strains of V. anguillarum and 25 of V. harveyi) and 22 Photobacterium damselae subsp. piscicida strains isolated from European seabass (ESB) and gilthead seabream (GSB) during natural outbreaks occurred in Italy and in Mediterranean area (Croatia, Albania and Tunisia) in the last 14 years (**Table 21**).

No repetitions (same fish / tank / farm / outbreak) isolated in the same seasonal period / year have been included in this list. All the strains were obtained from the culture collections held by the Fish Pathology Laboratory of MTSPV Service, DIMEVET, Bologna, Italy.

Table 21. Strains isolated and selected for the epidemiological cut-off values

ID Strain	Isolation date	Strains species	Fish species	Farm	Country
305/C/02	13/11/2002	*Vibrio anguillarum*	ESG	Land	Italy
68/05	11/03/2005	*V. anguillarum*	ESG	Land	Italy
82/05	25/03/2005	*V.anguillarum*	ESG	Land	Italy
372/05	16/11/2005	*V.anguillarum*	ESG	Cage	Italy
31/06	03/02/2006	*V.anguillarum*	ESG	Land	Italy
136/C/06	05/04/2006	*V.anguillarum*	ESG	Land	Italy
77/09	13/03/2009	*V.anguillarum*	ESG	Land	Italy
135/09	08/05/2009	*V.anguillarum*	ESG	Cage	Italy
158/B/09	27/05/2009	*V.anguillarum*	ESG	Land	Italy
84/10	19/04/2010	*V.anguillarum*	ESG	Cage	Croatia
246/D/10	23/10/2010	*V.anguillarum*	ESG	Cage	Italy
65/A/11	05/04/2011	*V.anguillarum*	ESG	Land	Italy
83/A/11	29/04/2011	*V.anguillarum*	ESG	Land	Italy
104/B/12	01/06/2012	*V.anguillarum*	ESG	Cage	Italy
117/13	02/05/2013	*V.anguillarum*	ESG	Cage	Italy
189/13	10/07/2013	*V.anguillarum*	ESG	Cage	Italy

440/14	26/11/2014	*V.anguillarum*	ESG	Cage	Italy
449/A/14	28/11/2014	*V.anguillarum*	GSB	Cage	Italy
4/B/15	13/01/2015	*V.anguillarum*	ESG	Cage	Italy
73/15	20/03/2015	*V.anguillarum*	ESG	Land	Italy
121/15	05/05/2015	*V.anguillarum*	ESG	Cage	Italy
130/F/15	18/05/2018	*V.anguillarum*	ESG	Cage	Italy
188/15	13/07/2015	*V.anguillarum*	ESG	Land	Italy
199/A/16	16/06/2016	*V.anguillarum*	ESG	Cage	Italy
167/C/17	19/05/2017	*V.anguillarum*	ESG	Land	Italy
141/17	19/04/2017	*V.anguillarum*	ESG	Cage	Italy
13/18	26/01/2018	*V.anguillarum*	GSB	Land	Italy
177/04	24/06/2004	*P. damselae piscicida*	ESG	Land	Italy
235/04	08/09/2004	*P. damselae piscicida*	ESG	Land	Italy
299/C/04	27/10/2004	*P. damselae piscicida*	ESG	Cage	Italy
79/05	23/03/2005	*P. damselae piscicida*	ESG	Land	Italy
114/05	02/05/2005	*P. damselae piscicida*	ESG	Land	Italy
256/05	30/08/2005	*P. damselae piscicida*	ESG	Land	Italy
314/05	07/10/2005	*P. damselae piscicida*	ESG	Land	Italy
82/06	07/03/2006	*P. damselae piscicida*	GSB	Land	Italy
262/08	31/10/2008	*P. damselae piscicida*	GSB	Cage	Tunisia
243/10	19/10/2010	*P. damselae piscicida*	ESG	Cage	Italy
97/14	01/04/2014	*P. damselae piscicida*	GSB	Land	Italy
325/C/14	25/09/2014	*P. damselae piscicida*	ESG	Cage	Italy
352/B/14	03/10/2014	*P. damselae piscicida*	ESG	Land	Italy
395/14	28/10/2014	*P. damselae piscicida*	ESG	Cage	Italy
396/14	28/10/2014	*P. damselae piscicida*	GSB	Cage	Italy
399/14	28/10/2014	*P. damselae piscicida*	ESG	Land	Italy
277/A/15	28/10/2015	*P. damselae piscicida*	GSB	Land	Italy
278/16	26/08/2016	*P. damselae piscicida*	ESG	Cage	Italy
305/15	25/11/2015	*P. damselae piscicida*	ESG	Cage	Italy
335/15	22/12/2015	*P. damselae piscicida*	ESG	Cage	Italy
340/16	06/10/2016	*P. damselae piscicida*	GSB	Land	Italy
342/A/16	07/10/2016	*P. damselae piscicida*	GSB	Land	Italy
209/B/11	01/12/2011	*V. harveyi*	ESG	Land	Italy
180/12	19/09/2012	*V. harveyi*	ESG	Land	Italy
212/13	17/07/2013	*V. harveyi*	GSB	Land	Italy
92/14	28/03/2014	*V. harveyi*	ESG	Land	Italy
471/14	11/12/2014	*V. harveyi*	ESG	Cage	Italy
194/A/16	09/06/2016	*V. harveyi*	ESG	Cage	Italy
205/16	22/06/2016	*V. harveyi*	GSB	Cage	Albania
230/A/16	13/07/2016	*V. harveyi*	GSB	Land	Italy
231/F/16	14/07/2016	*V. harveyi*	GSB	Cage	Italy
251/16	26/07/2016	*V. harveyi*	ESG	Cage	Italy
259/16	26/07/2016	*V. harveyi*	GSB	Land	Italy
278/16	26/08/2016	*V. harveyi*	ESG	Cage	Italy

314/16	20/09/2016	*V. harveyi*	ESG	Land	Italy
337/16	05/10/2016	*V. harveyi*	GSB	Cage	Albania
363/F/16	25/10/2016	*V. harveyi*	GSB	Land	Italy
383/16	09/11/2016	*V. harveyi*	ESG	Land	Italy
422/C/16	07/12/2016	*V. harveyi*	GSB	Land	Italy
140/17	19/04/2017	*V. harveyi*	ESG	Land	Italy
179/B/17	26/05/2017	*V. harveyi*	ESG	Land	Italy
192/B/17	13/06/2017	*V. harveyi*	GSB	Cage	Italy
215/A/17	04/07/2017	*V. harveyi*	ESG	Cage	Italy
216/17	06/07/2017	*V. harveyi*	GSB	Cage	Italy
234/17	21/07/2017	*V. harveyi*	ESG	Cage	Italy
236/17	27/07/2017	*V. harveyi*	ESG	Cage	Italy
215/C/2018	08/11/2018	*V. harveyi*	ESG	Land	Italy

The *Vibrio* species were firstly identified by API 20E system (Biomerieux) and biochemical keys reported by Noguerola and Blanch (2008). The codes obtained were: 3047524–3047526 and 4344525-4346125 for *V. anguillarum* and *V. harveyi,* respectively. Additionally, agglutination latex tests with Mono-Va kit (Bionor, Skien, Norway) for *V. anguillarum* and the 16S rDNA PCR for *V. harveyi* (Oakey *et al*., 2003) were performed. With regard to *P. damselae* susp. *piscicida* all the strains were identified by API20 E system (Biomerieux) (code 2005004), by agglutination latex test Mono-Pp (Bionor, Skien, Norway) and by 16S-*ureC* PCR (Osorio *et al.*, 2000). The whole genome sequences (NGS) performed by the partner AQUAxprs has confirmed the dientification of the isolates. The MICs are being tested for five different antibiotic molecules (flumequine, oxitetraciclyne, florfenicol, amoxicillin and trimethoprim/sulfadiazine) and the whole genome sequences (NGS) are being analyzed to detect any genes of resistance to the same antibiotics.

5.4.7 MIC assays

MICs were determined for all strains of *V. anguillarum* and eight (8) of *V. harveyi* using the protocol recommended by the CLSI guideline VET04-A2 (CLSI 2014b) for macrodiluition assays. Antibiotics were selected among the most used in aquaculture: flumequine (UB), oxytetracycline (OT), florfenicol (FFC), amoxicillin (AMX), trimethoprim/sulphamethoxazole (SxT).

Antibiotic powders (Sigma-Aldrich, St. Louis, USA) were weighed, dissolved in appropriate solvents to obtain stock solutions (5120µg/mL) and stored at -80°C. Each antibiotic stock solution was diluted 1:10 into Cation-Adjusted Mueller Hinton Broth (CAMHB, Oxoid, Basingstoke, UK) to obtain 512 µg/mL concentration. The preparation of each microtiter plate was carried out with 12 two-fold serial dilutions of each antibiotic stock solution. The final antibiotic concentrations ranging between 0.015 µg/mL and 256 µg/mL (0.015/0.29-32/608 µg/mL for SxT antibiotic) were obtained. Isolates were sub-cultured twice on Trypticase Soy Agar (TSA)+1.5% NaCl (Oxoid, Basingstoke, UK). After overnight incubation at 25°±1C, two or more colonies were picked from TSA agar plates and transferred into sterile saline solution (0.85% w/v) obtaining a 0.5 McFarland turbidity suspension. In order to adjust the concentration to about 10^6cfu/mL bacterial suspensions were diluted in CAMHB. Fifty microliters of the final suspension were dispensed onto microtiter wells containing 50 µL of each antimicrobial agent dilutions. The reference strain *E. coli* ATCC 25922 was used as quality control. Then each

microplate was incubated under aerobic conditions at 28±2°C for 24-28 hours (CLSI, 2014). The MIC_{50}, MIC_{90} values and MIC range of each antimicrobial agent were also determined (**Table 22**).

Table 22. Minimum Inhibitory Concentrations (MIC) values of 35 Vibrio isolates from from sea bass and seabream (MIC_{50} and MIC_{90}, MIC range).

N° strains	Strain	Drug	MIC $_{50}$ µg/mL	MIC $_{90}$ µg/mL	MIC range
27	*V. anguillarum*	UB	0.12	0.25	0.06-0.25
		OT	0.5	0.5	0.25-64
		FFC	0.5	0.5	0.25-1
		AMX	8	8	4-16
		SxT	0,03/0.59	0.06/1.119	0.03/0.59-0.5/9.5
8	*V. harveyi*	UB	0.5	0.5	0.25-0.5
		OT	0.25	0.5	0.25-0.5
		FFC	1	1	0.5-1
		AMX	>256	>256	>256
		SxT	0.12/2.38	0.12/2.38	0.12/2.38
35	*Vibrio* spp. *(V. anguillarum + V. harveyi)*	UB	0.12	0.5	0.06-0.5
		OT	0.5	0.5	0.25-64
		FFC	0.5	1	0.25-1
		AMX	8	>256	4>256
		SxT	0.06/1.19	0.12/2.38	0.03/0.59-0.5/9.5

Legend: UB (flumequine), OT (oxytetracycline), FFC (florphenicol), AMX (amoxicillin), SxT (trimethoprim/sulphamethoxazole).

The MIC values have still to be defined for the other *V. harveyi* strains and all the *P. damselae* subsp. *piscicida* strains already selected and identified in order to determine a provisional Epidemiological cut-off value by using a automated and freely available Excel spreadsheet calculators to apply the normalized resistance interpretation (NRI) method (Kronvall, 2010) [available at http://www.bioscand.se/nri/ with permission from the patent holder, Bioscand AB, TÄBY, Sweden (European patent No 1383913, US Patent No. 7,465,559)] and ECOFFinder MS (available at http://clsi.org/standards/micro/ecoffinder/) (Baron et al., 2017).

However, since indicated as recently necessary by Smith (2019) in a reference document on performance of antimicrobial susceptibility testing programmes relevant to aquaculture and aquaculture products (FAO Fisheries and Aquaculture, Circular No. 1191), to support the value of Epidemiological cut-off obtained by us it has become indispensable to increase the number of bacterial strains to be submitted to MIC studies. In fact, at least 100 wild type isolates generated by at least three independent laboratories are required for each bacterial species.

In fact, on the basis of Smith's recommendations, for setting a new ECVs it's important to consider:

- *the source of strains sets:* the susceptibility measures, MIC, distributions for WT members of a species are assumed to be the same independent of the geographical location or the host from which they were obtained;
- *the quantity and quality of data:* the Aquatic Working Group of CLSI is currently considering a proposal that 100 WT isolates generated from at least three independent laboratories with no one laboratory contributing over 50 percent of the observations would be sufficient. All the data must have been established by use of the relevant standard test protocol.

For these reasons, following also what emerged from the workshop "Transnational study to define Epidemiological cut-off values (ECV) for *Vibrio* and other aquatic bacteria" held by Smith at EAFP conference in Porto, Portugal, on 12 September 2019 and as agreed with WP3 leader, a MedAid-PerformFISH "concerted action" has been started in order to participate in a wide European study to determine ECV for *Vibrio anguillarum* and *V. harveyi* strains collected in the Mediterranean basin. Unfortunately, this collaborative research activity was stopped due to Covid-19 pandemia and has been postponed beyond M36, together with the implementation of the last phase of the development of the Perform-TEST.

Furthermore, in order to improve further differentiation of wild-type from non-wild-type isolates, the whole genome sequencing performed by AQUAxprs colleagues within task 3.3 activities on strains from ESG and GSB (see Table 21) is being analyzed to detect any gene of resistance to the selected antibiotics, in collaboration with Dr Guido Cordoni (University of Surrey, Guildford, UK).

At this regard, 148 sequences from 74 strains (27 *V. anguillarum*, 25 *V. harveyi* and 22 *Photobacterium damselae* subsp. *piscicida*) were obtained using Illumina platform. R1 and R2 sequences of each strain were assembled with the software shovill (spades assembler option) and the high-performance computing facility of the University of Surrey. Once obtained the contigs we annotated them using the software PROKKA. The files will be released on relevant public repositories (NCBI, EBI) once a paper on major peer reviewed journal will be published.

Different comparative genomics approaches are being performed in order to properly assess the phylogeny of our strains:

1) Whole genome phylogeny (pangenomics) (Roary/Scoary software)

2) Targeted features phylogeny (Laboratory data, data coming from contigs such as MLST (MLST software), AMR (Abricate), virulence factors (Pathfinder). All these data will be organised in a binary table and phylogenetic trees will be calculated by using appropriate distance and character based (Bayesian) algorithms (PAST and BEAST software).

Unfortunately, due to the Covid-19 pandemic not all activities have been completed. Only when ECV for each bacterial species and selected antibiotics will be defined, it will be possible to determine the amount (µg/ml) of antibiotic supplement for Perform-plates in order to finalize the setup of the method.

5.5 Multiplex PCR for simoultanous detection of gilthead sea bream enteric parasites

Although not explicitly included in DoA, in the light of the increasing importance of some "cryptic" enteric parasites in farmed gilthead seabream, such as the microsporidian *Enterospora nucleophila* and the apicomplexan *Cryptosporidium* sp., a new diagnostic method for their simultaneous detection and identification (multiplex PCR) is being developed within PerformFISH project, also based on results from ParaFishControl project, completed on March 31st, 2020. Unfortunately, the Covid-19 pandemic caused a forced halt of the activities before the method was fully developed, which must necessarily be postponed beyond month 36.

6 Final considerations

Diagnosis of fish diseases is a complex process that includes several steps, from clinical and anatomopathological observations up to various laboratory techniques aimed to ascertain the cause/causes of disease. If done properly, diagnosis represents a crucial element in fish health management, not only to conduct an effective control of a disease outbreak but also to implement appropriate prevention and surveillance plans. For these reasons, a reliable diagnosis of fish diseases must be based on the correct and specific identification of the pathogens by laboratory methods.

The OIE-World Organization for Animal Health, through the continuously updated and freely accessible online Diagnostic Manual of Aquatic Animal Diseases (https://www.oie.int/en/standard-setting/aquatic-manual/access-online/), provides standard procedures for effective laboratory testing for specific fish diseases with relevance at global level. However, for specific Mediterranean scenario, among these only VNN is of relevance for Mediterranean marine fish. Therefore, a relevant effort on diagnostics on the most relevant diseases in the Mediterranean finfish aquaculture was included in PerformFish objectives.

Therefore, the improvement and strengthening of diagnostic methods for major European sea bass and gilthead sea bream pathogens are key elements to face the recurrent or emergent diseases that can undermine productivity and sustainability of Mediterranean mariculture. For this reason, several WP3 activities in PerformFish project have aimed at improving already available diagnostic methods or developing new techniques that may be of support to industry for a rapid and reliable detection of the main pathogens of European sea bass and gilthead sea bream.

In particular, as a response of the fast and relevant epidemiological changes in Betanodavirus, a new technique for the simultaneous detection of different Betanodavirus strains, including emerging reassortants, has been set up with a multiplex PCR approach, relatively rapid and cost-effective if compared with standard virological/molecular assays. The development of a one-step multiplex RT-PCR assay to detect the presence of NNV and to discriminate between RGNNV, SJNNV genotypes from the RGNNV/SJNNV and SJNNV/RGNNV reassortant strains and to identify possible co-infections can represent a useful tool for routine diagnosis and surveillance programs.

Concerning main bacterial pathogens of European sea bass and gilthead sea bream, i.e. *Vibrio anguillarum* and *Photobacterium damselase* subsp. *piscicida*, the improvement of molecular characterization and identification of *Vibrio anguillarum* strains circulating in the Mediterranean

area and of MALDI-TOF-MS application for the identification of both pathogens provides useful elements for confirmatory diagnosis and epidemiological studies. These new developments will also allow a much efficient assessment of the recently found changes in pathogenicity of several strains, mainly found in *Photobacterium damselae* subs *piscicida* detected in East and West Mediterranean.

The preliminary setup of a molecular probe-based biosensor for *Vibrio anguillarum* detection may have an interesting application in the field to detect promptly its presence in critical production sites such as hatcheries, where *V. anguillarum* outbreaks can be devastating and more complex bacteriological protocols are required.

Also of interest for a field application, a method to test the antibiotic sensitivity of bacterial strains isolated during disease outbreak has been designed and preliminarily developed (Perfom-TEST) in order to support the industry to take fast and efficient decisions for antimicrobial therapeutics (see also PerformFISH deliverable 3.4).

Furthermore, a multiplex PCR to detect simultaneously different enteric parasites of gilthead sea bream, such as *Enterospora* and *Cryptosporidium*, is being developed to improve the diagnostic capacity towards these cryptic parasites both for routine diagnosis and for surveillance plans on fry and juveniles.

All these new improvements in diagnostic techniques increase the diagnostic capacity in different aspects (sensitivity, available spectrum of pathogens in a single probe, diagnostic speed…) and also the TRL of some of these techniques. However, all these new techniques should be used together with all the other available diagnostic techniques also reviewed in this document. Only with suitable diagnostic protocols under efficient disease surveillance programs in farms and with precise and in real-time information available for the fish health specialists it will be possible to have early warnings, precise identification and fast and efficient reactions to keep infectious and parasitic diseases of reared Mediterranean finfish species under control. Finally, we would like to highlight the relevance of the interconnection of diagnostics with all the different aspects of the fish health and disease management (disease risk assessment, epidemiology, prevention, welfare and therapeutics) also displayed in PerformFish WP3.

Laboratory research activities delayed by Covid-19 pandemic will resume and be completed as soon as allowed by the improvement of the sanitary restrictions, and further scientific papers related to the different aspects of diagnostics developed in PerformFish project are underway and will be published in the next months.

7 List of Tables

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9 References

SECTION 4.1 VIRAL ENCEPHALOPATHY AND RETINOPATHY (VNN) BY BETANODAVIRUS

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SECTION 4.3 PHOTOBACTERIOSIS BY PHOTOBACTERIUM DAMSELAE SUBSP. PISCICIDA

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SECTION 5.3 MALDI-TOF MS FOR V. ANGUILLARUM AND PHOTOBACTERIUM DAMSELAE SUBSP. PISCICIDA IDENTIFICATION

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SECTION 5.4 DEVELOPMENT OF A FIELD ANTIBIOTIC SENSITIVITY TEST - "PERFORM-TEST"

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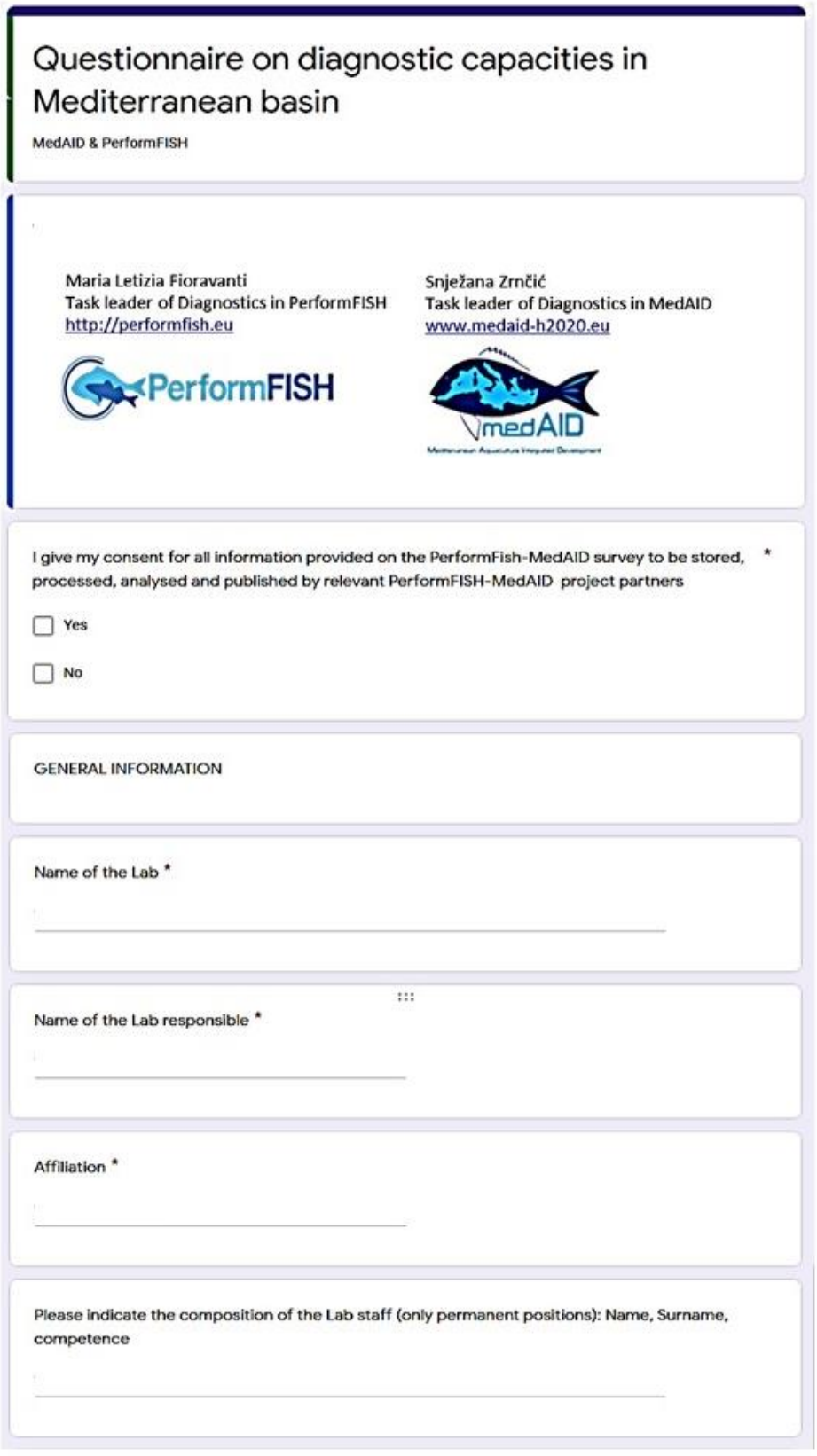
10 Annex 1. Questionnaire on diagnostic capacity in the Mediterranean aquaculture



Questionnaire on diagnostic capacities in Mediterranean basin

MedAID & PerformFISH

Maria Letizia Fioravanti
Task leader of Diagnostics in PerformFISH
http://performfish.eu

Snježana Zrnčić
Task leader of Diagnostics in MedAID
www.medaid-h2020.eu

I give my consent for all information provided on the PerformFish-MedAID survey to be stored, processed, analysed and published by relevant PerformFISH-MedAID project partners *

- [ ] Yes
- [ ] No

GENERAL INFORMATION

Name of the Lab *

Name of the Lab responsible *

Affiliation *

Please indicate the composition of the Lab staff (only permanent positions): Name, Surname, competence

Is your Lab private or public? *

- ◯ Private
- ◯ Public

Which is your main field of activity in fish health diagnostic? *

- ◯ Diagnostics for research
- ◯ Diagnostics for clinical purposes
- ◯ Both

If both, please specify in which proportion

_____

Do you receive fish in the Lab or do you collect fish samples in the field? *

- ◯ In the Lab
- ◯ In the field
- ◯ Both

If both, please specify in which proportion

_____

SPECIFIC DIAGNOSTICS INFORMATION

Kind of diagnostic activities carried out in your Lab

Clinical examination / Necropsy

Clinical examination *

- ○ yes
- ○ No

Necropsy *

- ○ yes
- ○ No

Parasitology

Do you commonly perform parasitological exams in your Lab? *

- ○ yes
- ○ no

If yes, please specify methodology

- ☐ Fresh mount examination
- ☐ Stained smears
- ☐ Molecular methods
- ☐ Electron microscopy (TEM, SEM)
- ☐ Other

Level of taxonomic identification

- ○ Species
- ○ Genus
- ○ Family
- ○ other

Specify the staining methods you use for detection of certain parasites

Specify the parasite and molecular methods you use for diagnostics

Specify the EM you use for detection of certain parasites

Please specify which parasites are more commonly diagnosed in European sea bass in your Lab

Please specify which parasites are more commonly diagnosed in gilthead sea bream in your Lab

Bacteriology

Do you commonly perform bacteriological exams in your Lab? *

- ○ Yes
- ○ No

Which methods are used in your Lab for isolation of bacteria?

- ☐ Isolation of bacteria on non-selective media
- ☐ Isolation of bacteria on selective media

Please specify which non-selective media

Please specify which selective media

Organs used for bacteriological exams

Phenotypical identification

- ○ yes
- ○ no

If yes, please specify:

- ☐ Traditional biochemical tests
- ☐ Commercial systems (e.g. API)

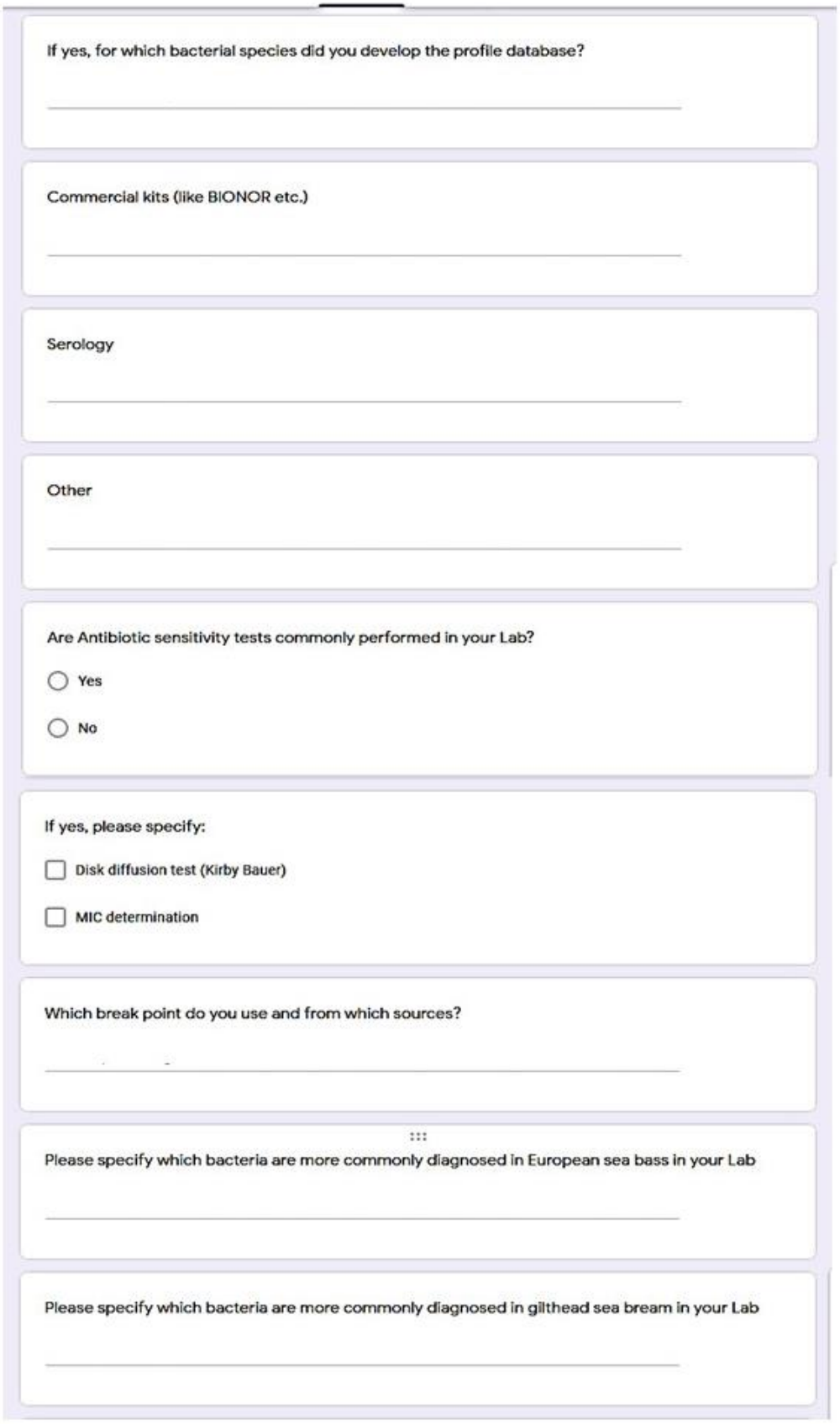
Molecular identification

- ○ yes
- ○ No

Specify molecular methods you use to identify particular bacteria (specify bacteria)

MALDI-TOF

- ○ yes
- ○ No



If yes, for which bacterial species did you develop the profile database?

Commercial kits (like BIONOR etc.)

Serology

Other

Are Antibiotic sensitivity tests commonly performed in your Lab?

- ○ Yes
- ○ No

If yes, please specify:

- ☐ Disk diffusion test (Kirby Bauer)
- ☐ MIC determination

Which break point do you use and from which sources?

Please specify which bacteria are more commonly diagnosed in European sea bass in your Lab

Please specify which bacteria are more commonly diagnosed in gilthead sea bream in your Lab

Virology

Do you commonly perform virological exams in your Lab? *

- ◯ Yes
- ◯ No

Which methods are used in your Lab for diagnostics of viral diseases?

- ☐ Cell culture isolation
- ☐ Molecular techniques
- ☐ TEM
- ☐ Altro...

Please specify which cell lines are used in your Lab and for which viruses

Please specify which molecular techniques are used in your Lab and for which viruses

Please specify which viruses are more commonly diagnosed in European sea bass in your Lab

Please specify which viruses are more commonly diagnosed in gilthead sea bream in your Lab

OTHER DIAGNOSTIC METHODS

Do you commonly perform histology in your Lab? *

- Yes
- No

If yes, for which pathogens?

Main staining techniques

Do you perform immunohistochemistry in your Lab?

- Yes
- No

If yes, for which pathogens?

Do you perform In situ hybridization in your Lab?

- yes
- No

If yes, for which pathogens?

Do you perform immunological methods in your Lab?

- ◯ yes
- ◯ No

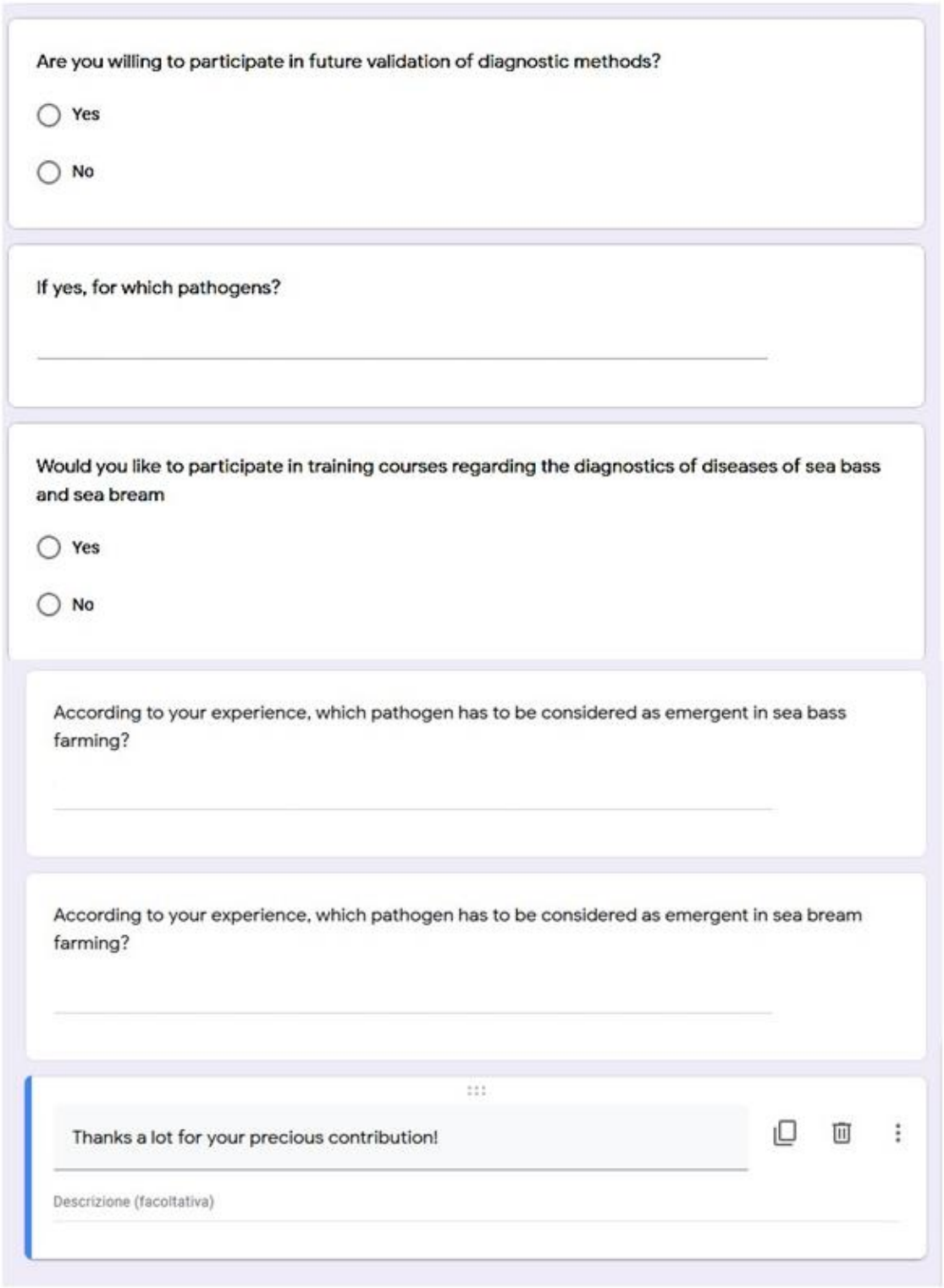
If yes, which methods and for which pathogens/purposes?

Do you perform analysis by NGS

- ◯ Yes
- ◯ No

If you have any other information on your diagnostic activities to share with us, please, describe (for example other methods applied in your Lab or other diagnostic fields such as toxicology, nutritional/metabolic disorders, etc.)

ADDITIONAL INFORMATION


Are you willing to participate in future validation of diagnostic methods?
Yes
No
If yes, for which pathogens?
Would you like to participate in training courses regarding the diagnostics of diseases of sea bass and sea bream
Yes
No
According to your experience, which pathogen has to be considered as emergent in sea bass farming?
According to your experience, which pathogen has to be considered as emergent in sea bream farming?
Thanks a lot for your precious contribution!
Descrizione (facoltativa)