WORKSHOP REPORT

# Vibriosis in aquaculture. 16th EAFP Conference, Tampere, Finland, 4th September 2013

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## Introduction

Aquaculture in brackish and marine water is growing worldwide (FAO, 2014), including new cultured species. Various *Vibrio* infections occur, and cause significant problems, in fish, crustacean and shellfish (Austin and Austin, 2007). *Vibrio anguillarum*, *V. salmonicida*, *V. ordalii* and *V. vulnificus* are among the pathogens that lead to the biggest losses in aquaculture all over the world (Toranzo et al., 2005; Sandlund et al., 2010; Sitjà-Bobadilla et al., 2014).

*Vibrio* species are ubiquitous in aquatic ecosystems. They are thermo dependent bacteria and are able to persist in seawater (Marco-Noales et al., 1999). Some of the *Vibrio* spp. are potential zoonotic to humans for years, causing risk of zoonosis in aquaculture professionals and in consumers of aquaculture products (Amaro and Biosca, 1996; Austin, 2010).

To address the issues of concern on vibriosis, a workshop on "Vibriosis in aquaculture "was organized at the 2013 EAFP Conference. In this workshop, 65 experts participated. The newest field observations and research findings on vibriosis in aquaculture were presented, with the aim to illustrate the size of the problem in various geographic regions of Europe, try to define risks, novel techniques in diagnostics, and discuss therapy and preventive measures, including use of vaccines.

## Vibriosis in warm water fish

### *Vibriosis in sea bream, sea bass and meagre in Spain*

Spanish Mediterranean warm water fish culture is located off shore in cages. Cultured species are mainly sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), and to a lesser degree

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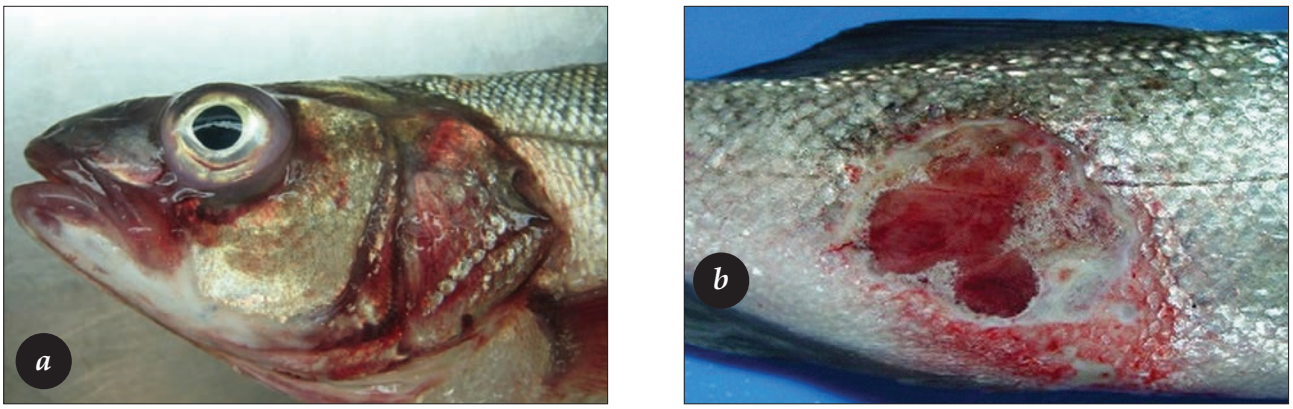


**Figure 1.***Vibrio anguillarum* infection in sea bass. A: Exophthalmus lesions, haemorrhages (picture by M. Isern). B: Tail ulcers (picture by S. Zrnčić).

meagre (*Argyrosomus regius*)(APROMAR 2012). Since 2005, *V. anguillarum* serotype O1 has caused diseases in mainly sea bass (Figure 1a) at 13ºC to 24ºC, but also in meagre in off shore cages. The monthly mortality varied from 1% in large fish (1 kg) to 30% in medium sized fish (150 g), without the right treatment. Vibriosis outbreaks also occurred in hatcheries and subsequent juvenile facilities (Frans et al., 2011). *V. anguillarum* serotype O2 was isolated as secondary pathogen in diseased sea bream suffering winter disease, frequently associated to *Pseudomonas anguilliseptica*. Early detection and quick application of antibiotics by effective oral treatment (flumequine, oxytetracycline, sulphonamides) were basic to reduce the vibriosis impact. However, vaccination was found the best and cost effective strategy to avoid vibriosis in seabass.

***Vibriosis in sea bass in Croatia***

Sea bass cultivation in Croatia started in parallel with farming in other Mediterranean regions, currently at fifty farms of different size (Katavić, 1994). Disease control is inevitable for economic production (Le Breton, 1999). Vibriosis caused by *V. anguillarum* serotype O1 is a limiting factor in sea bass culture in the Mediterranean region (Toranzo et al., 2005; Sitjà-Bobadilla et al., 2014) and still is among the most damaging diseases at Croatian farms (Figure 1b),with total mortalities from 20% - 50% during the on-growing period, if proper treatment is not applied.

Twenty years of epizootiological studies revealed that environmental conditions preceding vibriosis outbreaks have changed. Until 2004, acute forms occurred only during rapid increase of sea water temperatures, from 17 to 19ºC and a decrease in autumn. The last few years acute outbreaks occurred even in summer, while in winter, chronical forms of mixed infections by *V. anguillarum* and *Tenacibaculum maritimum* appeared. The outbreaks are usually controlled by antibiotic treatments via feed. Resistance to oxytetracycline and potentiated sulphonamide was observed. Therefore, efforts are made to implement preventive programmes of nonspecific immunomodulation and vaccination.

***Vibrio vulnificus in eel, in Spain and in the Netherlands***

*V. vulnificus* (Vv) can be pathogenic for humans, shrimps and fish (Farmer, 1979; Tison et al., 1982).The bacterium is very heterogeneous, comprising of 3 biotypes (Oliver, 2006) and

more than 9 serovars (Ser). Biotype 2 (Vv BT2), with a worldwide distribution, is the only biotype related to epizootics of fish (mainly eel) (Fouz et al., 2010; Høi et al., 1998). It is frequently isolated from fish sites in temperate and tropical regions.

Vv BT 2 originated from Asia, arrived in Spain in 1989, and spread to other European countries in the 90ies. It is the etiological agent of vibriosis of warm water fish, a hemorrhagic septicemia that mainly affects eel (*Anguilla anguilla*), tilapia *(Oreochromis niloticus),* derbio *(Trachinotus ovatus),*and sea bass *(Dicentrarchus labrax),* but also rainbow trout *(Oncorhynchus mykiss),* especially in fish culture (Dalsgaard et al., 1999; Li et al., 2006; Chen et al., 2006; Pedersen et al., 2008; Longyant et al., 2008). Vv Ser E was first isolated from eel (Tison et al., 1982), and may sporadically cause human septicemia (Amaro and Biosca, 1996).

In Spain, use of lower salinities (0.1-0.2 %) in the water of fish farms to prevent for and inhibit Vv outbreaks resulted in occurrence of new serovars of Vv in fish in 2000 (Ser A & I), which have spread over Europe since 2004 (Fouz et al., 2006, 2010). Biotype 2 is polyphyletic and probably emerged in the fish farming environment from commensal strains (Sanjuán et al., 2011). Eel vibriosis (Figure 2) in brackish water is mainly due to serovar E, showing external ulcers, but in freshwater due to serovars A or I, showing jaw degradation as a differential clinical sign (Fouz et al., 2006).

In the Netherlands, during 1996-2009, 23 outbreaks of Vv were diagnosed at eight indoor eel farms (Haenen et al., 2014). One eel farmer suffered a necrotic fasciitis by Vv (Dijkstra et al., 2009). The simultaneously isolated zoonotic strains (ST 112) of Vv from eels from that eel farm and from its eel farmer were identical (Haenen et al., 2014).

## Vibriosis in cold water fish

### *Vibriosis in cold water fish in Norway*

Norway has a big production of Atlantic salmon, and minor production of rainbow trout, Atlantic cod *(Gadus morhua)*, halibut *(Hippoglossus hippoglossus)* and Arctic char *(Salvelinus alpinus)* (Fisheries Do, 2013), and cleaner fish as wrasse *(Labroides dimidiatus)* and lumpfish *(Cyclopterus lumpus)*.

Vibriosis caused by *V. anguillarum*, *V. ordalii*, *Aliivibrio (Vibrio) salmonicida, V. splendidus, V. logei* and *V. tapetis* is still a problem in Norwegian fish farming (Johansen, 2013).

*V. anguillarum*, serotypes O1, O2a, O2a-biotype II, O2b are commonly isolated: serotype O1 from rainbow trout, salmon and cleaner fish, and serotype O2b from cleaner fish, lumpfish and cod (Johansen, 2013). The new sero-subgroup O2a-biotype II has also been found in cod (Mikkelsen et al., 2011). Vibriosis outbreaks in cod farming by *V.anguillarum* are triggered by stressors as high temperature (>14°C), handling and vaccination.

In 2012, after a time gap several new outbreaks of coldwater vibriosis caused by *Aliivibrio salmonicida* occurred in salmon farms in the northern part of Norway. The disease has been under control by vaccination since 1987, and it was speculated that this re-occurrence was due to increased infection pressure or inadequate vaccine regimes (Colquhoun et al., 2013). These new isolates were similar to previous ones shown by 16S rRNA

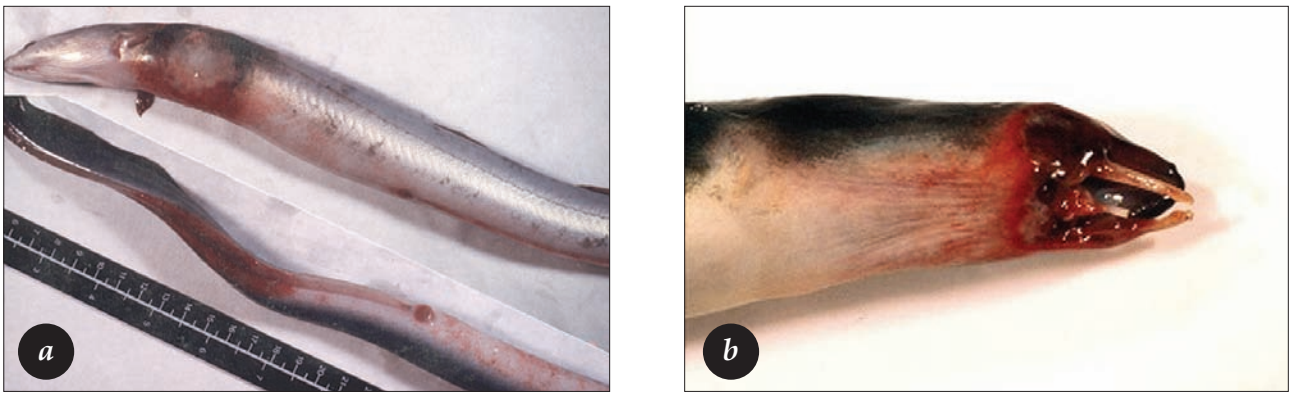


**Figure 2.** Eel with a *Vibrio vulnificus* infection (pictures by B. Fouz and C. Amaro). A: Eel vibriosis in brackish water due to *V. vulnificus* biotype 2 serovar E. External ulcers are the differential clinical sign. B: Eel vibriosis in freshwater due to *V. vulnificus* biotype 2 serovar A or I showing jaw degradation as a differential clinical sign.

sequencing, immunoblotting and vaccine studies. *Aliivibrio salmonicida* has also been isolated from cod larvae (Mikkelsen et al., 2011).

## Vibriosis in marine molluscs

In Europe, mollusc culture is regularly affected by various disease outbreaks with high losses, as reported at annual meetings of EU Reference Laboratory annually (http://www.eurl-mollusc.eu) and OIE (www.oie.int). Some *Vibrio* species grow in molluscs, and some were reported in association with disease and mortality among bivalve larvae, juvenile, and adult oysters (Table 1). As examples, *V. aestuarianus*, *V. splendidus* and *V. harveyi* were associated with massive mortality outbreaks in Pacific cupped oysters *(Cassostrea gigas)* reared in open sea tidal areas (Gay et al., 2004; Garnier et al., 2008; Saulnier et al., 2010). Many of the strains from the *V. splendidus*- and *V. harveyi*-clades are present in healthy oyster. Epidemiological studies of the isolated *V. splendidus*-related bacterial species showed a high genetic diversity, suggesting a polyphyletic nature of this bacterial group (Le Roux et al., 2002). Infection trials with *V. aestuarianus* under experimental conditions (immersion, cohabitation and injection) showed, that almost all strains isolated from moribund oysters were virulent. Only 100 injected bacteria could induce up to 95% oyster mortality. In moribund oysters, bacterial concentrations up to $10^7$ bacteria/ml in hemolymph were reached.

## Diagnostic methods, therapy and prevention of vibriosis

### *Diagnostic methods*

Isolation of *Vibrio* spp. from fish can be done on marine agar, BHIA, TSA supplemented with 5% (v/v) blood and NaCl at 0.5 – 3.5 % (w/v), and on TCBS agar with incubation at 15-25°C (Austin and Austin, 2007). Species identification by biochemistry is difficult. Biotyping (Tison et al., 1982; Bisharat et al., 1999) and serological tests (Romalde et al., 1995) are used to subtype some *Vibrio* species: for *V. vulnificus* biotype 2, immunoblotting of O-antigen (Biosca et al., 1997) and agglutination tests (Fouz et al., 2006) are used. Serotyping of *V. anguillarum* in Norway and Denmark are used to evaluate the efficacy of vaccines and to distinguish between pathogenic and environmental bacteria.

A multiplex PCR assay that targets specific virulence-related sequences has been optimized for

**Table 1.** *Vibrio* species detected in diseased mollusc species in Europe and some third countries.

| *Vibrio species in mollusc species of stage* | *V. aestuarianus* | *V. splendidus clade* | *V. pectinicida* | *V. harveyi clade* | *V. tapetis* | *V. coraali ilyticus clade* | *V. tubiashii* | *V. fortis* |
|---|---|---|---|---|---|---|---|---|
| ***Crassostrea gigas*** | | | | | | | | |
| Larva | | Outside Europe [Japan,3] | | | | France [13] | England, US [15,16] | UK [19] |
| Spat | France [1,2] | | | France [8] | | | US [16] | |
| Adult | France [1,2] | France [1,4] | | | | | | |
| ***Crassostrea virginica*** | | | | | | | | |
| Juvenile | | | | | | | Outside Europe [USA,17,18] | |
| ***Ostrea edulis*** | | | | | | | | |
| Larva | | | | | | Spain [14] | UK [15] | |
| ***Venerupis decussatus*** | | | | | | | | |
| Larva | | Outside Europe [China, 5] | | Spain [5] | | | | |
| Adult | | | | | Spain, France [10, 11] | | | |
| ***Venerupis philippinarum*** | | | | | | | | |
| Seed | | France [4] | | | | | | |
| Adult | | | | | Spain, France [11, 12] | | | |
| ***Pecten maximus*** | | | | | | | | |
| Larva | | Norway [6] | France [7] | | | | | |
| ***Haliotis tuberculata*** | | | | | | | | |
| Adult | | | | France [9] | | | | |

[1] Garnier et al., (2007) *Microbial Ecol.* **53**, 187–196. [2] Garnier et al., (2008) *Syst Appl Microbiol* **31**, 358-365. [3] Sugumar et al.,(1998) *Dis Aquat Org* **33**, 111-118. [4] Gay et al., (2003) *J Shellfish Res* **22**, 1. [5] Gómez-Leónet al., (2005) *Appl. Environm Microbiol* **71**, 98-104. [6] Torkildsen et al., (2005) *Aquac Int* **13**, 575-592. [7] Lambertet al., (1998*) J Syst. Bact.* **48**, 481-487. [8] Saulnier et al., (2010) *Microbial Ecol* **59**, 787-798. [9] Nicolas et al., (2002) *Dis Aquat Org* **50**, 35-43. [10] Allam et al., (2001) *Developm Comp Immunol* **25**, 365-375. [11] Novoa et al., (1998) *J Invertebr Pathol* **71**, 34-41. [12] Borrego et al., (1996) *Dis Aquat Org* **46**, 480-484. [13] Kesarcodi-Watson et al., (2012) *Aquac* **344–349**, 29–34. [14] Prado et al., (2005) *Dis Aquat Org* **67**, 209-215. [15] Jeffries, (1982) *Aquac* **29**, 201-226. [16] Elston et al., (2008) *Dis Aquat Org* **82**, 119-134.[17] Tubiash et al., (1965) *J Bact* **90**, 1036-1044. [18] Tubiash et al., (1970) *J Bact* **103**, 272-273. [19] Thompson et al., (2003) *Int J Syst Evol Microbiol* **53**, 1495-1501.

diagnosis of *V. vulnificus* (Sanjuán and Amaro, 2007). An adapted (Multi-Locus Sequence Typing) MLST method was used for typing *V. vulnificus* isolates from outbreaks in Dutch eel farms (Haenen et al., 2014), and another MLST method was developed for characterization of the *V. anguillarum* sero-sub group O2a biotype II in Norway (Mikkelsen, unpubl. results).

MALDI-TOF (MALDI = Matrix-assisted-laser-desorption/ionization, TOF =Time-of-Flight) is a new, cheap, fast and efficient protein based technique for diagnosis and subtyping of bacteria a.o. (Dare, 2006; Dieckmann et al., 2010). The most important human *Vibrio* species are in the data base, but the data base needs to be extended with more of the aquatic *Vibrio* species. A joint project with participation of SVA (Sweden), DTU Vet (Denmark) and CVI (the Netherlands) deals with this area.

For molluscs, for typing of *V. splendidus*-related species from Pacific cupped oysters *(Crassostrea gigas)* biochemistry failed, and DNA gyrase subunit B (*gyrB*) gene sequencing was used. Several strains clustered, but no known *Vibrio* species were found (Le Roux et al., 2004). Taxonomic analysis of unidentified isolates with gene sequencing, fluorescent amplified-fragment length polymorphism (FAFLP) fingerprinting, DNA-DNA hybridization and biochemical tests defined new species (Le Roux et al., 2002; Thompson et al., 2005; Beaz-Hidalgo et al., 2010). Additionally, histopathology, *in situ* hybridization and Transmission Electron Microscopy (TEM) are used.

***Therapy***

Antibiogram results of vibriosis by *V. anguillarum* in sea bream and sea bass in Spain were stable among outbreaks, cages and season. Flumequine, oxytetracyclines, sulfonamides (+trimethoprim) and florfenicol were effective treatments via feed in summer. However, during winter months, due to low feed intake and usually low concentration of antibiotics in premixes, fish cannot reach effective levels of antibiotics. Therefore, in winter, for off shore cage treatments, there is a need for adapted premixes with higher concentrations of antibiotics.

In Croatia, recently the number of resistant *V. anguillarum* isolates increased. As a result, a surveillance program of antimicrobial resistance has started at marine fish farms for potentiated sulphonamides, oxytetracycline, flumequine and florfenicol by disc diffusion test and minimal inhibitory concentrations (MIC). In Denmark all *V. anguillarum* serotype O1 and O2 were tested against potentiated sulphonamides and oxolinic acid and no resistance was seen so far.

***Prevention***

Vaccination of sea bass against *V. anguillarum* serotype O1 was effective, by immersion in hatchery followed by revaccination, either by immersion or injection, with a relative percentage of survival of 99% in large scale trials in Spain. An oral vaccine, Aquavac Vibrio Oral® for *Vibrio* is registered in the EU for trout, and is used under cascade for sea bass, with variable results in Croatia. In Spain it is not registered as a premix, so, feed factories are not allowed to use it.

In practice, almost all sea bass fingerlings are injected with vaccines from MSD, Novartis, Pharmaq, Hipra or Fatro, prior to on-growing, thereby avoiding the use of antibiotics. Injection procedures need to be optimized for use in

inland facilities before transferring the fish to offshore production, as so far i.p. vaccination is not an option off shore. Additionally, oral vaccine formulations for off shore application need to be optimized. As vibriosis spreads very rapidly in open offshore cages (Noga, 2011), even fish two miles away will show parallel outbreaks of vibriosis within appr. 10 days after a first outbreak. Therefore, communication between farmers on biosecurity strategies is important to control the disease, especially in unvaccinated fish.

In Spain, vaccination of eels by prolonged immersion in three doses against vibriosis by Vv Bt 2 has been highly effective, with over 90% survival (Amaro et al., 2000; Fouz et al., 2001; Esteve-Gassent et al., 2003) since 2000.

In Denmark, *V. anguillarum* causes disease in rainbow trout farms, if the fish are not vaccinated. The triple vaccine (with 2 serotypes of *V. anguillarum* and *Aeromonas salmonicida*) by injection seems to be effective against vibriosis.

In Norwegian cod farming, by including *V. anguillarum* O2a-biotype II in the vibriosis vaccine, high protection against the three sero-subgroups of *V. anguillarum* (Mikkelsen et al., 2007; 2011) was found. Multicomponent vaccines are used in salmon and in cod, which reduced use of antibiotics in salmon. In cod farming, vibriosis is still a big problem. Cod fry > 3 g respond well to dip vaccination in high concentrated antigen, but upgrading to industrial scale seems to be a challenge. Larger cod > 30 g obtained high protection after injection of an oil-based multicomponent vaccine including *V. anguillarum* O2a and O2b, and *Aeromonas salmonicida* subsp. *achromogenes.* To avoid vibriosis in cod larvae good hygiene and appropriate feeding regimes are practiced.

In mollusc culture in France, control consists of limiting oyster transfers during mortality, application of rearing practices which reduce disease spread and impact (right densities and regular surveillance), and selection of mollusc strains which are less susceptible to *Vibrio* species.

In farmed penaeid shrimp (like Pacific white shrimp *Litopenaeus vannamei* and Black tiger shrimp *Penaeus monodon*) in SE-Asia, the bacterin, *AquaVac Vibromax*® (MSD Animal Health) is registered for use. Laboratory and field trials were done, with a micro-encapsulated vaccine during 2 to 4 hours into freshly hatched *Artemia* spp. (Wongtavatchai et al., 2010; Powell et al., 2011). This revealed, that 1) AquaVac Vibromax® was the first widely tested product that showed benefits of vaccination for penaeid shrimp, as it improved the response against *V. parahaemolyticus* in shrimp; 2) Shrimp homogenates showed natural antibacterial activity against *V. anguillarum*; 3) Vibromax® increased the antibacterial activity towards some pathogenic bacteria; 4) AquaVac Vibromax® activated penaeid shrimps resisted negative effects of vibriosis on growth and feed conversion, resulting in higher production.

## Discussion

During the workshop discussion, some additional points were raised. Vibriosis occurs in various aquaculture species and systems in cold and warm geographic regions. *V. anguillarum* is easy to identify to species, but identification of other *Vibrio* spp. is difficult, due to continuous description of new species. Recently the MALDI-TOF is being adapted for identification of fish bacteria. The Bruker® database needs to

be extended with protein spectra of pathogenic bacteria from aquaculture. It was stressed, that also Asian isolates of well characterized *Vibrio* species from aquaculture animals need to be included in the MALDI-TOF database.

Concerning therapy, antibiotic sensitivity testing, according to CLSI (Clinical and Laboratory Standards Institute) norms, Mueller Hinton (MH) agar was recommend for use. As most *Vibrio* spp. are salt dependent, NaCl needs to be added to MH for *Vibrio* spp. growth, at the right temperature (CLSI).

Following antibiotic treatments, development of resistance of *Vibrio* spp. to various antibiotics has been found. This would make future treatments of fish ineffective, and might be a risk for consumers and farmers.

Secondary *Vibrio* species infections sometimes play a role. In Croatia, no problems related to *V. harveyi* were seen, but in Spain, this species was mostly found as a secondary bacterium in disease of non-vaccinated sea bass fingerlings, often primarily caused by *V. anguillarum* or *Photobacterium damselae* subsp. *piscicida.*

In Italy, *V. harveyi* harmed cage cultured sea bass, and vaccination was recommended (A. Manfrin, during discussion). *V. anguillarum* was diagnosed in the eighties in rainbow trout, when farmers fed fresh sea fish to these trout, causing vibriosis. A vibriosis vaccine protected the trout. However, the vaccine is not used anymore, as farmers stopped feeding fresh sea fish. In sea bass, vibriosis problems remain, although the situation improved through better husbandry and vaccination, but effective vaccines against *Vibrio* species are still needed.

In general, although there are effective vibriosis vaccines to some *Vibrio* species, developing new efficient ones is difficult, as *Vibrio* strains may change very fast, and several *Vibrio* species play a role in disease.

## Conclusion

In conclusion, the audience underlined the need for development and availability of effective vaccines against vibriosis caused by various *Vibrio* spp., thereby to reduce the amount of used antibiotics, which might also stop the development of antibiotic resistance seen in some areas. Furthermore, at fish farms, biosecurity must be increased. For molluscs production, vibriosis resistant genetic mollusc lines should be selected.

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