The role of opportunistic bacteria in marine cold-water larval cultures

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Scientific environment

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Nina Sandlund

Dedikert til

Liv

-Verdens beste lillesøster

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Abstract

The cultivation of marine larvae is often associated with high rates of mortality and is regarded as a bottle-neck in maintaining stable levels of production of juveniles for on-growing. In the course of the past few decades a great deal of effort has been put into increasing the production of farmed marine species. In spite of this, production is still low, compared to the production of salmonids, partly due to problems that arise during the early stages of life. The mortality problem is thought to be caused by bacteria that originate either from larval or live feed cultures. These are systems with high densities of biological waste and debris which may contribute to the growth of opportunistic bacteria. *Vibrio* spp. have often been suggested as causal agents. Because of the complex microbiological composition involved in larval and live feed cultures, and the small size of the larvae and feed organisms, isolating bacteria and describing their roles may be demanding. In order to identify pathogenic bacteria, reliable infection models are needed. The aim of this thesis is to enhance the knowledge of the bacteria associated with the cultivation of marine species. Experimental model organisms were great scallop *Pecten maximus*, cod *Gadus morhua*, halibut *Hippoglossus hipposlossus*, and turbot *Scophthalmus maximus*.

The present work utilizes the multi-dish system as a model for bath challenge experiments using several species of marine larvae (**Papers I**-**III**, **V**). The method is reliable and efficient both as means of minimizing the number of larvae needed and for saving time and work space. It also provides individual control. In **Paper I**, six candidates of bacteria pathogenic to great scallop larvae were tested. In a similar experiment with cod larvae, 53 bacterial strains were tested for virulence (**Paper II**). Both studies included two controls i.e. unchallenged larvae as negative control and one larval group challenged with a known pathogen *Vibrio pectenicida* (**Paper I**) and *Vibrio anguillarum* serotype O2α (**Paper II**) as positive control. In addition to the positive controls, *Vibrio splendidus* (**Paper I**), four different strains of *V. anguillarum* and *Carnobacterium* sp. strain were verified as highly pathogenic (**Paper II**). The attempt to serotype the *V. anguillarum* strains produced inconclusive results (**Paper**

II), and further studies are needed. The study described in **Paper II** also show that most bacteria associated with diseased fish is not a primary cause to disease.

Studies of the susceptibility of cod, halibut and turbot larvae to various serotypes of *V. anguillarum* and *Vibrio* spp. produced high mortality in all groups challenged with the O2α serotype. Cod and halibut larvae also suffered high mortalities when challenged with O1 (**Paper V**). The immunohistochemical examinations of larvae challenged with serotype O2α showed little or no pathology, leading to the hypothesis that extracellular products or toxins were killing the larvae (**Paper III**, **V**). Serotype O1 caused severe pathology (**Paper V**).

Bioencapsulation using rotifers were successfully used to administer *V. anguillarum* and *Vibrio logei* to cod larvae. The immunohistochemical examination revealed the presence of bacteria within the gastrointestinal tract of challenged larvae (**Paper IV**). However, mortality in these groups could not be related to exposure of bacteria as there was no increased mortality compared to the control groups. *V. logei* has been frequently isolated from cod larval cultures suffering from high mortality, thus suggested as a causative agent to disease. However bath challenge (**Paper II**) and bioencapsulation of the bacterium in live feed (**Paper IV**) did not have any negative effects on the cod larvae. It cannot be ruled out that under different circumstances the bacterium could be pathogenic. The absence of mortality in groups orally challenged with *V. anguillarum* serotype O2α (**Paper IV**) is in contrast to the bath challenge experiments in which mortality increased a few days post-challenge (**Papers II**, **III**, **V**). No conclusive explanations were found regarding this matter.

Sammendrag

Produksjon av marine larver er ofte forbundet med høy dødelighet og er sett på som en av flaskehalsene for å få til en stabil produksjon av yngel. Over de siste par tiårene har det blitt satset på å få fram en marin oppdrettsnæring. Til tross for dette er produksjonen fortsatt lav sammenlignet med produksjon av laksefisk, mye på grunn av problemene i de tidlige livsstadiene. Problemene med høy dødelighet er antatt å skyldes bakterier som enten har sitt opphav i larve- eller levendefôrkulturene. Dette er systemer med høy tetthet av biologisk materiale og avfall som sannsynligvis bidrar til vekst av opportunistiske patogener. Infeksjoner med *Vibrio* spp. blir ofte satt i sammenheng med den høye dødeligheten. Det mikrobielle samfunnet i larve- og levendefôrkulturer er komplekse og sammensatte, noe som kompliserer arbeidet med å identifisere mulige agens. Det er derfor nødvendig med gode og forutsigbare smittemodeller. Formålet med dette studiet var å øke kunnskapen om bakterielle infeksjoner i marin larve produksjon. Eksperimentelle modellorganismer var larver av kamskjell *Pecten maximus*, torsk *Gadus morhua*, kveite *Hippoglossus hippoglossus* og piggvar *Scophthalmus maximus*.

Dette studiet bruker et brønn-brett system som bad-smitte modell (**Artikkel I-III**,**V**). Metoden er pålitelig og effektiv, både med hensyn på reduksjon av antall forsøksdyr, tidsbesparende og romkapasitet. I tilegg kommer individuell kontroll. I **Artikkel I** ble seks mulige kamskjell-larve-patogene bakterie isolater virulenstestet. Et lignende forsøk utført på toskelarver testet 53 bakterie isolater for virulens (**Artikkel II**). I begge studiene ble det brukt to ulike kontroller, en usmittet negativ kontroll og en positiv kontroll i form av larvegrupper smittet med en kjent patogen *Vibrio pectenicida* (**Artikkel I**) og *Vibrio anguillarum* serotype O2α (**Artikkel II**). I tilegg til de positive kontrollene, forårsaket *Vibrio splendidus* (**Artikkel I**), fire ulike isolater av *V. anguillarum* og et *Carnobacterium* sp. isolat høy dødelighet. De fire *V. anguillarum* ble forøkt serotypet, men resultatene var ikke entydige. Videre studier er derfor nødvendige. I tillegg viser studiet beskrevet i **Artikkel II** at mesteparten av bakterieisolatene fra syke fiskelarver ikke kan regnes som primær årsak til sykdom.

Studier for å se på mottagelighet for ulike *V. anguillarum* serotyper og *Vibrio* spp. viste at torsk-, kveite- og piggvarlarver er mottakelige for *V. anguillarum* serotype O2α. Smitte forårsaket høy dødelighet hos alle artene. I tillegg var dødeligheten høy i torsk og kveite larvegruppene smittet med O1 serotypen (**Artikkel V**). De immunhistologiske undersøkelsene viste lite histopatologi i larver smittet med O2α-serotypen i motsetning til grupper smittet med O1 serotypen. Denne mangelen på histopatologi kan skyldes av at larvene dør som følge av utskillelse av ekstracellulære produkter og toksiner (**Artikkel III**, **V)**.

Inkorporering av *V. anguillarum* og *Vibrio logei* i rotatorier ble brukt som metode for og oral smitte av torskelarver. Immunhistologiske undersøkelser bekreftet at bakteriene ble overført til larvene ved at bakterier ble observert i larvenes mage og tarmkanal (**Artikkel IV**). Likevel, dødeligheten i disse gruppene var ikke høyere enn i kontrollgruppene og kunne dermed ikke relateres til smitten. *V. logei* har ofte blitt isolert fra larvegrupper av tosk med høy dødelighet og dermed satt i sammenheng med sykdomsutbrudd. Verken badsmitte (**Artikkel II**) eller oral administrering (**Artikkel IV**) av bakterien har ført til øket dødelighet. Det kan like vel ikke utelukkes at *V. logei* under andre forutsetninger ville kunne være patogen. Fraværet av dødelighet i de *V. anguillarum* smittede gruppene (**Artikkel IV**) står i sterk kontrast til badsmitteforsøkene, hvor bakterien forårsaket høy dødelighet (**Artikkel II**, **III**, **V)**. Ingen konkret forklaring ble funnet ble funnet på dette.

Introduction

Norwegian aquaculture has become an important industry in the course of the past 20 years. Large-scale aquaculture started about 40 years ago with salmon, *Salmo salar*, and rainbow trout, *Oncorhynchus mykiss*. Production of those species expanded and new species began to be cultivated. In 2007, the total export value of grow-out fish produced in the Norwegian aquaculture industry was estimated to be approximately NOK 19 billion and a production volume of over 700,000 tonns (Kjønhaug 2008). The production of marine aquaculture species is small compared to that of salmonids. In 2007 the exported value of cod, *Gadus morhua*, was approximately NOK 170 millions, and halibut, *Hippoglossus hippoglossus*, NOK 62.2 million. The EU is the main the export market. Historically, marine species, in particular cod, have been of great economic importance in Norwegian fisheries for over 300 years (Vollan 1956). In spite of efforts to establish a marine fish farming industry, high mortality during the early life stages has resulted in an unstable supply of cod and halibut fry for on-growing. Even so, farmed production of cod and halibut has increased in the course of the past few years. Bivalve farming of mussels, *Mytilus edulis*, oyster, *Ostrea edulis*, and great scallop, *Pecten maximus*, is a small industry in Norway, with an annual production of a few thousand tonnes (Directorate of Fisheries, http://www.fiskeridir.no). Great scallop is a highly valued commodity and market prizes are high due to their excellent quality and large biomass. One commercial hatchery in Norway, Scalpro (Rong, Øygarden, Hordaland County), produce great scallop. Spat production has been highly variable over the last years due to water quality problems.

Mortalities in marine larval cultures occur randomly and frequently and the causative agents are not always identified. This thesis addresses only bacterial infections as causes of disease and mortality. However, it should be noted that virus infections cause severe losses, such as irido-like viruses and herpes-like viruses in bivalve larval cultures (reviewed by Le Pennec et al. 2003, and Batista et al. 2007), and nodavirus in turbot, *Scophthalmus maximus*, and halibut hatcheries (Johansen et al. 2004, Nerland

et al. 2007, respectively). The nutritional value of larval feed has been studied as an important factor to sustain larval growth, development and survival (reviewed by Kvåle et al. 2007).

Rearing marine larvae is complex. It involves various live feed cultures, and rearing protocols are highly variable. The various live feed cultured and rearing regimes essential in commercial production of the four aquaculture species, great scallop, cod, turbot, *Scophthalmus maximus*, and halibut involved in this study, will therefore be introduced.

Rearing marine larvae

Large-scale aquaculture of marine species is a relatively young industry, and the methods used when rearing of marine larvae are not as standardised as the rearing protocols used for salmonid fish. The used of water treatment, water flow in tanks, live feed cultures and enrichment methods, feeding regimes and densities in larval tanks vary among the commercial operators. Such differences in rearing regimes are likely to cause variations in tank environment among the hatcheries. The following paragraphs concerning "*Rearing of marine larvae*" therefore include only a selection of protocols.

Live feed cultures

Great scallop, cod, turbot, and halibut larvae all require live feed as part of their feeding regime. In nature, marine larvae feed on algae and zooplankton (e.g. copepods). In large-scale aquaculture rotifers and *Artemia* spp. have replaced natural zooplankton, due to the easiness of culture and availability (see further details in paragraphs below). Live feed cultures with their high densities of organic matter have been associated with high rates of mortality in larval cultures, as they represent a significant quantity of the bacterial load on the larvae (reviewed by Austin 2006). Larval intestinal microflora is reported to be similar to that of live feed cultures

(Munro et al. 1994, Eddy & Jones 2002). Commercial enrichments used in live feed production of cod larvae influence the total number of bacteria (colony-forming units) in the enrichment cultures and the gastrointestinal tract of the larvae (Korsnes et al. 2006). The composition of the bacterial population associated with cod larvae difffers among hatcheries (Verner-Jeffreys et al. 2003b), probably influenced by the enrichment and rearing protocols utilised. The various live feed cultures used to rear marine larvae are described below.

Algae

Different types of algae are used during the production of marine larvae, either as a direct food source, added to rearing water ("the green water technique") or food for live prey. The alga needs to be easily cultured and be of the right size (2-15 µm) (Reitan 2005, Muller-Feuga et al. 2003b). Intensive rearing of great scallop and fish larvae uses algae such as *Tetraselmis* spp. *Chaetoceros* spp., *Pavlova lutheri*, and *Isochrysis* spp., among others (Christophersen et al. 2006, Muller-Feuga et al. 2003b). The mixture protocol of algal cultures differ in terms of species and ratio, with nutrient value and composition, mainly the content of lipids and fatty acids, being an important aspect (Muller-Feuga et al. 2003a, reviewed by Reitan et al. 1997). Algae are reared in transparent plastic bags or tubes to which nutrients and carbon dioxide are added and which are exposed to a light source. The rearing temperature is around 20-25°C. Algae are either grown in batch cultures, i.e. the whole culture is harvested, or in continuous cultures, where a limited volume is harvested during the late growth phase.

In marine fish larvae cultures, algae are not used as a direct food source, but are added as green water (reviewed by Reitan et al. 1997). A recent study that compared survival and feeding incidence of cod larvae reared in green water consisting of *Isochrysis galbana* versus clear water, showed that the addition of algae in conjunction with low light intensity produced the highest feeding incident during start feeding. However, this difference was not significant. Significantly improved survival

was found in cod larval tanks to which green water was added (van der Meeren et al. 2007).

Benefits reported from addition of microalgae (reviewed by Reitan et al. 1997 and Palmer et al. 2007) are:

- That algae may be used as a direct food source, through active uptake by the larvae
- That algae may serve as an indirect food source in which the nutrient value of the live prey is sustained
- Improved water quality thanks to the production of oxygen and removal of metabolic by-products like nitrogenous substances
- Microbial control by production of antibacterial substances
- Increased feeding incidence, possibility due to modification of light conditions

Algae are commercially available as fresh and frozen products if the facilities for algal growth are not avaiable in the hatchery.

Rotifers

Several species of rotifers are used in production of marine larvae, with *Brachionus plicatilis* the most common (Olsen 2004). Their rapid reproduction, body size and relatively slow motility compared to fish larvae, have contributed to their usefulness as suitable prey. Rotifers alone do not have sufficient nutrient value (Olsen 2004), but their capability as filter-feeding organisms has been utilised by bioencapsulation of algae, yeasts, and even beneficial bacteria to improve their nutrient value and general quality as live feed. The use of yeasts, with the addition of oil to increase fat content, in rotifer production has fallen in Norwegian hatcheries. Several farmers report a preference for the use of algae instead of yeast, as rotifer cultures have a better reproduction rate and are easier to maintain. Rotifers are robust and tolerate wide variations in temperature and salinity, as long as these parameters are changed gradually. Rotifers are thus suitable for mass production as live feed. Rotifer cultures

are usually reared around 20-26°C and at salinities of around 20-25 ppt (Hagiwara et al. 2007), Olsen 2004), but commercial cod hatcheries often use 30-35 ppt salinity. Rotifer culture densities vary widely, but 3000 rotifers ml^{-1} are commonly found in cultures ready for harvesting (Reitan 2005). Rotifers can be grown in either batch, or continuous culture.

Artemia spp.

Artemia or brine shrimp nauplii (the early life stage of *Artemia*) have been used to culture all species of cold water fish larvae. The most widely used species is *A. franciscana* (Olsen et al. 2004). As with rotifers, filter-feeding capacity is exploited to improve the nutrient value and general quality as live feed. Commercially available oil emulsion products like Super Selco and DHA Super Selco are commonly used as enrichments to increase the content of essential n-3 fatty acids (HUFA) (Olsen 2004). Another advantage is their easy availability. *Artemia* are commercially available as cysts that are hatched at the farm, making it easier to maintain stable access to larval food compared to rotifers, which need to be cultured. The temperature for cultivation of *Artemia* is around 28°C, with salinity 33-35 ppt, while densities may range from 100-300 individuals ml^{-1} (Reitan 2005, Olsen 2004). Juvenile *Artemia* are sometimes used for feeding turbot and halibut larvae that require larger prey than cod larvae.

Larvae

The four aquaculture species used as experimental models in this thesis require differences in rearing regimes, and are therefore described separately below.

Great scallop

In the only commercial scallop hatchery in Norway, Scalpro AS, great scallop larvae are hatched and reared at 18°C in 450-800 l conical tanks at a concentration of 5-10 larvae ml^{-1} (Torkildsen & Magnesen 2004). Development from egg to 20 mm fry takes around 180 days via seven distinct developmental stages. The larvae settle around day 30 as postlarvae (Hovgaard et al. 2001).

The larval cultures are given a mix of micro algae as described above. The species of algae used at the Scalpro AS facility are *Pavlova lutheri*, *Isochrysis* sp. *Skeletonema costatum* and *Chaetoceros mülleri* (Christophersen et al. 2006).

Cod

The production of cod larvae in Norway started in the 1880s at Flødevigen research station in southern Norway, where larvae were hatched and released into the sea. About 20 years ago large-scale farming started (Øiestad 2005). In intensive production the eggs are held in conical cylindrical up-welling tanks or incubators in the dark at 7-8°C. The up-welling system enables the eggs to float freely and makes it easier to remove dead eggs. Newly hatched larvae are transferred to feeding tanks and the temperature is raised to between 9 and 12°C (Olsen et al. 2004). Start-feeding with rotifers usually begins around three days post hatch and the rotifer diet lasts for about 25-30 days, depending on temperature (Fig. 1). Cod are recommended to be fed large rotifers (180-320 µm) (Reitan 2005). Typical larval densities are 30-40 larvae l^{-1}. (Olsen et al. 2004). A period with *Artemia* may be used prior to dry feed or as a supplement during the change from rotifers to formulated feed. A transition period is used when diets are changed in order to ease the changeover between the various feed and to ensure that most larvae have access to the right size of prey (Fig 1.). Most Norwegian cod farmers have stopped using *Artemia* and go directly from rotifers to formulated feed.

Turbot

The only commercial turbot farm in Norway, Stolt Sea Farm Øye, Kvinesdal, in the County of Aust-Agder, is now merely operated as a broodstock station. Until recently, the facility was operated as a producer of turbot fry, using intensive production protocols (J. Stoss, Stolt Sea Farm Øye, pers.comm). In European hatcheries, turbot eggs are normally kept in up-welling incubators and hatched at temperatures of around 12-14°C. During the first five days post-hatch (p.h.), the temperature is gradually increased to around 18-20°C. Addition of algae (green water) and feeding with rotifers starts at day one p.h. and lasts for around12-14 days (Fig. 1). Larvae are

held in tanks of various sizes with densities ranging from 10-100 larvae l^{-1}. Densities of 20-40 larvae l^{-1} are most common (Olsen et al. 2004). During the green water period the water is kept stagnant (Olsen et al. 2004, Stoss et al. 2004). As the larvae grow the water flow is increased. The larvae are usually fed *Artemia* nauplii after two weeks. Weaning, or change of diet to formulated feed, is usually around days 22-25 p.h. As with cod, a transition period is used to ease the transition between the feeds (Fig. 1) (Olsen et al. 2004).

Halibut

Like cod and turbot eggs, halibut eggs are kept in up-welling tanks in the dark. Halibut larvae hatch around 16 days (82 d°) post-fertilisation. The larvae are premature and have the longest yolk-sac stage of the fish species discussed in this study. In farming facilities, halibut yolk-sac larvae are kept in tall dark silos at 5-6°C until start-feeding (Kjørsvik et al. 2004,) in order to mimic their natural environment (Fig. 1). These tanks also use up-welling water flow. During the yolk-sac stage, the larvae are fragile and sensitive to environmental changes, and the dark environment in the silos provides the stability needed. The larvae are usually transferred to start-feeding tanks around day 40 p.h (Fig. 1). At this stage the temperature is gradually raised to around 9-12°C (Kjørsvik et al. 2004). When the larvae are transferred to first-feeding tanks, sand or clay is added to colour the water during the live feed stage (Fig. 1) (Van der Meeren pers. comm.). Green water may also be used. Larval densities are normally between 1-10 larvae l^{-1} (Olsen et al. 2004). Large rotifers (300 µm) can be used as feed during the first few days, but *Artemia* nauplii are usually used as start feed. Later on, *Artemia* juveniles may be used before weaning or as a supplement during the weaning process. The addition of dry feed usually starts around day 80 p.h. (Fig. 1) (Olsen et al. 2004, Stoss et al. 2004).

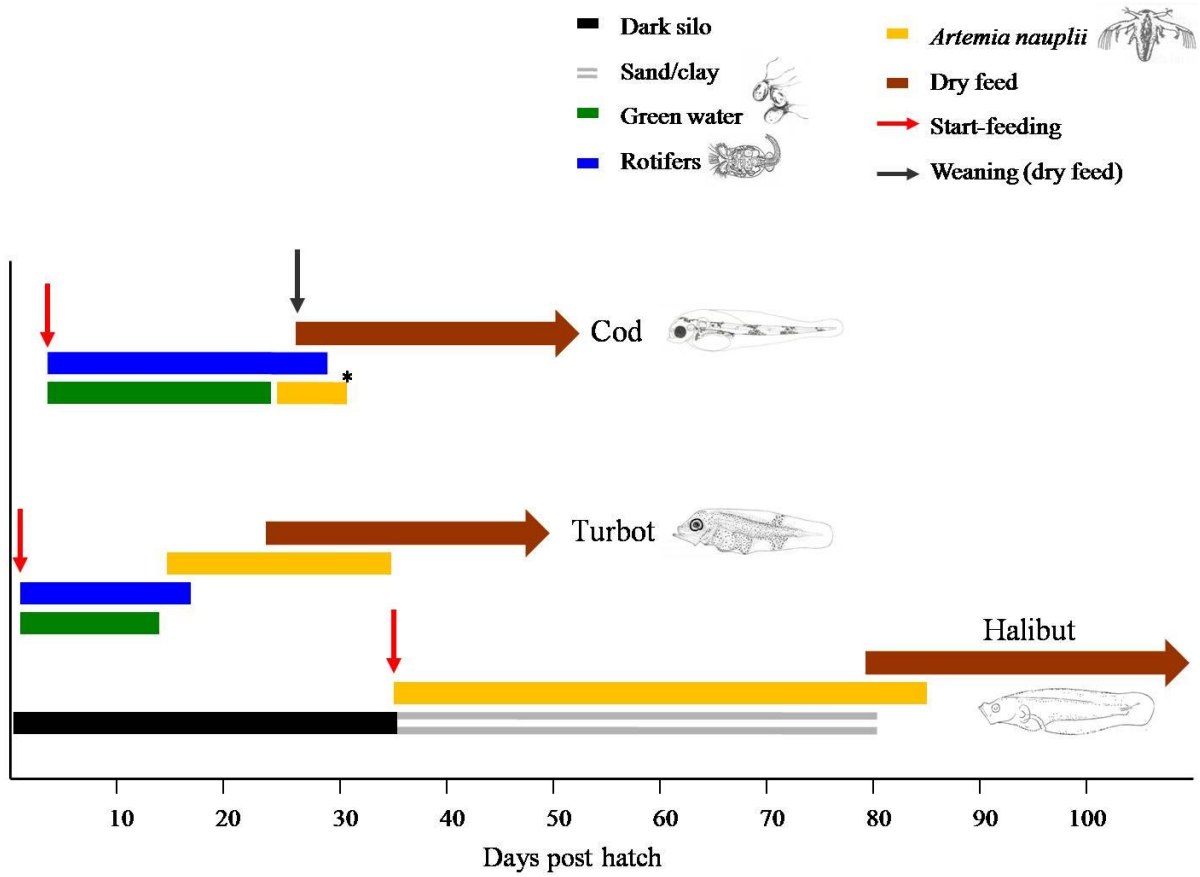

Figure 1. Overview of general feeding regimes used in commercial hatcheries for the three species, halibut *Hippoglossus hippoglossus*, turbot *Scophthalmus maximus* and cod *Gadus morhua*. Green water; addition of microalgae. The figure is based on Olsen et al. 2004, Stoss et al. 2004 in addition to communication with fish farmers. * = *Artemia* is not always used. Drawing of turbot larvae (at the end of the yolk-sac stage) from Jones (1972), cod larvae (at the end of the yolk-sac stage) from Fridgeirsson (1978) and halibut larvae from Russel 1976. Drawing of algae (*Isochrysis* sp.) by Stein Mortensen and drawings of rotifer and *Artemia* nauplii by Anonymous.

Identification of bacteria associated with marine larval cultures

The isolation and identification of bacterial strains from environmental samples or larvae are complicated by the presence of a wide variety of bacterial strains. This often results in a complex selection of bacterial strains. In subadult and adult fish, bacteria are commonly isolated from haematopoietic tissue, i.e. head kidney, spleen and liver. In larvae, small size and undifferentiated tissue complicate such protocols, causing high risk for contamination, hence homogenisation of whole surface disinfected larvae is commonly used. In order to make marine larval production more efficient and to reduce the mortality problem, it is vital to obtain as much knowledge as possible about the normal and infectious bacterial flora.

Phenotypic characterisation

Phenotypic characterisation provides information about the physical and biochemical characteristics of an organism. In dealing with unknown bacteria, descriptions of colony morphology, growth on different media, motility, Gram staining and description of shape all provide rapid information about the bacterial strain involved. To isolate marine bacteria, marine agar (MA), blood agar added salt (BA), and thiosulphate citrate bile sucrose agar (TCBS) are often used. BA provides information about the ability of the bacterium to lyse red blood cells (haemolytic capacity). TCBS, a *Vibrio*-specific medium originally developed for isolation of human pathogenic *Vibrio* strains, is now also widely used for the isolation of environmental strains (reviewed by Harwood et al. 2004).

Commercial biochemical tests like API 20E, originally developed for identification of human pathogens, is becoming more frequently used to identify fish pathogens when a rapid diagnostic method is needed (reviewed by Popovic et al. 2007). API 20E was developed for the identification of *Enterobacteriaceae* and/or non-fastidious, Gram-negative bacteria based on the bacterium's metabolism. It is standardised, easy to perform and the results are avaiable within 48 hours. However, as pointed out by

Popovic et al. (2007) the method needs to be modified for use with marine bacteria, especially as far as incubation temperature and duration are concerned.

Genotypic characterisation

Genotypic characterisation provides information that cannot be obtained via phenotypic characterisation. In the course of the past couple of decades, sequencing of bacterial genes has become a common method for identifying unknown bacteria. Amplification and sequencing of the 16S rRNA gene revolutionized taxonomy and identification of prokaryotes (Giovannoni et al. 1990) and has since been widely used as a molecular clock to estimate phylogenetic relationships among bacteria. The reason for its widespread use is its ubiquity, evolutionary stability and essential function in the protein synthesis system. 16S rRNA forms part of the small ribosomal subunit, 30S. In the course of the past few years, additional genes have been utilised in phylogenetic studies. Basing identification of bacterial strains on one gene alone is usually not sufficient (reviewed by Thompson et al. 2004b). Polymorphisms (Moreno et al. 2002), and heterogeneity (Dahllöf et al. 2000, Case et al. 2007) have shown to be as common in the 16S rRNA gene, and additional genes are therefore needed for phylogenetic studies. Examples of genes suggested as good phylogenetic markers are *GyrB* (Le Roux et al. 2004), *recA* (Thompson et al. 2004a) and *rpoB* (Dahllöf et al. 2000, Case et al. 2007).

Bacteria associated with marine larval cultures

Normal bacterial flora

Even though the species discussed in this thesis are diverse in terms of development, rearing and feeding regimes, their farmers face similar challenges, for example the production of live feed cultures and maintenance of a stable tank environment. The main obstacle is probably the high rates of mortality that are too frequently experienced during the larval stages. Larvae are exposed to bacteria from hatching onwards, as bacteria are naturally present in the environment (reviewed by Hansen & Olafsen 1999 and Olafsen 2001). Knowledge of the normal microflora is essential if we are to fully understand the shifts in microflora that occur during a disease outbreak and identify possible pathogenic bacteria. Being water-filtering organisms, scallop larvae ingest bacteria through feeding, and fish yolk sac larvae as they drink water (Mangor-Jensen & Adoff 1987). The epiflora of eggs and the water flora are important for the first bacterial colonisation of the gastrointestinal tract (reviewed by Ringø & Birkbeck 1999), while at start-feeding the microflora shifts and is affected by the exogenous feed (Bergh et al. 1994, Munro et al. 1994, Verner-Jeffreys et al. 2003b, Brunvold et al. 2007). Studies of bacteria associated with reared yolk-sac larvae show dominance of oxidative Gram-negative rods, and after the onset of first feeding, vibrios dominate (Blanch et al. 1997, Verner-Jeffreys et al. 2003a). Jensen et al. (2004) suggested that halibut larvae possess a relatively distinct and specific normal microflora, regardless of their geographical origin. However, results were more variable in fed larvae than in yolk-sac larvae, possibly due to differing live-feed protocols. As the water source and water treatment differ among the hatcheries, it cannot be out ruled that the normal flora will also differ among rearing facilities. Results suggesting this were found by Verner-Jeffreys et al. (2003b), who studied the microflora at three different halibut hatcheries.

Infectious bacteria

Disease in a hatchery can affect both egg and larval stages. Disease is often related to changes in water quality and environmental parameters that can lead to growth of

opportunistic and pathogenic bacteria. Verification of a bacterium's virulence involves *in vivo* challenge studies (Table 1). Challenge studies involving early life stages can be divided into two main groups, bath challenge and oral administration by bioencapsulation of the bacterium in live feed. Challenge experiments and probiotic studies, involving challenges with pathogenic bacteria, on marine bivalves and fish larvae, are listed in Table 1.

The bacterial flora of marine fish eggs seems to be dominated by the genera *Cytophaga*, *Flavobacterium*, and *Flexibacter* (some strains have been renamed *Tenacibaculum* spp.) (Hansen & Olafsen 1989, Verner-Jeffreys et al. 2003b). *Tenacibaculum ovolyticum* (formerly named *Flexibacter ovolyticus*) is known to cause disease in halibut eggs (Hansen et al. 1992). Good disinfection and rearing routines (e.g. removal of dead eggs) reduce outbreaks of disease during the egg phase.

A common experience in hatcheries is a sudden collapse in larval cultures. Changes in mortality may appear overnight and at random among tanks. The critical periods are several, although start-feeding (reviewed by Yúfera & Darias 2007) and change in diets seems to be high-risk periods. Frequent observations are that larvae appear apathetic and stop feeding, followed by a collapse in the larval culture. Live feed cultures, which introduce large amounts of organic matter to larval cultures, are a possible source of pathogenic bacteria (reviewed by Battaglene & Cobcroft 2007). Bacterial enteritis may be common, as has been described in rearing of striped trumpeter, *Latris lineata*, larvae (reviewed by Battaglene & Cobcroft 2007) and larval cultures of Japanese flounder, caused by *Vibrio ichthyoenteri* (Muroga 2001). These reports are similar to what is experienced in hatcheries of cod, halibut and turbot. In farming of gilt-head sea bream larvae, *Sparus aurata*, mortalities related to abdominal swelling caused by a bacterial infection have been reported (Sedano et al. 1996). Typical clinical signs in cod larvae are dilations in the swim bladder and intestine. The term "floaters", is used of these larvae due to their characteristic way of floating on the side or upside-down in the water surface. Observations imply bacterial infection (van der Meeren et al. 2005) and *Vibrio logei* have been suggested as a

causative agent (Egil Karlsbakk unpubl. results, Institute of Marine Research). Although other genera, such as *Aeromonas*, *Alteromonas* and *Pseudomonas*, besides *Vibrio* are associated with disease and mortality in larval cultures, *Vibrio* spp. infections dominate and have been most studied (see Table 1 for more details)

Table 1. Overview of challenge experiments and probiotic studies, involving challenge with pathogenic bacteria and virus on marine larvae. I.P injection = intraperitoneal injection

Species	**Bacterial strain(s) or Virus**	**Challenge model(s)**	**Reference**
halibut *Hippoglossus hippoglossus*	*Flexibacter* spp. NCIMB 13127, NCMBI 13128, *Vibrio anguillarum* NCMBI 6, *Vibrio fisheri* ATCC 7744, *Vibrio* strain HI-10448	bath challenge	Bergh et al. 1992
halibut, cod *Gadus morhua*	*Flexibacter ovolyticus* NCMBI 13127, *Vibrio* sp. HI-10448, V. anguillarum HI-1360	bath challenge	Skiftesvik & Bergh 1993
halibut, turbot *Scophthalmus maximus*	*A. salmonicida* subsp. *salmonicida,*	bath challenge	Bergh et al. 1997
halibut and cod	*F. ovolyticus* NCMBI 13127 and 13128, *A. salmonicida* subsp. s*almonicida*, *V. anguillarum* strain HI 10448,	bath challenge	Bergh 2000
halibut	Strain 4:44, and PB52	bioencapsulation in rotifers	Makridis et al. 2000
halibut	Vibrio strains PB 1-11 and PB 6-1	bioencapsulation in *Artemia* sp.	Makridis et al. 2001
halibut	*V. anguillarum* and others	bath challenge	Verner-Jeffreys et al. 2003a
turbot	*Lactic Acid Bacterium* (LAB),*Vibrio* sp.	bath challenge and bioencapsulation in rotifers (Probiotic study)	Gatesoupe 1994
turbot	*V. anguillarum* 91079, *Vibrio alginolyticus*, *Aeromonas* sp. strain C39 .	bioencapsulation in rotifers	Munro et al. 1995
turbot	*V. anguillarum*	bioencapsulation in *Artemia* sp.	Grisez et al. 1996
turbot	*Vibrio splendidus*, *Vibrio* strain E	bath challenge/bioencapsulation in rotifers (Probiotic study)	Gatesoupe 1997

Species	Bacterial strain(s) or Virus	Challenge model(s)	Reference
turbot	*Vibrio splendidus*	bath challenge	Gatesoupe et al. 1999
turbot	*Vibrio* spp.	bath challenge, (probiotic study)	Huys et al. 2001
turbot	*Vibrio pelagicus*	Bath challenge	Villamil et al. 2003
turbot	*Roceobacter* sp., *V. anguillarum* strain 90-11-287, *V. splendidus* DMC-1	bath challenge, (probiotic study)	Hjelm et al. 2004
turbot	*V. splendidus* spp., *V. alginolyticus* spp.	bioencapsulation in rotifers	Thomson et al. 2005
turbot	*V. anguillarum*	bath challenge, bioencapsulation in rotifers	Planas et al. 2005
turbot	*Roceobacter* sp., *V. anguillarum*	bath challenge, bioencapsulation in rotifers (probiotic study)	Planas et al. 2006
Cod, herring *Clupea harengus*	*V. fisheri*, *V. salmonocid*, *Flavobacterium* sp.	bath challenge	Olafsen & Hansen 1992
flounder *Paralichthys olivaceus*	*Vibrio spp.*	bioencapsulation in rotifers and *Artemia* sp.	Muroga et al. 1990
flounder	*Vibrio ichthyoenteri* (strains F-2, FK-1), *Edwardsiella tarda*, *V. anguillarum*	bioencapsulation in *Artemia* sp.	Kim et al. 2004
gilt-head seabream *Sparus aurata*	*Vibrio* spp.	bioencapsulation in rotifers and *Artemia* sp.	Sedano et al. 1996
European sea bass *Dicentrarchus labrax*	*V. anguillarum*	bath challenge	Kotzamanis et al. 2007
pollack *Pollachius pollachius*	Probiotic strain *Pediococcus acidilactici*	bioencapsulation in *Artemia* (Probiotic study)	Gatesoupe 2002
Colorado River cutthroat trout *Oncorhynchus clarkii pleuriticus*	*Lactobacillus* sp.	bioencapsulation in *Artemia* sp. (Probiotic study)	Arndt & Wagner 2007
Pacific threadfin *Polydactylus sexfilis* and Amberjack *Seriola rivoliana*	*Vibrio* spp. and *Pseudoalteromonas* spp.	bath challenge	Verner-Jeffreys et al. 2006
grouper *Epinephelus coioides*	Nervous Necrosis Virus (NNV)	bioencapsulation of recombinant *E. coli* expressing NNV capsid protein gene in *Artemia* sp. I.P injection of virus suspention (vaccination study)	Lin et al. 2007
Pacific herring *Clupea pallasii*	Viral Haemorrhagic Septicaemia Virus (VHSV)	bath challenge	Hershberger et al. 2007

Species	Bacterial strain(s) or Virus	Challenge model(s)	Reference
great scallop *Pecten maximus*	*Vibrio* spp.	bath challenge	Nicolas et al. 1996
great scallop	*Roseobacter* sp.	bath challenge	Ruiz-Ponte et al. 1999
great scallop	*V. splendidus* spp., *Aeromonas* spp./ *Pseudoalteromonas* spp.	bath challenge	Torkildsen et al. 2005
Chilean scallop *Argopecten purpuratus*	*V. anguillarum*- related (VAR)	bath challenge	Riquelme et al. 1995
Chilean scallop	*Vibrio alginolyticus*, *V. splendidus*, *Aeromonas hydrophila*	bath challenge	Riquelme et al. 1996b
Chilean scallop	VAR, *Alteromonas haloplanktis*	bath challenge (probiotic study)	Riquelme et al. 1996a
Chilean scallop	*Vibrio* spp. *Pseudoalteromonas* spp. and others	bath challenge (probiotic study)	Riquelme et al. 1997
Chilean scallop	Inhibitor producing strains, 11, C33 and 77	bath challenge	Riquelme et al. 2000
Chilean scallop	Inhibitor producing *Vibrio* C33, *Pseudoalteromonas* sp.	bath challenge	Riquelme et al. 2001
Pacific oyster *Crassostrea gigas*	*Vibrio* spp., *Aeromonas* spp.	bath challenge	Garland et al. 1983
Pacific oyster	Herpes-like virus	bath challenge, inoculation with virus suspention made from diseased larvae	LeDeuff et al. 1996
Pacific oyster	*Vibrio splendidus*, VAR, *Vibrio* spp.	bath challenge	Sugumar et al. 1998
Pacific oyster	*Vibrio tubiashii*, *Aeromonas media* strain A199	bath challenge (probiotic study)	Gibson et al. 1998
Pacific oyster	*V. alginolyticus*	bath challenge	Nakamura et al. 1999
Pacific oyster	*Vibrio* spp.	bath challenge	Estes et al. 2004
Pacific oyster	*V. tubiashii*	bath challenge	Elston et al. 2004
Pacific oyster, flat oyster *Ostrea edulis*	*Vibrio* spp.	bath challenge	Jeffries 1982
flat oyster	*Pseudomonas* spp., *Vibrio* spp. amongst others	bath challenge	Lodeiros et al. 1987
American oyster *Crassostrea virgincia* eggs and larvae	*Vibrio* spp. *Pseudomonas spp.* (supernatant, heat-killed and washed bacteria)	bath challenge	Brown 1973
American oyster eggs and larvae	*Vibrio* sp. (supernatant, heat-killed and washed bacteria)	bath challenge	Brown & Losee 1978

Species	Bacterial strain(s) or Virus	Challenge model(s)	Reference
American oyster	*Vibrio spp.*	bath challenge	Elston & Leibovitz 1980
American oyster	*Vibrio spp.* (VAR, *V. splendidus* like)	bath challenge	Brown 1981
clam *Mercenaria mercenaria*, American oyster, flat oyster, bay scallop *Aequipecten irradians*, shipworm *Teredo navalis*	27 Gram-negative motile rod strains, *Aeromonas* sp. *Vibrio* sp.	bath challenge	Tubiash et al. 1965
scallop *Argopecten ventricosus*, scallop *Nodipecten subnodosus*, penshell *Atrina maura*, Pacific oyster	*Vibrio alginolyticus*	bath challenge	Luna-González et al. 2002
giant clam *tridacna gigas* larvae	*Vibrio* spp.	bath challenge	Sutton & Garrick 1993
Manila clam *Tapes philippinarum*, great scallop, Pacific oyster	*Vibrio* sp.	bath/ co-habitation challenge	Nicolas et al. 1992
red abalone *Haliotis rufescens* larvae and post-larvae	*V. alginolyticus*	bath challenge	Anguiano-Beltran et al. 1998
abalone *Haliotis diversicolor supertexta* post-larvae	*V. paraheamilyticus* and others	bath challenge	Cai et al. 2006a
abalone post-larvae	*V. alginolyticus* and others	bath challenge	Cai et al. 2006b
shrimp *Penaeus modon*	*V. harveyi* like and *Photobacterium phosphoreum*like	bath challenge	Prayitno & Latchford 1995
shrimp *Penaeus indicus*	*V. harveyi*	bath challenge (vaccination study)	Alabi et al. 1999
rock lobster *Jasus verreauxi* phyllosoma	*V. harveyi*	bath challenge	Diggles et al. 2000
tropical rock lobster *Panulirus ornatus*	*Vibrio spp.*	bath challenge and bioencapsulation in *Artemia* sp. (probiotic/virulence study)	Payne 2006

Vibrio infections

Vibrios are commonly found in large amounts in the marine environment and are also associated with live feed organisms such as rotifers and *Artemia* (Verdonck et al. 1997, Eddy & Jones 2002, respectively). Some of these vibrios are known to be opportunistic bacteria (Reviewed by Thompson et al. 2004b). More specifically, *Vibrio pectenicida* and *Vibrio splendidus* have been associated with mortalities in great scallop hatcheries (Nicolas et al. 1996, Lambert et al. 1998, Torkildsen et al. 2005). *V. splendidus* and *V. splendidus*-related strains are globally distributed and are frequently involved in disease in a wide variety of species, i.e. the cold water coral *Eunicella verrucosa* (Hall-Spencer et al. 2007), carpet shell clam *Ruditapes decussatus* larvae (Gómez-León et al. 2005), Pacific oyster *Crassostrea gigas* larvae and adults (Sugumar et al. 1998, Gay et al. 2004, respectively), turbot larvae (Gatesoupe et al. 1999, Thomson et al. 2005) and corkwing wrasse *Symphodus melops* (Bergh & Samuelsen 2007). A bacterium that has frequently been isolated in cod hatcheries suffering high mortalities is *Vibrio logei*, which is thus assumed to be one of the causative agents of collapse in larval batches (Egil Karlsbakk unpubl. results, Institute of Marine Research). *V. logei* is particularly associated with luminescence and is symbiotic with *Vibrio fisheri* in light organs in sepiolid squid (Fidopiastis et al. 1998, Nishiguchi et al. 1998). Findings of *V. logei* associated with cod larvae were also reported in recent work by Brunvold et al. (2007) and McIntosh et al. (2008), in studies of microbial communities in cod hatcheries using denaturing gradient gel electrophoresis (DGGE).

Classical vibriosis - Vibrio anguillarum

Vibrio anguillarum was first described as pathogenic to eel *Anguilla anguilla* about 100 years ago. A description of the disease probably dates as far back as the early 1700s from diseased eel on the north-east coast of Italy (reviewed by Egidius 1987).

In the present study, *V. anguillarum* was included in all fish larval (challenge) experiments because of its well-known virulence to cod, turbot and halibut (reviewed

by Egidius 1987, and Bergh et al. 2001, respectively). The bacterium was used either as an additional (positive) control group or for descriptive histopathological studies.

V. anguillarum is Gram-negative, rod-shaped, oxidise-positive, motile, with growth between 15-37°C and requiring the addition of salt in growth media (Austin & Austin 2007). The bacterium's strong affinity for iron may cause anaemia in chronically infected fish. The virulence plasmid pJM1 codes for iron transportation proteins and siderophores (Crosa 1980, Actis et al. 1985, Actis et al. 1986). However bacterial strains without the pJM1 plasmid are pathogenic, suggesting chromosome-mediated virulence characteristics (Lemos et al. 1988, Wiik et al. 1989). Other essential virulence mechanisms are flagella (motility) (Milton et al. 1996), chemotaxis (Larsen et al. 2004) and extracellular products (Lamas et al. 1994a, 1994b).

The opportunistic pathogen causes haemorrhagic septicaemia in a wide range of cold- and warm-water fish species (reviewed by Toranzo et al. 2005). It is part of the normal marine flora, and outbreaks often occur when water temperature rises quickly (Reviewed by Toranzo & Barja 1990). *V. anguillarum* has also been associated with live feed cultures used in rearing of the Japanese flounder *Paralichthys olivaceus*, and is thus assumed to transfer the bacterium to the larvae (Mizuki et al. 2006).

V. anguillarum is a heterogeneous species divided into serotypes, and so far more than 23 serotypes have been described (Pedersen et al. 1999). Serotypes O1 and O2 are most frequently isolated as pathogenic strains, where O1 is associated with salmonids and O2 is mostly associated with cod (Larsen et al. 1994). The O2 serotype is divided into two subgroups O2α and O2β, of which O2α is the dominant serotype of the two (reviewed by Toranzo & Barja 1990). Serotype O3 is usually isolated from diseased eel (Pedersen et al. 1999), but recently the O3 serotype has also been isolated from diseased salmonids (i.e. Atlantic salmon *Salmo salar*, Pacific salmon *Oncorhynchus kisutch* and rainbow trout *Oncorhynchus mykiss*) in Chile (Silva-Rubio et al. 2008).

Vaccination against vibriosis, primarily *V. anguillarum* serotype O1, O2α and O2β, has been successfully done in commercial farming of salmonid fish for almost 20 years (reviewed by Sommerset et al. 2005). However, vaccination regimes in marine fish farming have not provided the same reliable protection, with the result that outbreaks of vibriosis are still frequent, causing significant losses to the aquaculture industry all over the world. In 2007, 19 Norwegian cod farming sites were diagnosed with vibriosis caused by various serotypes of *V. anguillarum* (Hellberg et al. 2008). Recent results have shown that *V. anguillarum* strains isolated from diseased cod differ from known serotypes biochemically, serologically and genetically (Mikkelsen et al. 2007). Mikkelsen et al. have suggested that these isolates belong to a new sero-subtype other than O2 α and O2 β and address the question of vaccination and bacterial isolates used in cod vaccines.

It should be noted that *V. anguillarum* was suggested to be reclassified as *Listonella anguillarum* in the mid-80s (Macdonell & Colwell 1985). Since 1986 *Listonella* has been on the "List of Prokaryotic Names with Standing in Nomenclature" (http://www.bacterio.cict.fr/l/listonella.html). On the other, hand there is still some debate regarding this change in nomenclature, with the result that both names are used in recent published work (Planas et al. 2006, Mikkelsen et al. 2007, Sugita et al. 2008)

Preventing disease

As described above, bacteria cause significant losses in marine larval cultures. Prophylactic management is probably the most important means of promoting good health conditions in a farming situation. Key issues are water treatment and good cleaning routines, including disinfection of equipment, hygienic zones with limited transportation of equipment and people. The importance of a stable microbiological environment in larval tanks has been pointed out by several authors (Vadstein et al. 2004, Verner-Jeffreys et al. 2004, reviewed by Battaglene & Cobcroft 2007).

Rearing systems

In aquaculture facilities the quality of the water supply is of great importance, as pathogens may enter the farming facility via the water. Norway has implemented a directive concerning treatment of inlet and outlet water to aquaculture facilities with guide-lines regarding filtration and disinfection (FKD: 1997-02-20 nr 192). Removal of particles by filtration is utilised to improve the efficiency of UV radiation. Ozone is also added as a disinfection method. Ozone is highly reactive and breaks down organic particles into smaller components by oxidation. Biofilters are used for "biological cleaning of organic nitrogenous compounds" and are often used in recirculation systems. Essential for a biofilter is that the surface area should be as large as possible relative to volume (Uglenes et al. 2005, Weaton 1977). Skimming is a method for removing the smallest particles from the water. Microscopic bubbles are passed through the water column, producing a surface layer of organic matter that is easily removed. The method is often used in conjunction with ozone (Uglenes et al. 2005). These are all frequently used methods to prevent or minimise the occurrence of pathogenic agents such as parasites, bacteria and viruses in inlet water. Differences in water flow in rearing systems have been tested in both fish (Verner-Jeffreys et al. 2004) and scallop hatcheries (Andersen et al. 2000, Torkildsen & Magnesen 2004, Christophersen et al. 2006, Magnesen et al. 2006). Recirculation of water may result in a stable microflora and better survival of halibut larvae (Verner-Jeffreys et al. 2004). In rearing of great scallop larvae a different approach to water treatment has

led to fewer problems with mortality, hence more stable spat production. The key factor in this process seems to have been the introduction of a flow-through system (Andersen et al. 2000, Christophersen et al. 2006). The results show that optimal rearing conditions will vary among hatcheries and species.

Administration of beneficial bacteria - probiotics

Probiotics, in Greek meaning "for life", have been defined as "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health" (Gatesoupe 1999) and "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1989). The idea is to add bacteria that provide a stable microflora in the gastrointestinal tract and that have a beneficial effect on the health of the host. It should be remembered that aquatic animals interact with "microbiota" not only in the gastrointestinal tract, but throughout the whole body surface, including the gills. The beneficial effects of probiotics can include disease treatment and, more importantly, prevention of disease outbreaks, as probiotic bacteria may minimize the opportunity for opportunistic bacteria to proliferate. Probiotics may also improve nutrient digestion and absorption (reviewed by Vine et al. 2006, Kesarcodi-Watson et al. 2008, and Tinh et al. 2008). Concerning cold-water fish larvae, *in vivo* studies with turbot, larvae have been performed with promising results, using a *Roseobacter* strain (Hjelm et al. 2004, Planas et al. 2006). *Roseobacter* has also proven beneficial in farming of great scallop larvae, with increasing larval survival (Ruiz-Ponte et al. 1999). However, this positive effect was not seen when the pathogenic bacteria *Vibrio pectenicida* A496 was added. In the rearing of cod larvae, addition of *Lactobacillus plantarum* resulted in lower bacterial counts and lower presence of bacteria such as *Pseudomonas* and *Cytophaga/Flexibacter*-like species. The latter is frequently associated with egg microflora (Bergh 1995) and mortality in early life stages (Hansen et al. 1992).

Also in bivalve cultures the benefits of probiotic bacteria have been investigated (reviewed by Kesarcodi-Watson et al. 2008). In a study by Riquelme et al. (2001)

(Table 1) two strains of inhibitor-producing bacteria (IPB), *Vibrio* sp. and *Pseudomonas* sp., were added to tanks of Chilean scallop *Argopecten purpuratus* larvae, in order to compare survival with larval groups treated with antibiotics. Although survival rates in IPB-treated groups were higher, no significant differences were found. This addresses another important aspect, the use of probiotics as supplements or substitutes for antibacterial agents (reviewed by Vine et al. 2006 and Tinh et al. 2008). Minimising the use of antibiotics is a key factor in attempts to limit the development of resistance.

Immunity

The larval period is associated with an environment that is believed to contain a heavy bacterial load. During the larval stages, cod, turbot and halibut lack a fully competent immune system, which makes larvae vulnerable to disease. The combination of these factors complicates the rearing of marine larvae. Bivalves do not develop a specific immune system and the haemocytes are the essential cells involved in the immune response (reviewed by Dyrynda et al. 1995). Nevertheless specific expression of possibly immune related genes, as a result of bacterial challenge, has been reported in mussels, *Mytilus galloprovincialis*, and carpet-shell clams *Ruditapes decussatus* (Cellura et al. 2007, Gestal et al. 2007, respectively). For cod, the unspecific immune system (Complement factor 3, C3) is expressed at the egg stage (three days post-fertilisation and onwards) (Lange et al. 2004, Magnadóttir et al. 2004, Magnadóttir et al. 2005) while the specific immune system (immunoglobulin M, IgM) is believed to be competent by around two months post-hatch (Schrøder et al. 1998). Preliminary results of current research on halibut larvae have shown that the specific immune system (B-cell marker IgM and T-cell markers CD8α, CD8β and CD4) is expressed around day 73 post hatch and onwards (Sonal Patel, Institute of Marine Research, unpublished results). To the best of my knowledge no such immune studies have been performed in turbot.

The development of vaccines has been critical for the development of the aquaculture industry (reviewed by Sommerset et al. 2005). One of the criteria for successful vaccination in an organism is its ability to produce immunological "memory", i.e. a specific immune response. Since the larvae are not immunocompetant at early developmental stages, vaccination is not an option. However, stimulation of the unspecific immune system by addition of immunostimulants in the form of a dietary supplement has been proposed in order to maintain good health and increase survival rates in larval cultures (reviewed by Bricknell & Dalmo 2005). Examples of immunostimulants are bacterial peptidoclycan and lipopolysaccharides (LPS), polysaccharides and β-glucans. In larval cultures of cod bacterial LPS, various polysaccharids and β-glucans have been tested, but the results have been variable. However, LPS (originating from *Aeromonas salmonicida* spp.) and β-glucans (originating from *Chaetoceros mülleri*) gave promising results (reviewed by Magnadóttir et al. 2006, Skjermo et al. 2006, respectively).

Aims of the study

Bacterial infections are a major obstacle to the rearing of marine larvae. Larval rearing systems consist of high densities of organic matter and heavy bacterial loads as a consequence of high larval densities and the addition of live feed cultures. Such environments may contribute to the proliferation of opportunistic bacteria and outbreaks of disease. Verification of a causative agent is often difficult due to the complexity of the microflora found in these systems. Key issues to improve marine larval rearing protocols are therefore to understand the microflora, which bacteria are involved in infections, and how the bacteria infect. The general aim of this study was to enhance our knowledge of bacteria associated with marine cold-water larvae and to identify virulent strains.

Specific aims for this study were:

- To use known pathogens in order to study infection and histopathology in bivalve and fish larvae, by means of immunohistochemistry (*Vibrio pectenicida* and *Vibrio anguillarum*, respectively).

- To further study bacteria that have been suggested to be pathogenic (i.e. *Vibrio splendidus*-like LT-06, *Pseudoalteromonas*-like strain LT-13 and *Vibrio logei*) by means of challenge experiments and immunohistochemistry.

- To describe differences in susceptibility to and infection caused by various *Vibrio* spp. among cod, halibut and turbot, by means of challenge experiments and immunohistochemistry.

- To investigate differences in infection between bath challenge and oral administration of a pathogen.

List of papers

Paper I Sandlund N, Torkildsen L, Magnesen T, Mortensen S, Bergh Ø (2006) Immunohistochemistry of great scallop *Pecten maximus* larvae experimentally challenged with pathogenic bacteria. Dis Aqua Org 69: 136-173

Paper II Sandlund N & Bergh Ø (in press) Screening and characterisation of potentially pathogenic bacteria associated with Atlantic cod *Gadus morhua* larvae: Bath challenge trials using a multidish system. Dis Aqua Org

Paper III Engelsen AR, Sandlund N, Fiksdal IU, Bergh Ø (in press) Immunohistochemistry of Atlantic cod larvae *Gadus morhua* experimentally challenged with *Vibrio anguillarum*. Dis Aqua Org

Paper IV Sandlund N, Brunvold L, Patel S, Espedal PG, Olsen RH, Otterlei E, Sandlund L, Bergh Ø (Submitted) Immunohistochemical examinations of Atlantic cod larvae, *Gadus morhua*, exposed to *Vibrio* spp. through bioencapsulation in live feed. Aquaculture

Paper V Sandlund N, Rødseth OM, Knappskog D, Fiksdal IU, Bergh Ø Comparative susceptibility of turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus*, and cod, *Gadus morhua* yolk-sac larvae challenged with different serotypes of *Vibrio anguillarum* and *Vibrio* spp. Manuscript

Summary of papers

Paper I describes a study that tested the virulence of bacterial strains associated with mortality in great scallop *Pecten maximus* larvae. *Vibrio pectenicida* was used as a positive control in the experiment. The study confirmed that the *Vibrio splendidus*-like strain LT 06 is pathogenic to great scallop larvae. In addition to increased mortality, immunohistochemical studies showed bacterial cells in lumen and in mucosal cells. Immunohistochemical examinations of larvae exposed to *V. pectenicida* showed positive immunostaining, however no free bacterial cells were observed. This led to the assumption that extracellular products or toxins killed the larvae. The *Pseudoalteromonas*-like strain LT 13, which is assumed to be pathogenic to scallop larvae, did not cause increased mortality and no histopathology was observed. It was therefore suggested that the bacterium is not a primary cause of disease, but merely an opportunist or secondary pathogen.

Paper II describes a screening study performed on cod yolk sac larvae, testing virulence among various bacterial strains isolated from diseased marine larvae, mostly cod, and other marine fish. The results showed that most bacterial isolates did not have a negative effect on the larvae and were therefore not regarded as primary pathogens. Five strains caused mortality that was significantly higher than in the unchallenged control group, four were identified as *Vibrio anguillarum* and one as *Carnobacterium* sp. However the serotyping of the four *V. anguillarum* strains gave inconclusive results, suggesting they belong to a different serotype than O1, O2 and O3, which are usually associated with disease in fish.

Paper III describes histopathology in cod yolk sac larvae challenged with a high (10^6 CFU ml^{-1}) or low (10^4 CFU ml^{-1}) dose of *V. anguillarum* serotype O2α. Larvae exposed to the high challenge dose suffered high mortality but the immunohistochemical examinations revealed scarce histopathology limited to the gastrointestinal tract. It was therefore suggested that extracellular products or toxins excreted from the bacterium were involved in the increased mortality.

Paper IV presents a study in which cod larvae were exposed to *V. anguillarum* and *Vibrio logei* through bioencapsulation in rotifers. No increase in mortality compared to the unchallenged control was observed in the challenged groups. Although reisolation of *V. anguillarum* and *Vibrio logei* from exposed larvae was unsuccessful, immunohistochemical examinations revealed presence of the bacterial strains in the gastrointestinal tract, showing that the bacteria were successfully transferred to the larvae. The larval culture in one of the control tanks experienced a sudden collapse, in which most larvae died within 3-4 days. Isolation of bacteria from diseased larvae together with immunohistochemical examinations led to the conclusion that *V. splendidus* was the causative agent.

Paper V describes differences in susceptibility to various serotypes of *V. anguillarum* among cod, halibut and turbot yolk sac larvae. All three species suffered high mortality when exposed to serotype O2α while cod and halibut were also affected by the O1 serotype. Indications that the two serotypes use different infections strategies were found during the immunohistochemical examinations. Examinations revealed severe histopathology in larvae exposed to the O1 compared to the groups exposed to O2α. Positive immunostaining, necrotic tissue and bacterial cells were seen in several organs throughout the whole larvae. In contrast, the O2α-infected larvae showed limited histopathology, and bacterial cells were limited to the gastrointestinal tract. The *V. splendidus* and *Vibrio salmonicida* strains did not have negative effects on the larvae.

Discussion

Marine larvae reared in hatcheries are exposed to an environment very different from their natural habitat. In nature, marine larvae inhabit the pelagic waters grazing on algae and zooplankton. This dynamic and diverse environment is in sharp contrast to the larval and live feed oligo-cultures in the hatcheries.

In commercial aquaculture, cost-effectiveness is crucial, thus densities in live feed and larval cultures are kept high. This environment with high abundance of biomass (larvae, food, detritus and green water) gives pathogenic and opportunistic bacteria increased possibilities to proliferate and thereby cause disease. In such high densities of larvae and organic matter an outbreak of disease may develop rapidly. Marine larvae are hatched at an early immature stage without a fully developed immune system to deal with infections, and no efficient treatments are yet available.

The microbial environment in larval rearing systems

A common experience in hatcheries is an unanticipated and sudden outbreak of disease. Such an incident is described in **Paper IV**, in which a *Vibrio splendidus* infection terminated one out of six identical tanks populations. This observation gives room for speculation that in spite of identical rearing regimes, in reality every larval unit has its own microflora and that small shifts may cause drastic changes. Microbial control in rearing systems have been pointed out as essential in rearing of marine larvae (Skjermo & Vadstein 1999). Obtaining a favourable balance in the micro flora between r-selected (fast-growing, which many are opportunistic) and K-selected (slow-growing, primarily non-opportunistic) bacteria, thus appear to be an important challenge. The use of matured water has been suggested as a method approach for improving the rearing environment of marine larvae (Vadstein et al. 2004). Matured water is considered to be inhabited by K-selected species forming a more stable and controlled system (Vadstein et al. 2004). A study showed up to76% higher survival rates in halibut, *Hippoglossus hippoglossus*, and turbot, *Scophthalmus maximus*, yolk-

sac larval groups reared in matured water than in groups reared in filtered water. The matured water also seemed to have a positive effect on the growth of turbot larvae (Skjermo et al. 1997).

As the production of marine larvae is associated with high rates of mortality, different approaches have been tried to improve rearing conditions. Rearing protocols in the commercial hatcheries vary in terms of water treatment and flow systems, depending on their source of water and their facilities. Optimizing feed production protocols and rearing routines and improving in-let water quality will probably result in a better controlled environment in hatcheries, as has been reported successfully done in rearing of striped trumpeter, *Latris lineata*, (reviewed by Battaglene & Cobcroft 2007).

Variations such as stagnant water, flow-through systems and recirculation of the water may be used within the same facility and at different stages of the production cycle. In the Norwegian great scallop, *Pecten maximus*, hatchery, a flow-through system has been established and has proven to be efficient in terms of increase growth and survival (Andersen et al. 2000, Christophersen et al. 2006). In a study of water treatment in halibut larvae rearing, recycled water produced the highest survival rate (unless antibiotics were added) in addition to the most stable physical environment (Verner-Jeffreys et al. 2004).

Marine scallop and fish larvae are small; this makes it technically difficult to isolate bacteria from specific organs and a wide range of bacterial strains is therefore usually isolated during an outbreak of disease. As shown in the screening experiment (**Paper II,** and to some degree in **Paper I)**, most bacterial strains isolated in association with diseased larvae cannot be regarded as primary pathogens. This is in agreement with similar challenge studies performed on halibut and great scallop larvae (Verner-Jeffreys et al. 2003a, Torkildsen et al. 2005, respectively). The virulence test on halibut larvae included isolates from three different rearing regimes, i.e. disinfected eggs with either flow or recycled water, and non-disinfected eggs in recycled water (Verner-Jeffreys et al. 2003a), while Torkildsen et al. (2005) tested bacterial strains

isolated from algal cultures, eggs and larvae from the only great scallop hatchery in Norway, Scalpro AS. Given the differences in water source, water treatment and rearing regimes among hatcheries, it may be speculated that differences in microflora will also occur. Comparative studies are therefore valuable and different approaches need to be considered for different species and hatcheries.

A study by Jensen et al. (2004) suggested that halibut larvae possess a distinct and specific normal flora regardless of geographic region. On the other hand, both egg epiflora and larval microflora differed among halibut hatcheries (Verner-Jeffreys et al. 2003b). Although *Pseuodoalteromonas* and *Vibrio* species dominated the larval samples, the larval microflora was highly variable among larvae sampled from the same batches. The studies referred to above illustrate the difficulties in studying the microflora in marine larval rearing systems.

All the studies referred to above are based on the cultivation of bacterial strains. However not all bacteria will grow on agar. A method that circumvents cultivation, denaturing gradient gel electrophoresis (DGGE), has been successfully used to monitor the bacterial flora in great scallop (Sandaa et al. 2003, Sandaa et al. 2008) and cod *Gadus morhua* (Brunvold et al. 2007, McIntosh et al. 2008) hatcheries. As shown in **Paper IV**, identification of bacterial strains using cultivation and DGGE is not always consistent. On the other hand, the two methods will probably complement each other.

Bacterial infections in larval cultures

Live feed cultures as source of pathogenic bacteria?

Live feed cultures contain a heavy bacterial load and supply large quantities of biological material to the larval rearing system. This may contribute to growth of opportunistic bacteria. Consistency between microflora in live feed cultures and larval intestinal flora (Munro et al. 1994, Eddy & Jones 2002) and between microflora in live feed cultures and larval rearing water (Eddy & Jones 2002), have been reported. To support this it has been shown that as a food source is introduced, a shift in the larval microflora follows (Verner-Jeffreys et al. 2003b, Brunvold et al. 2007).

The high densities and the harvesting procedures of live feed cultures may contribute to shifts in microflora which can be transferred to the larvae. It can be speculated that the replacement of the harvested volume in live feed cultures creates a potential for proliferation of opportunistic bacteria causing this sudden shifts in microflora. In addition, these cultures are reared at higher temperatures than the larval species, and it can be speculated that the larvae are therefore introduced to bacteria not normally part of their normal flora.

Inconclusive results have been reported concerning the topic of live feed cultures, i.e algae, rotifers and *Artemia* spp., as a source of pathogenic bacteria. The intestine of larvae fed live feed has been found to contain a higher bacterial count than larvae and juveniles fed formulated feed (Savas et al. 2005). However, the quantity of bacteria is not necessarily correlated with survival (Munro et al. 1994, Verner-Jeffreys et al. 2004) but is probably more closely related to the species of bacteria involved. The *Pseudoalteromonas*-like strain LT-13 (**Paper I**) and the *Carnobacterium* sp. strain causing high mortality to cod larvae (**Paper II**) were originally isolated from an algal culture of *Tetraselmis* spp. (Torkildsen et al. 2005, **Paper II**, respectively). This could be regarded as a paradox in view of the beneficial aspects that have been reported regarding the addition of *Tetraselmis* spp. cultures; for example antibacterial activity (Tolomei et al. 2004, Makridis et al. 2006) and the ability to change the composition

of the microflora (Olsen et al. 2000, Salvesen et al. 2000). Salvesen et al. (2000) found that *Tetraselmis* sp. and *Pavlova lutheri* cultures had an overall low level of fast-growing opportunistic bacteria compared to cultures of *Nannochloropsis oculata*, *Skeletonema costatum, Chaetoceros mülleri* and *Isochrysis galbana*. Olsen et al. (2000) found that *Tetraselmis* sp. may change the gut bacterial flora in *Artemia franciscana* by reducing the number of haemolytic bacteria. By feeding the *Artemia* to first-feeding halibut larvae, this change in microflora was transferred to the larvae.

Commercial enrichments used in the production of live feed have been suggested to influence the growth rate of pathogenic bacteria. Korsnes et al. (2006) isolated *Vibrio anguillarum* from cod, *Gadus morhua*, larvae, fed rotifers enriched with a selection of four commercial enrichment diets. Verdonck et al. (1997) identified *V. anguillarum* in rotifer cultures. However this finding could not be related to mortality as the *V. anguillarum* strains were identified as belonging to serotypes not associated with disease.

It should be noted that identification, based on 16S rDNA analysis, of the cod larval pathogenic strain as *Carnobacterium* sp. may not be correct (**Paper II**). *Carnobacterium* sp. is Gram-positive while the isolate identified as *Carnobacterium* sp., stained Gram-negative and were weakly haemolytic.

Vibrio infections

Vibrio infections cause substantial losses in marine aquaculture. *Vibrio anguillarum* and *Vibrio splendidus* are often associated with disease in bivalve and marine fish farms (reviewed by Paillard et al. 2004, and Toranzo et al. 2005, respectively).

Vibrio anguillarum

Paper II adds information to support the theory that unknown serotypes of *V. anguillarum* may be responsible for vibriosis outbreaks in marine fish farms, as has recently been suggested by Mikkelsen et al. (2007). The attempt to serotype the four pathogenic strains identified as *V. anguillarum* through phenotypic and genotypic analysis gave inconclusive results (**Paper II**). Because of the wide variety of both

virulent and avirulent strains of *V. anguillarum* that have been isolated (Austin et al. 1995), the thorough identification and characterisation of strains involved in outbreaks of vibriosis should be emphasised in terms of vaccine development.

Bath challenge experiments with *V. anguillarum* HI-610 strain serotype O2α have resulted in significant increases in mortality (Samuelsen & Bergh 2004, Vik-Mo et al. 2005, Seljestokken et al. 2006, **Papers II, III, V**). In spite of this, little pathology and few bacterial cells were observed during the immunohistochemical examinations (**Papers III**, **V**). Intestinal damage and loss of erythrocytes are common during bacterial infection but variations are reported in both fish larvae and fry (reviewed by Ringø et al. 2007). It has been shown that *V. anguillarum* serotype O2 does not adhere to mucus as other fish pathogenic bacteria (Knudsen et al. 1999). This may explain why histological and immunohistological findings in **Papers III**, **IV** and **V** showed free bacterial cells in the lumen, whereas few were attached to the brush border in the gastrointestinal. Supernatant taken from cultures of *V. anguillarum* or *V. anguillarum*-like strains contain extracellular products (ECPs) known to be toxic to Chilean scallop, *Argopecten purpuratus*, and turbot larvae (Riquelme et al. 1995, Planas et al. 2005, respectively). This suggests that toxins are killing the larvae. This was also suggested to be the case in the scallop larval experiment based on observations of pathology and positive immunostaining in larval tissue, in spite of the absence of observations of free bacterial cells (**Paper I**). Bacteria may also be degraded, causing positive staining.

Differences in susceptibility to serotypes of *V. anguillarum* were observed among cod, halibut and turbot larvae (**Paper V**). Given that different serotypes of *V. anguillarum* are associated with different species of fish (Pedersen et al. 1999), this is not a unique finding. On the other hand, knowledge about such differences in larval stages, before a specific immune system has developed, is scarce. The portal of entry for *V. anguillarum* has been debated (Baudin Laurencin & Germon 1987, Kanno et al. 1989, Smith et al. 2004). This study did not provide conclusive results regarding the portal of entry for *V. anguillarum* strain HI-610 (**Paper III**), but it seems likely that

the bacterium is capable of surviving inside the gastrointestinal tract without causing disease (**Papers III –V**). In contrast to the O2α serotype strain, the O1 serotype showed extended pathology and necrotic tissue in the cod and halibut larvae examined. Dermis and muscular tissue in addition to the head area were most severely affected (**Paper V**). Grisez et al. (1996) reported septicaemia in turbot larvae exposed to *V. anguillarum* serotype O1 embedded in *Artemia*. Severe pathology caused by the O1 serotype was also observed in newly hatched spotted wolfish, *Anarhichas minor* (Ringø et al. 2006). However, these two studies did not include serotype O2α. The observed differences in infection seem to be linked to serotype rather than species, indicating that the two serotypes display different strategies for host infection.

Vibrio splendidus

Vibrio splendidus strains were originally isolated as a non-pathogenic environmental bacteria (Reviewed by Thompson et al. 2004b). It has however been shown that *V. splendidus* is pathogenic to both molluscs (Nicolas et al. 1996, Sugumar et al. 1998, Lacoste et al. 2001a, Le Roux et al. 2002, Gay et al. 2004, Gómez-León et al. 2005) and fish (Jensen et al. 2003, Thomson et al. 2005, Bergh & Samuelsen 2007, **Paper IV**) on several occasions. Before the experiment described in **Paper I**, Torkildsen et al. (2005) verified a *V. splendidus*-like strain, LT-06, as being pathogenic to Great scallop larvae in a Norwegian hatchery. These studies demonstrate *V. splendidus* as a worldwide problem, also in marine larval cultures.

The *V. splendidus* strain isolated from the diseased cod larvae (**paper IV**) seems to be serologically similar to the LT-06 strain. The antiserum against the LT-06 strain (**Paper I**) gave positive immunostaining of the cod larval isolate (**Paper IV**). Further studies are needed to verify this *V. splendidus* strain as pathogenic, i.e. challenge studies, and to characterise the differences between the two isolates. Preliminary results have revealed 99-100% similarity between the *gyrB* sequences of the two isolates (data not shown). The *V. splendidus* isolate used in the susceptibility study did not cause mortality to either turbot, cod or halibut larvae (**Paper V**). This

seemingly avirulent *V. splendidus* isolate should be implemented in the further studies suggested above.

Pseudoalteromonas sp. and *Vibrio logei* – opportunists or just secondary pathogens?

The incidents that led to the inclusion of the *Pseudolateromonas*-like strain LT-13 and *Vibrio logei* strains in this study (**Papers I**, **II, IV**) were the challenge experiment by Torkildsen et al. (2005) and the frequent isolation of *V. logei* in cod hatcheries suffering high mortality (Egil Karlsbakk, Institute of Marine Research, Pers. Comm.). Both bacteria were associated with mortality in larval cultures. However, the limited mortality observed in these experiments could not be related to the bacterial challenge (**Papers I**, **II, IV**). In both scallop larvae and cod larvae immunohistochemical examinations showed the presence of LT-13 and *V. logei* (**Papers I** and **IV,** respectively), but no indications of infection was observed. To support this, an experiment performed on cod (15 g) challenged by an intraperitoneal injection with *V. logei* (cell concentration $1x10^8$), resulted in death of only two out of 30 fish (Nylund et al. 2006). Moreover, a bath challenge experiment on cod (approximately 2.6 g) to test the virulence of a *V. logei*-like strain resulted in low mortality and the bacteria could not be reisolated from diseased fish (Mikkelsen et al. 2007). A study that monitored the presence of the LT-13 strain in a great scallop hatchery over a period of 11 months (Sandaa et al. 2008), showed that the observations of the bacterium were usually related to mortality. These results indicate that *V. logei* and LT-13 may be secondary pathogens or opportunists. It should also be noted that luminous bacteria (e.g. *V. logei*) are regarded as commonly present in the intestinal tract of a variety of marine species (reviewed by Hansen & Olafsen 1999)

The immunohistochemical observations of larvae challenged with *V. logei* showed that the bacterium was present in the gastrointestinal tract for a maximum of 72 hours post-challenge (**Paper IV**), suggesting that they were unable to colonise the gut. Similar experiments on turbot larvae showed that bacterial strains added through bioencapsulation in live feed display different abilities to colonise the gastrointestinal

tract (Makridis et al. 2000, Makridis et al. 2001). This could also be the case for the *V. logei* strain (**Paper IV**).

Strains of *V. logei* have been identified as symbiotic to the bacterium *Vibrio fisheri*, that possesses quorum sensing (QS), in sepiolid squids (Fidopiastis et al. 1998). QS systems are a complex communication system used for both species-specific and interspecies communication among bacteria by the release of signal molecules (Greenberg 2003). It can therefore be speculated that *V. logei* possesses the same characteristics as its symbiont. The QS system involves regulation of gene expression and is activated when the signal molecules reach a threshold concentration. There is some evidence that QS is essential for virulence (Diggle et al. 2007, Nelson et al. 2007). Well-known fish pathogenic bacteria such as *Vibrio salmonicida* (Nelson et al. 2007), *V. anguillarum* (Milton et al. 1997), and *Aeromonas salmonicida* (Rasch et al. 2007) posess this system. It would therefore be of interest to examine *V. logei* strains for the presence of a QS system and to determine whether this influences the role of the bacterium in outbreaks of disease. Taking this into consideration, it could further be suggested that the absence of mortality was caused by challenge doses of *V. logei* were too low or that additional bacteria need to be present.

Stress and infection

Opportunistic bacteria cause disease under certain conditions, such as when the host is stressed or the immune system is weakened. In a farming situation, where the animal is kept within a limited area, stress can be triggered by several factors. Examples of such factors are environmental changes (e.g.changes in oxygen levels, pollution or shifts in temperature), handling and presence of predators or high concentrations of algae and jelly-fish. It seems to be a general phenomenon that stress may induce immunosuppression (reviewed by Tort et al. 2004), which in turn could increase susceptibility to disease (reviewed by Magnadóttir 2006, and Ringø et al. 2007), as shown in juvenile oysters, *Crassostrea gigas* (Lacoste et al. 2001b), juvenile sea bass, *Dicentrarchus labrax* (Varsamos et al. 2006) and goldfish, *Carassius auratus* (Dror et al. 2006). These aspects complicate the search for pathogenic agents

when it comes to *in vivo* testing of virulence using a challenge model. Challenge models are controlled environments, in which variable factors, such as rearing conditions, are limited to a minimum in order to ensure that the added bacterium is responsible for any increased mortality. Assuming that *Pseudoalteromonas* strain LT-13 and *V. logei* are opportunists, it cannot be out ruled that under different circumstances the bacteria could cause disease.

Larval studies - methology

Larval challenge models

Survival in larval groups is usually unpredictable which complicates studies of early life stages. In the study of virulence, reliable challenge models are essential in order to achieve consistent and repeatable results. Challenge experiments usually contain one type of control, i.e. unchallenged control group replicates. However, two positive controls were implemented in the studies included in this thesis (i.e. *Vibrio pectenicida* (**Paper I**) and *Vibrio anguillarum* strain HI-610 serotype O2α, **Paper II**). By comparing the results to two control groups two extremes may be compared, thereby elucidating any intermediate results. This is particularly useful when studying unknown possible pathogens.

As overviewed in Table 1 (page 27-30), either bath challenge or oral administration through live feed is used in larval challenge studies. This thesis utilizes a multidish system for bath challenge studies (**Papers I-III, V**) and bioencapsulation in rotifers for oral administration of bacteria to cod larvae (**Paper IV**).

Multidish systems

The multidish challenge model used in **Papers I-III** and **V** is based on the principle of using small volumes for performing animal tests. This makes it the ideal method for studies involving larvae. Larvae are placed in a sterile environment without the possibility of aeration or feeding. The challenge system is "user-friendly" and may be used for a number of larval species. The model represents an "artificial environment" compared to the "real life" of a hatchery situation. Nevertheless, it is possible to keep larvae alive long enough to obtain necessary information, in this case concerning the virulence of a particular bacterium. As the experiments show, the bacteria verified as primary pathogens caused significant mortality in the course of 2-7 days post-hatch.

The most significant benefits and disadvantages regarding the challenge method are listed below.

Benefits of using a multidish system are:

- A highly controlled environment
- Individual control
- The possibility of performing screening tests with large amounts of bacteria
- That by challenging the egg stage information regarding the bacterium's ability of being pathogenic to eggs and/or larvae is obtained
- Less work than when working on larger fish

Disadvantages of using a multidish system are:

- That the duration of the experiments will be limited by the length of the yolk sac stage
- That it is not possible to feed the larvae
- That it is a highly artificial environment

Bioencapsulation

Bioencapsulation in live feed, i.e. rotifers and *Artemia*, is a method administrating bacteria or substances are administrated to larvae. This model has been used to test bacterial strains for virulence as well as *in vivo* probiotic abilities (see Table 1, page 27-30 for more details). In addition, live feed (*Artemia* sp.) has been used for oral administration of immunostimulants (Skjermo & Bergh 2004). This model represents an environment closer to a hatchery situation than the multi dish system can simulate. Larvae are fed and reared in a manner comparable to those of a commercial hatchery.

In commercial farming of marine species the green water technique is applied. This technique was not included in the bioencapsulation experiment (**Paper IV**). Furthermore, the tanks were small and had water exchange, and dead larvae and debris were easily removed. This may have produced an environment in which the

opportunistic bacterium *V. anguillarum* would not proliferate and be infectious. It could also be speculated whether the study should have lasted longer. However, in the turbot infection study of Planas et al. (2005), mortality started within 24-72 hours. Bioencapsulation as a challenge method, using both rotifers and *Artemia*, has been successfully performed with turbot larvae on several occasions (Makridis et al. 2000, Makridis et al. 2001, Planas et al. 2005, Planas et al. 2006). This indicates that the method needs to be further modified for cod larval experiments.

A different approach to the bioencapsulation experiment would be to use axenic larvae (Munro et al. 1995) and live feed, i.e. rotifers (Tinh et al. 2006) and *Artemia* sp. (Gomez-Gil et al. 1998, Marques et al. 2004). Using axenic larvae would presumably limit the risk of a collapse in a control group, as experienced in the study described in **Paper IV,** and other unpredictable incidents. On the other hand, the collapse in the control group that resulted in the isolation of *V. splendidus* turned out to present significant input to the experiment. In addition the experiment was a useful reminder that even in controlled (challenge) experiments, controlling all the factors involved in structuring a microbial community, is complicated. Looking back, the axenic approach could probably have been tested prior to the cod experiment. On the other hand it was desirable to run the experiment in as similar a fashion to a commercial farming situation as possible.

Immunohistochemistry - a useful tool in larval studies

Studying gross pathology in larvae is difficult due to size, which means that examinations of tissue samples by means of histological methods are more practical. Various histological methods are regularly used in the detection, diagnosis and verification of pathogenic agents and histopathological studies (Grisez et al. 1996, Mortensen et al. 2005, Timur et al. 2005). Immunohistochemistry (IHC) is based on the principle of specific antibody-antigen binding to verify the presence of a specific agent. The studies that make up this thesis used IHC to verify the presence of bacteria after challenge (**Papers I**, **III**-**V**). Using IHC for this purpose also improves the analysis in larval challenge studies as a supplementary method to reisolation of

bacteria (as shown in **Paper IV**). Detection of the added bacterium is also vital as a means of validating the challenge model, as it provides information regarding the hosts' uptake of the bacterium. Polyclonal antisera were used in the immunohistochemical studies presented in this thesis (**Papers I**, **III-V**). Antibodies of this type are not as specific as the monoclonal antibodies, which could result in false positive immunostaining on tissue samples. However, absorption of a polyclonal antiserum with closely related antigens will increase the specificity of the serum (Ross & Boulton 1972, Knappskog et al. 1993). The polyclonal antisera used in these studies were absorbed with the other bacteria included in that specific study or with closely related bacteria (**Papers I**, **III-V**) (e.g. the anti-*V. anguillarum* sera were absorbed with other serotypes of *V. anguillarum*). No cross reaction were found after testing on bacterial smears and tissue samples (e.g. anti *V. anguillarum* O1 serum was tested for cross-reactions on bacterial smears and tissue samples containing O2a and O2b).

Another limitation of using IHC and specific antisera, is that identification of other agents present will be at least difficult. This could result in the wrong diagnosis, for example if more than one pathogenic agent is present. The diagnoses of *V. splendidus* as the causative agent in cod larvae (**Paper IV**) however, were based on positive immunostaining of the bacterium in larval tissues samples as well as isolation of the bacterium from homogenised larvae.

Larvae tend to have poorly differentiated and developed tissues, which complicate histopathological studies. Even so, IHC has been successfully used to describe differences in histopathology in great scallop larvae (**Paper I**) and fish larvae (**Papers III-V**) from different challenge regimes.

Larval studies - results

The bacterium *V. anguillarum* strain HI-610 serotype O2α produced a significant increase in mortality the bath challenge experiments (**Papers II**, **III**, **V**). The lack of mortality in the "bioencapsulation experiment" was therefore not expected (**Paper IV**). The immunohistochemical examination verified the successful transfer of *V. anguillarum* and *V. logei* from the rotifers to the cod larvae (**Paper IV**). *V. anguillarum* was present in the gastrointestinal tract throughout the experiment, while *V. logei* apparently disappeared within 72 hours post-challenge. It has been verified that *V. anguillarum* orally administrated to juvenile turbot survive the passage through the gastrointestinal tract and proliferate in faeces (Olsson et al. 1998). This finding together with the immunohistochemical observations, indicates that the *V. anguillarum* strain was alive in the cod larvae (**Paper IV**). Dead bacteria would probably be either digested or expelled together with the digested food. As mentioned above, the minimal attachment of *V. anguillarum* to mucosal surfaces could be linked to serotype (Knudsen et al. 1999). This is in agreement with observations of larvae exposed to *V. anguillarum* by bath challenge, where few bacterial cells were seen in the gastrointestinal tract (**Paper II**). Repeating this experiment with serotype O1 would therefore be desirable. The absence of mortality seen in the *V. anguillarum* groups could also be related to the rearing volume and water exchange. Based on the suggestion that that toxins were the cause of death in the small-volume, stagnant multidish system (**Papers II**, **III**, **V**), it can be speculated that the concentration of toxins in the tank experiment was not sufficiently high (**Paper IV**).

Isolation of bacterial strains within the gastrointestinal tract requires surface disinfection prior to homogenisation of the whole larvae due to their small size. The attempt to reisolate *V. anguillarum* and *V. logei* was not successful. This indicates that the quantity of identified bacterial strains was too low. On the other hand, isolation of *V. splendidus*, the causative agent of the mortality in the control group, was successful.

Visual examination of immunostained larval tissue samples suggested that larvae infected with *V. splendidus* were extensively infected and large amounts of bacteria were observed in the gastrointestinal tract whereas larvae infected with *V. anguillarum* and *V. logei* were unaffected. This noticeable difference could be central when attempting to isolate the bacteria.

Isolation and characterisation of bacterial strains

The microflora associated with live feed and marine larval cultures is complex, heterogeneous and therefore complicated to study. Culture-dependant techniques are the most common approach, but as pointed out by Austin (2006) quantitative and qualitative aspects are rarely emphasised together and the relevance of such data could be questioned. Furthermore, the presence of anaerobic bacteria is ignored by most culture-dependant approaches.

The use of various types of growth medium will broaden the selection of bacterial strains, resulting in a wide variety of strains (**Paper II**). It can also be narrowed down by the use of specific media, such as the *Vibrio*-specific medium Thiosulphate Citrate Bile Sucrose agar (TCBS). In search of pathogenic bacteria, growth on blood agar, to test for haemolytic characteristics, could help to narrow down the search. Even though not all pathogenic bacteria are haemolytic (i.e. *Vibrio alginolyticus*, *Yersinia ruckeri* Buller 2004), this is a good starting point. Adjusting the growth temperature in relation to the environment should also be considered. Bacterial strains may be sensitive to high temperatures, such as the causative agent of cold-water vibrioisis, *Vibrio salmonicida*, which has a growth optimum at 15°C and a maximum growth temperature of 22°C (Egidius et al. 1986). The difference in optimal growth temperature can be exploited to enhance the selection of particular bacterial strains by using variations in incubation temperature. Combinations of various incubation methods will also assist in the process of obtaining a pure culture of the bacterial strain.

The use of phenotypic, genotypic or serological characterisation alone rarely provides sufficient information to identify a bacterial strain. On the other hand, using a combination of the various methods provides a more reliable identification. Phenotypic and genotypic variations may be found among closely related isolates, as shown for *V. splendidus*-related (Le Roux et al. 2002) and *Vibrio alginolyticus* (Snoussi et al. 2008) strains. This was also found among the pathogenic *V. anguillarum* strains (HI 21412, HI 21413, HI 21414 and HI 21429) tested in **Paper II**. *V. anguillarum* strains are described as haemolytic when grown on blood agar (BA) (Buller 2004). The four strains were weakly haemolytic and did not show a clear zone around the colonies (**Paper II**). Further yellow colonies were produced on TCBS (**Paper II**). According to Buller (2004) *V. anguillarum* strains show variations in growth on TCBS. In terms of isolating pathogenic vibrios from environmental samples (Lotz et al. 1983), TCBS was not found to be sufficient by itself but rather a supplement.

The API 20E test has been used as a phenotypic test for various fish pathogenic bacteria, including *V. anguillarum* (Austin & Austin 2007). The A/L/O test (ADH, LDC and ODC included in the API 20E test, respectively) identifies *V. anguillarum* strains as +/-/- (Alsina & Blanch 1994). The four strains from the screening experiment gave a similar A+/L-/O- profile (**Paper II**). Even so variations among the four strains were observed when using the API 20E test. HI 21413, HI 21414 and HI 21429 tested negative for utilization of arabinose while HI 21412 tested positive. Further strains HI 21412, HI 21413 and HI 21414 showed variable test results for citrate utilization, while HI 21429 tested negative (**Paper II**).Variations among *V. anguillarum* isolates have been reported (Austin & Austin 2007). As reviewed by Popovic et al. (2007), limitations and inconsistent API results are frequently reported when testing marine bacteria. None-the-less it was concluded that API can be a useful tool when modifications are being made. As discussed above, the serological analysis of the four strains was inconclusive (**Paper II**) and further studies need to be performed.

Sequencing the 16S rRNA and *gyrB* genes of the four strains showed 98-99% similarity to various *V. anguillarum* isolates (**Paper II**). A minimum of 97% resemblance in gene sequence has been suggested for certain identification (Hagström et al. 2000, Hagström et al. 2002). Polymorphism and heterogeneity in the 16S rRNA gene have been reported (Dahllöf et al. 2000, Moreno et al. 2002). As reviewed by Thompson et al. (2004b), the 16S rRNA gene has been found to be appropriate for allocation of bacterial strains into families but not sufficient to allocation on the species level, hence the decision to use two genes. The decision to use the *gyrB* gene as an additional gene was based on the previous results suggesting that this gene would be a good phylogenetic marker (Le Roux et al. 2004).

Conclusive remarks and future perspectives

This study has described four *Vibrio anguillarum* strains, isolated from moribund cod larvae, as highly pathogenic to cod larvae. This confirms vibriosis and *V. anguillarum* to be a problem in cod hatcheries. It has further been suggested that these strains belong to other serotypes than O1, O2 and O3, usually associated with disease in fish (Toranzo et al. 2005). The assumption of new serotypes was based on the inconclusive serotyping results and the fact that a recent study put forward a similar suggestion (Mikkelsen et al. 2007). Further biochemical, serological and phylogenetic studies of the four *V. anguillarum* isolates are needed. Such studies should include optima and limitations regarding growth and tests of the presence of virulence plasmids, in addition to a thorough serotyping scheme. *In vivo* virulence studies with these *V. anguillarum* strains on juvenile cod, in addition other cultured fish species, should be of interest for vaccine development for the aquaculture industry.

The incident of the collapse of one of the control groups presented in **Paper IV** resulted in the suggestion of *Vibrio splendidus* as pathogenic to cod larvae. However the bacterium was only isolated form moribund larvae, hence *in vivo* virulence studies are needed to confirm the isolate as pathogenic. Such studies should also include immunohistochemical examinations of challenged larvae to compare the results with the histological findings of **Paper IV**.

Few of the bacterial isolates tested for virulence caused an increase in larval morality, suggesting that most bacteria associated with disease are not primary pathogens. Further studies of bacterial populations in hatcheries, similar to those performed by Jensen et al. (2004) , Verner-Jeffreys et al. (2003b), Brunvold et al. (2007), McIntoch et al. (2008) and Sandaa et al. (2003, 2008) are therefore needed. Thorough studies comparing the microflora in larval batches that experience high survival or low survival rates might provide more clues about the dynamics of the bacteria involved in larval disease. In addition, studies of the interactions between micro flora and abiotic parameters are vital to obtain a full picture of collapse in marine larval cultures.

Examples of abiotic parameters are:

- water treatment and water quality
- temperature (fluctuations)
- handling
- feeding regimes and nutrient value

Looking at changes in the microflora in relation to such parameters, will probably help to optimise production protocols and limit the incidence of disease outbreaks.

One topic identified as challenging is the nutritional value of the live feed used in production of both bivalve (Muller-Feuga et al. 2003b) and fish (Olsen 2004) larval cultures. In a hatchery situation, larval diets are based on a few food organisms, which could result in an inadequate nutrient composition. Protein (reviewed by Kvåle et al. 2007) and fatty acid (Morais et al. 2007, Garcia et al. 2008) requirements of fish larvae are hard to meet. In rearing of halibut this is often associated with problems of malpigmentation and abnormal eye migration (reviewed by Hamre et al. 2007). A recent study of sea bass, *Dicentrarchus labrax*, larvae showed that diets that contain different levels of fish protein hydrolysates caused variations in the composition of larval microbiota and susceptibility to challenge with *V. anguillarum* (Kotzamanis et al. 2007). However, it was not established whether or not this was caused by stimulation of the immune system or antagonism between *V. anguillarum* and other bacteria. In the view of this fact, it would be of great interest to combine challenge and nutritional studies to investigate susceptibility to disease, i.e. to compare susceptibility to disease among larval groups fed a natural zooplankton diet and an enriched rotifer and *Artemia* spp. diet.

Final remark; The problems of the aquaculture industry can only be solved through close cooperation between the farmers, the authorities and research will be crucial. Such interdisciplinary cooperation is the only way to ensure sustainable growth of the marine aquaculture industry.

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Paper I

Sandlund N, Torkildsen L, Magnesen T, Mortensen S, Bergh Ø (2006) Immunohistochemistry of great scallop *Pecten maximus* larvae experimentally challenged with pathogenic bacteria. Dis Aqua Org 69: 136-173

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Immunohistochemistry of great scallop *Pecten maximus* larvae experimentally challenged with pathogenic bacteria

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ABSTRACT: Three challenge experiments were carried out on larvae of the great scallop *Pecten maximus*. Larvae were bath-challenged with *Vibrio pectenicida* and 5 strains resembling *Vibrio splendidus* and one *Pseudoalteromonas* sp. Unchallenged larvae were used as negative controls. The challenge protocol was based on the use of a multidish system, where the scallop larvae (10, 13 and 15 d post-hatching in the 3 experiments, respectively) were distributed to 2 ml wells with stagnant seawater and exposed to the bacterial cultures by bath challenge. Presence of the challenge bacteria in the wells was verified by polymerase chain reaction (PCR). A significantly increased mortality was found between 24 and 48 h in most groups challenged with *V. pectenicida* or *V. splendidus*-like strains. The exception was found in larval groups challenged with a *Pseudoalteromonas* sp. LT 13, in which the mortality rate fell in 2 of the 3 challenge experiments. Larvae from the challenge experiments were studied by immunohistochemistry protocol. Examinations of larval groups challenged with *V. pectenicida* revealed no bacterial cells, despite a high degree of positive immuno-staining. In contrast, intact bacterial cells were found in larvae challenged with *V. splendidus*. In the case of larvae challenged with the *Pseudoalteromonas* sp., positive immuno-staining was limited to visible bacteria inside the digestive area and cells of the mucosa. The experiments confirm that *V. splendidus* and *V. pectenicida* are pathogenic to scallop larvae, and that the *Pseudoalteromonas* strain is probably not a primary pathogen, although it cannot be ruled out as a secondary pathogen.

KEY WORDS: *Pecten maximus* · *Vibrio splendidus* · Immunohistochemistry · Challenge experiments · Larvae

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INTRODUCTION

The great scallop *Pecten maximus* L occurs naturally along the coasts of Europe, north to the Lofoten islands in Norway. Due to good prices on the European market, suitable environmental conditions and a harvestable wild population, potential for commercial scallop production in Norway has been identified, and attempts to cultivate the species are currently underway in Norway and France (reviewed by Bergh & Strand 2001).

Aquaculture of the great scallop in Norway is based upon hatchery-produced spat. This production is commonly associated with highly variable survival during the larval stages. Mortalities of up to 100% during the early life stages have frequently been experienced at the only Norwegian hatchery, and the total annual production of 2 mm spat has been limited to about 2 million since 1996 (Torkildsen & Magnesen 2004).

Opportunistic bacteria are probably the primary cause of the mortalities (Nicolas et al. 1996, Robert et al. 1996, Torkildsen et al. 2000, 2002, 2005). This is a well-known problem in bivalve hatcheries, and several bacteria, especially *Vibrio* species, cause diseases in bivalves. *Vibrio pectenicida* has been isolated from moribund *Pecten maximus* larvae in French hatcheries (Lambert et al. 1998). In cultivated northern Chilean scallop *Argopecten purpuratus*, vibriosis caused by

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V. anguillarum (Riquelme et al. 1995) and *V. alginolyticus* (see Riquelme et al. 1996a,b) has caused high mortality. *V. splendidus* was the causative agent of annual summer mortalities of the Pacific oyster *Crassostrea gigas* in France (Lacoste et al. 2001, Le Roux et al. 2002, Waechter et al. 2002). The causative agent of brown ring disease (BRD), *V. tapetis* (Borrego et al. 1996) caused high mortalities in Manila clams *Ruditapes philippinarum*, especially in France. Mortalities up to 100% caused by *V. tapetis* occurred in a challenge study of Manila clams (Allam et al. 2002). Other bacterial species causing disease in bivalves are *Aeromonas hydrophila* (Riquelme et al. 1996a), *Chlamydia* (Leibovitz 1989), *Chlamydia*-like organisms (Morrison & Shum 1982, Renault & Cochennec 1995, Hine & Diggles 2002) and *Rickettsia*-like organisms (Elston 1986, Le Gall et al. 1988, 1991, Wu & Pan 1999).

However, bacteria are also a part of the diet of bivalves, which feed on microalgae, other microplankton, bacteria and particulate organic material (Hovgaard et al. 2001). When Samain et al. (1987) studied water quality, they found that feed particle sizes between 0.22 and 1 µm significantly improved the growth of *Pecten maximus* larvae and suggested that bacteria are an important part of the food supply. Comparable results were found in studies of the Chilean scallop *Argopecten purpuratus*. Larval growth and survival increased when this species was cultivated in water filtered though a 5 µm filter, compared with filtration though a 0.22 µm filter (Riquelme et al. 1997). In hatcheries, scallop larvae are normally fed various species of algae, typically *Pavlova lutheri*, *Isochrysis galbana*, *Skeletonema costatum*, *Chaetoceros calcitrans* and *Tetraselmis suecica*, in different combinations and concentrations (Ruiz-Ponte et al. 1999, Riquelme et al. 2001, Torkildsen & Magnesen 2004). The different algal cultures and the scallop larvae are associated with different bacterial communities (Sandaa et al. 2003). The use of bacterial supplements, as an addition to algal diets, was suggested by Douillet & Langdon (1993) and Douillet (1993a,b). By using ^{14}C-labelled live or heat-killed bacteria, Douillet (1993a,b) demonstrated that Pacific oyster, *Crassostrea gigas*, larvae can digest and assimilate bacterial carbon. Crosby et al. (1990) found similar abilities in the American oyster *C. virginica*. In early studies of the blue mussel *Mytilus edulis*, Birkbeck & McHenery (1982) concluded that mussels are capable of selecting lysozyme-sensitive bacteria for subsequent processing. Douillet & Langdon (1993) demonstrated that the addition of a particular bacterial strain, CA2, possibly an *Alteromonas* sp., enhanced the growth and survival of Pacific oyster larvae. The optimal bacterial concentration of CA2 was 10^5 cells ml^{-1} (Douillet & Langdon 1994). *P. maximus* larvae showed significantly lower mortality rates when cell extracts of *Roseobacter* strain BS107, were added to larval cultures (Ruiz-Pointe et al. 1999).

Since the literature cited above clearly documents that bacteria in bivalve cultures may represent both threats and valuable food components, it is important to describe the action of different bacteria found in larval cultures. An understanding of the processes involved in larval mortality is essential. The aim of the present study was to investigate the action of bacteria suspected of being pathogenic to scallop larvae. Larval groups were challenged with bacterial isolates from *Pecten maximus* larvae, resembling *Vibrio splendidus* and *Pseudoalteromonas* (Torkildsen 2004). Mortality was compared to that of larvae challenged with the known pathogenic bacterium *V. pectenicida* (Lambert et al. 1998) and an unchallenged control group. Immunohistochemistry was employed to assess uptake of the bacteria and their effect on larval tissues.

MATERIALS AND METHODS

Broodstock, oocytes and larvae. Scallop larvae were produced according to standard cultivation procedures at the scallop hatchery Scalpro A/S, Rong, near Bergen, as described by Torkildsen & Magnesen (2004). Broodstock originated from the County of Hordaland in western Norway (60° N). Oocytes were collected and hatched at the hatchery in April 2001. Spawning was induced by thermal shock, and the oocytes were fertilised as described by Gruffydd & Beaumont (1970). Larvae were kept in 800 l tanks with stagnant seawater at 18 ± 1°C taken from the nearby fjord at a depth of 60 m. The water was filtered through a 1 µm filter bag and renewed 3 times a week. Larvae were fed a diet consisting of mixed monocultures of the algae *Isochrysis galbana* (Parke) Tahitian strain, *Pavlova lutheri* (Droop), *Chaetoceros calcitrans* (Takano)/*C. mulleri*, *Skeletonema costatum and Tetraselmis suecica* at a ratio of 3:2:3:1:1 with a total concentration of 50 cells µl^{-1}.

Bacteria. The following bacteria were selected for challenge experiments: LT 06, LT 13, LT 21 and LT 73, isolated by Torkildsen et al. (2000, 2002, 2005) and PMV 18 and PMV 19 (C. Lambert unpubl.). LT 13 belongs to the genus *Pseudoalteromonas*, while the others (PMV 18, PMV 19, LT 06, LT 21 and LT 73) resemble *Vibrio splendidus* (Torkildsen et al. 2005). Challenge experiments included a negative control (unchallenged control) and a positive control challenged with *V. pectenicida* strain A496, a known pathogen of great scallop larvae (Lambert et al. 1998), referred to as challenged control. All bacteria were

stored at –80°C in a 20% glycerol/marine broth (Difco 2216) stock. They were incubated at 18°C and grown on petri dishes with Difco 2216 marine agar (MA) for 48 h. Colonies of the bacteria were transferred to Erlenmeyer flasks with 50 ml of marine broth (MB) (Difco 2216) and shaken at 90 rpm in a shaking incubator (INFORS AG) for 48 h. PMV 18 grows slower than the other strains and was therefore grown for 72 h. Bacteria were harvested by centrifugation (Heraeus Sepathec Megafuge 1.0 R) at 2772 × *g* for 10 min at 4°C, washed twice in phosphate-buffered saline (PBS) and resuspended in PBS. The number of live cells in the suspensions was determined by counting colony-forming units (CFU). Each bacterial suspension (100 µl) was plated on MA and grown for 48 h. Colonies were then counted, and total cell concentration was determined (Table 1). For each experiment, 2 parallel counts were made.

Challenge experiments. Three almost identical challenge experiments were performed. Scallop larvae were exposed to bacteria at different ages post hatching, 10 d in challenge Expt I, 13 d in Expt II and 15 d in Expt III. The multidish tray containing the control group of Expt I was lost during handling, and thus this experiment did not include a negative control. Larvae were transferred to 24-well polystyrene multidishes (Nunc) with 2 ml of sterile seawater (SSW) (28 ppt salinity), with about 20 to 40 larvae in each well. Ideally, the number of larvae per well should have been identical, but priority was given to minimizing handling. Given the small size (100 to 150 µm) and fragility of the larvae, an approximately equal number of larvae per well combined with a large number of replicates was considered preferable to the risk of losing larvae due to physical handling. To each well (except for the unchallenged control) 100 µl of the above-mentioned 48 h bacterial suspension (PBS+bacteria) was added. The cell concentrations of the bacterial suspensions were approximately 10^8 cells ml^{-1}, except for 3 suspensions that had a cell concentration of 10^7 cells ml^{-1} (Table 1). One multidish (i.e. 24 wells) was used for each bacterial strain. Inoculations were repeated with 10-fold and 100-fold dilutions of the bacterial culture. The larvae were incubated at 16°C in an air-conditioned room. Live and dead larvae were counted after 24 and 48 h using an inverted stereoscopic microscope (Leitz DM IL). Six wells were counted for each dilution and bacterial strain. Non-swimming larvae and larvae lying passively on the bottom were counted as dead. In some wells it was difficult to determine the exact number of live and dead larvae, especially when they were swimming rapidly. To reduce the possibility of error, the wells were counted twice. At the end of each counting session, 12 wells were emptied with a pipette, and larvae were fixed in 4% phosphate-buffered formaldehyde for further processing for immunohistochemistry. This procedure did not discriminate among live, moribund and dead larvae. The 12 wells that were emptied included the 6 counted wells.

Table 1. Cell concentration (cells ml^{-1})in all bacterial suspensions for each experiment, based on viability counts. CFU = colony-forming units on plates 1 and 2. 100 µl was added to each plate. A496 = *Vibrio pectenicida*. The bacterial strains PMV18, PMV 19, LT 06, LT 21 and LT 73 resemble *Vibrio splendidus*, while LT 13 resembles *Pseudoalteromonas*

Bacteria	Expt	Dilution plated	CFU Plate 1	CFU Plate 2	Cell conc.
A496	I	1.00×10^7	3	3	3.00×10^8
PMV 18	I	1.00×10^6	9	7	8.00×10^7
PMV 19	I	1.00×10^7	1	4	2.50×10^8
LT 06	I	1.00×10^7	3	3	3.00×10^8
LT 13	I	1.00×10^7	4	6	5.00×10^8
LT 21	I	1.00×10^7	4	2	3.00×10^8
LT 73	I	1.00×10^7	3	3	3.00×10^8
A496	II	1.00×10^6	3	5	4.00×10^7
PMV 18	II	1.00×10^7	1	3	2.00×10^8
PMV 19	II	1.00×10^7	3	6	4.50×10^8
LT 06	II	1.00×10^7	3	4	3.50×10^8
LT 13	II	1.00×10^7	5	7	6.00×10^8
LT 21	II	1.00×10^7	5	6	5.50×10^8
LT 73	II	1.00×10^7	4	4	4.00×10^8
A496	III	1.00×10^6	6	5	5.50×10^8
PMV 18	III	1.00×10^7	4	6	5.00×10^7
PMV 19	III	1.00×10^7	9	8	8.50×10^8
LT 06	III	1.00×10^7	8	9	8.50×10^8
LT 13	III	1.00×10^7	7	6	8.50×10^8
LT 21	III	1.00×10^7	5	5	5.00×10^8
LT 73	III	1.00×10^7	9	8	8.50×10^8

For bacterial samples, 100 µl water was taken from randomly selected wells from each group, and plated on MA. The remaining larvae were returned to the air-conditioned room. The counting procedure lasted about 15 min. Bacterial colonies grown from the wells were inoculated in Erlenmeyer flasks with 50 ml MB, incubated at 18°C and shaken at 90 rpm for 48 h. The cultures were frozen at –80°C in 20% glycerol until the polymerase chain reaction (PCR) was performed. Three counts were made for each experiment.

Statistical considerations. The survival/mortality data were not normally distributed; non-parametric tests were used for statistical analyses. Testing for several proportions ($\alpha = 0.025$, critical region: $\chi^2 < 12.832$ for $\nu = 5$ df) (Walpole et al. 2002) was performed to test the homogeneity among the 6 wells that were counted for each larval group, after 24 and 48 h. A 2×2 contingency table ($p < 0.05$, df = 1), performed

in Statistica v 5.0 (StatSoft), was used to test for mortality differences between the 2 control larval groups and the challenged test larval groups.

Polymerase chain reaction (PCR), DNA amplification. This method was used to verify that the bacteria present in the wells actually were LT 06 and LT 13. Specific primers were used for LT 06 f1: 55–82 (ACAGTAACAATCCTTCGGGTGCG), r1: 452–476 (TCAAGAGGCGCCGCTATTAACTAC) and LT 13 f1: 55–80 (AACAGAAAGTAGCTTGCTACTTGGC), r1: 435–460 (TCACAGCTAGCAGGTATTAACTACT). The fragment size produced by the LT 06 primers is 417 bp and the fragment produced by the LT 13 primers is 405 bp. The PCR was performed in reaction mixtures of 25 µl in 0.2 ml 8-strip PCR tubes (Axygen). The mixture contained 14.85 µl of distilled water, 3 µl of $MgCl_2$, 2.5 µl of PCR buffer, 2.5 µl of 1.25 mM dNTP (Promega Madison), 0.5 µl of each primer, 0.15 µl of *Taq* polymerase (Promega) and 1 µl of bacterial sample. The mixture without bacterial sample was used as a negative control, and LT 06 and LT 13 were used as positive controls. Reactions were carried out in a Gene Amp, PCR systems 9700 (Perkin Elmer) with an initial denaturation step of 94°C for 2 min, 35 cycles of denaturation (94°C for 1 min), annealing (62°C for 30 s), extension (72°C for 1 min) and final extension at 72°C for 5 min. Amplified DNA, 5 µl, was examined by horizontal 1% agarose (SeaKem LE). Gene Mass Ruler DNA ladder mix (MBI Ferments), 2 µl, was used as a nucleic acid standard. Visualisation was obtained by UV illumination after staining with ethidium bromide.

Immunohistochemistry. All microscope slides used for testing the different bacteria against the antiserum were coated with a diluted Poly-L-Lysine solution (Sigma Diagnostics). Coating was carried out to allow the bacteria to attach to the slides during the staining procedure.

Antisera were made for the bacterial strains *Vibrio pectenicida* (anti-A496), LT 06 (anti-LT 06) and LT 13 (anti-LT 13) and were produced according to the method of Oeding (1957). Formaldehyde-killed, washed bacteria were administered by intravenous injection to the rabbits. The polyclonal rabbit antisera were absorbed by the method of Knappskog et al. (1993), to minimize the possibility of cross-reaction. Each absorbed antiserum (anti-A496, anti-LT 06 and anti-LT 13) was tested for cross-reaction with bacterial strains and larval tissue samples prior to the immunohistochemistry. The dilution used on tissue samples of each antiserum was determined by testing a range of antiserum dilutions on bacterial samples.

Larval samples fixed in 4% phosphate-buffered formaldehyde were dehydrated in ethanol and embedded in paraffin. Larvae were sectioned at 3 µm (Leica Jung Biocut 2035), incubated at 58°C for 30 min, dewaxed in xylene, rehydrated in a series of ethanol baths and washed in running water. The absorbed polyclonal rabbit antisera, anti-A496, anti-LT 06 and anti-LT 13, were diluted 1:10 in Tris-buffered saline (TBS) with 2.5% bovine serum albumin (BSA). To prevent non-specific antibody binding, sections were blocked by using 5% BSA in Tris-buffered formaldehyde for 20 min. Avidine-biotin-alkaline phosphatase complex (ABC/AP) reaction kit (DAKO A/S) and New Fuchsin Substrate system (Dako) were used to visualise positive staining. Shandon's haematoxylin was used for counterstaining. At each stage of staining, 2 controls were used. Unchallenged larvae were used as negative controls and bacterial smears on microscope slides were used as positive controls. The same procedure was used to stain both larvae and bacteria. During the staining procedure, tissue sections and bacterial samples were kept separately in order to prevent cross-contamination. To ensure complete staining of all the larvae, each microscope slide was totally covered with the immunohistochemistry kit. All incubations were performed at room temperature (20°C) in a humidity chamber. A Leica DMBE microscope equipped with a Leica Wild MPS52 phototube was used to photograph the sections. Films used were Fujichrome 100 and Fujichrome 200.

RESULTS

Challenge experiments

Survival and mortality data were pooled if there were no significant differences among the 6 wells. From these results, 8 out of 46 of the chi-square statistical analyses rejected the H_o hypothesis, i.e. rejected that all populations had equal survival rates. We concluded that there were differences in mortality among the 6 wells (Tables 2 & 3).

In most challenged larval groups, mortality (i.e number of non-motile larvae) was higher at 48 than at 24 h after challenge (Table 3). The exception was larval groups challenged with LT 13, in which the mortality rate decreased in Expts II and III. In the unchallenged control group, there was no difference between 24 and 48 h (Table 3). Mortality within the unchallenged control larval groups was higher in Expt III than in Expt II.

Mortality in the unchallenged control larval groups was between 18.2 and 31.4% (average mortality was approximately 25%) (Table 3). In challenged control larval groups (larvae challenged with *Vibrio pectenicida*) the mortality rate varied between 17.6% and 65.7% (average mortality 37%) (Table 3). The difference in mortality rates between the 2 control groups was only significant in Expt III after 24 h (2×2 contin-

Table 2. Treatments found to be significantly different in mortality. The bacterial strains LT 21, LT 73 and PMV 19 resemble *Vibrio splendidus*, while LT 13 resembles *Pseudoalteromonas*

Expt	Time (h)	Bacteria	χ^2
I	24	LT 72	14.87
I	48	LT13	16.43
I	48	LT21	13.87
II	24	LT73	15.75
II	48	PMV 19	14.71
III	24	LT2 21	14.85
III	48	PMV 19	11.25
III	48	LT21	20.64

gency table, p = 0.0162). At that time, mortality rates in all challenged groups were significantly different from the unchallenged control larval group (Table 3).

Larvae challenged with LT 06 suffered an average mortality rate of approximately 40%, ranging between 28.6 and 64.7% (Table 3). Mortality in these groups was significantly different from the mortality in the unchallenged control larval groups in Expt III after 24 h (2 × 2 contingency table, p = 0.0110). When compared with mortality in the challenged control larval groups, a significant difference in mortality was found in Expt I after 24 h of challenge (2 × 2 contingency table, p = 0.0051).

In general, the challenge with LT 13 resulted in low mortality in most larval groups and the average percentage mortality was lowest of all challenged groups (approximately 29%). Mortality in these larval groups was the same as or less than in the unchallenged control groups (Table 3), and was significantly different only in the Expt III after 24 h (2 × 2 contingency table, p = 0.0286). Mortality in larval groups challenged with LT 13 was significantly different from the challenged control larval groups in Expts II and III, 48 h post-challenge (2 × 2 contingency table, p = 0.0019 and p = 0.0258, respectively)

Challenge with LT 21 caused an average mortality rate of approximately 45% (Table 3), and mortality was significantly different from that in the unchallenged controls in Expt II, 24 and 48 h after the challenge (2 × 2 contingency table, p = 0.0294 and p = 0.0000, respectively) and Expt III after 24 and 48 h (2 × 2 contingency table p = 0.0010 and p = 0.0000). In comparison with the challenged control larval groups, the difference in mortality was significant in Expt I, 24 h post challenge (2 × 2 contingency table, p = 0.0002) and in Expt II and III after 48 h of challenge (2 × 2 contingency table, p = 0.0126 and p = 0.0026, respectively).

Challenge with the bacterial strain LT 73 resulted in high mortality in all challenge experiments, especially in Expt III after 48 h of challenge (86.4%) (Table 3). Average mortality was approximately 49%, which was the highest of all groups. The mortality was significantly different from that in the unchallenged control larvae in Expt II after both 24 and 48 h (2 × 2 contingency table, p = 0.0349 and p = 0.0000, respectively), and in Expt III after 24 and 48 h of challenge (2 × 2 contingency table, p = 0.0127 and p = 0.0000, respectively). When compared to the mortality experienced in the challenged control larval groups, significant differences were found in Expts I and III after 24 h (2 × 2 contingency table, p = 0.0000 and p = 0.0162, respectively).

PMV 18 caused an average mortality of approximately 38% (Table 3). Mortality in larval groups challenged with PMV 18 and the unchallenged control larval groups was significantly different in Expts II and III after 24 h (2 × 2 contingency table, p = 0.0000 and p = 0.0001, respectively). Compared with the challenge control larval groups, the mortality in larval groups challenged with PMV 18 was significantly different in Expt I at both 24 and 48 h (2 × 2 contingency table, p = 0.0245 and p = 0.0009) and in Expt II after 24 and 48 h

Table 3. Percent mortality of challenge Expts I, II and III including unchallenged and challenged control groups. Challenged control: larval groups challenged with *Vibrio pectenicida*. ND: no data (Challenge Expt I did not include an unchallenged control group). *Significantly different from the unchallenged control larval groups (p < 0.05). ^Significantly different from the challenged control larval groups (p < 0.05). The bacterial strains PMV18, PMV 19, LT 06, LT 21 and LT 73 resemble *Vibrio splendidus*, while LT 13 resembles *Pseudoalteromonas*

Expt	Time (h)	Unchallenged control	Challenged control	PMV 18	PMV19	LT 06	LT 13	LT 21	LT 73
I	24	ND	17.6	29.4^	36.8^	31.5^	26.3	33.3^	36.5^
I	48	ND	65.7	40.7^	75.0	64.7	50.5	65.9	53.0
II	24	18.2	24.1	60.6*^	18.5	28.6	18.0	27.2*	27.9*
II	48	19.3	26.6	12.8^	18.8	29.4	10.4^	39.9*^	46.8*^
III	24	31.4^	46.4*	50.0*	40.8*	44.3*	42.6*	47.1*	43.8*
III	48	31.0	39.3	36.9	49.6*^	39.7	23.6^	53.6*^	86.4*^
Average		20.5	36.6	38.4	39.9	39.7	28.6	44.5	49.1

(2×2 contingency table, $p = 0.0000$ and $p = 0.0005$, respectively).

Larvae challenged with PMV 19 experienced an average mortality of approximately 40% (Table 3). These larval groups were significantly different from the unchallenged control larval groups in Expt III after 24 and 48 h (2×2 contingency table, $p = 0.0481$ and $p = 0.0003$, respectively). When compared to larval groups challenged with *Vibrio pectenicida*, significant differences in mortality were found in Expt I after 24 h (2×2 contingency table, $p = 0.0002$) and Expt III after 48 h (2×2 contingency table, $p = 0.0370$).

PCR verified that the randomly sampled bacteria taken after each counting (24 and 48 h) and challenge experiment, were LT 06 and LT 13 that were used for challenge (Figs. 1 & 2). The use of specific primers against these 2 bacterial strains produced fragments similar in size to the fragment size produced by the primers LT 06 (417 bp) and LT 13 (405 bp). Some variations in the intensity of the fragments were detected.

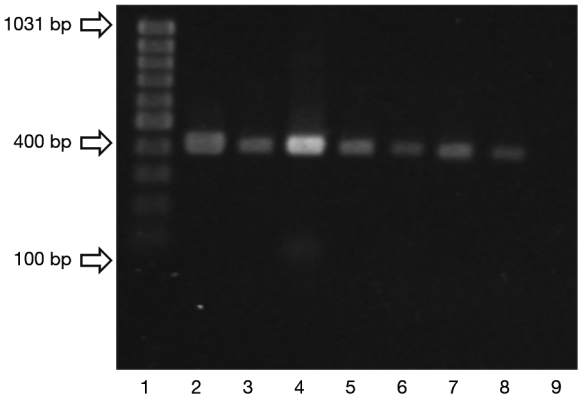


Fig. 1. Agarose gel electrophoresis photo of PCR-amplified 16S rDNA in bacterial isolates sampled from the 24-well multidishes challenged with LT 06. Lanes 2 to 7: bacterial isolates taken from the 24-well mulitidishes from the different challenge experiments randomly sampled after each count; Lane 8: LT 06 used as positive control; Lane 9: Strain LT 13 used as negative control; Lane 1: standard marker. All templates are diluted 1:100

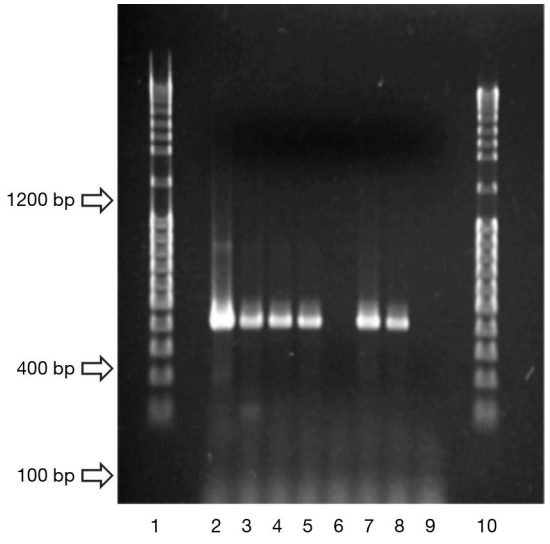


Fig. 2. Agarose gel electrophoresis photo of PCR amplified 16S rDNA in bacterial isolates sampled from the 24-well multidishes challenged with LT 13. Lanes 2 to 5: bacterial isolates taken from the 24-well multidishes from the different challenge experiments randomly sampled after each count; Lane 6: bacterial strain LT 06 used as negative control; Lanes 7 and 8: LT 13 used as positive control; Lane 9: core mix with no template; Lanes 1 and 10: standard markers. All templates are diluted 1:100

Immunohistochemistry

Formaldehyde fixation produced contraction of the larvae prior to embedding, complicating the reading of slides and interpretation of immunohistochemical staining. The structure of larvae, before and after fixation, is shown in Fig. 3. Fig. 3c was drawn from Fig. 4, and represents an explanation of Figs. 4 to 13.

Cross-reactions among the 3 antisera, anti-A496, anti-LT 06 and anti-LT 13 were not found, either when tested directly on bacteria or on larval tissue samples. The unchallenged control larvae did not display positive immuno-staining when stained with anti-A496 and anti-LT 06 (Fig. 4). However, approximately 50% of the unchallenged control larvae displayed specific immuno-staining inside the stomach and digestive area and endothelial cells when stained with anti-LT 13 serum (see Fig. 13). This staining was generally weaker than in larvae challenged with LT 13. The morphology of all control larvae was normal and displayed no signs of tissue damage (Fig. 4). The immunohistochemical examinations showed no relationship between pathogenesis and larval age.

Immuno-staining revealed large differences among the 3 larval groups; challenged control larval groups (larvae challenged with *Vibrio pectenicida*) and larval groups challenged with LT 06 or LT 13. Larvae challenged with *V. pectenicida* and LT 06 displayed similarities when immuno-stained (Figs. 5 & 8) and showed similar signs of infection in comparison with larvae challenged with LT 13. In addition, most larvae were positively stained on the outer shell and mantle surfaces.

All challenged control larvae examined displayed positive immuno-staining. Infection was located in oesophagus, stomach and rectum, spreading in the areas around the digestive mass, especially the endothelial mucosa, and to the surrounding tissues. In most of the larvae, necrotic tissue and pycnotic cells could be observed (Figs. 5 & 6). In challenge Expt III a

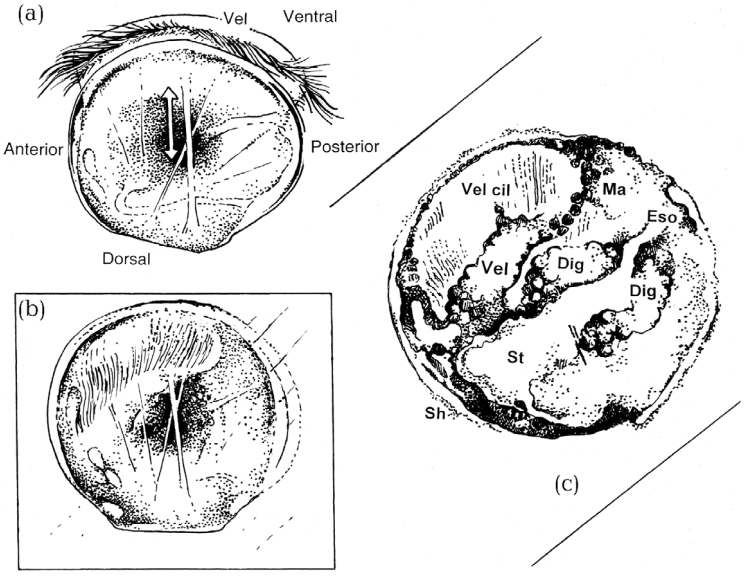


Fig. 3. *Pecten maximus*. Orientation and tissues seen in the sections (see Figs. 4–13). (a) illustrates the larva prior to fixation and how the velum contracts (arrows). (b) illustrates the larvae after fixation, with the contracted velum inside the thin shell, folded inwards with a longitudinal furrow and ciliar rim, underlying the mantle lobes. The longitudinal section that cuts through the approximate centre of the larva (indicates orientation of the larva section, cut to illustrate [c]), includes parts of stomach and gut. The ventral part includes parts of the contracted velum and mantle. (c) Tissues and body compartments of the larva corresponding to the larva shown in Fig. 4 (where strong staining covers the details and makes difficult any differentiation between the mantle cells, the thin epithelium of the velum roof and the larger cells of the velum basis). Lines indicate counter-clockwise turning, to the same orientation as Fig. 4. Eso: esophagus; St: stomach; Dig: digestive cells; Sh: shell matrix; Vel cil: velum cilia; Ma: mantle cells

clear difference was seen in the positive immuno-staining between 24 and 48 h (Figs. 6 & 7). All larvae challenged for 48 h were generally infected and appeared positively immuno-stained in most or all tissues (Fig. 7). This clear difference was not found in the 2 previous challenge experiments or in larval groups challenged with LT 06 and LT 13. Despite the positive intracellular immuno-staining of mucosal cells (Fig. 6, arrow), no positive identification of bacterial cells was confirmed in any of the individuals examined, either inside the digestive mass or intracellularly in epithelial cells.

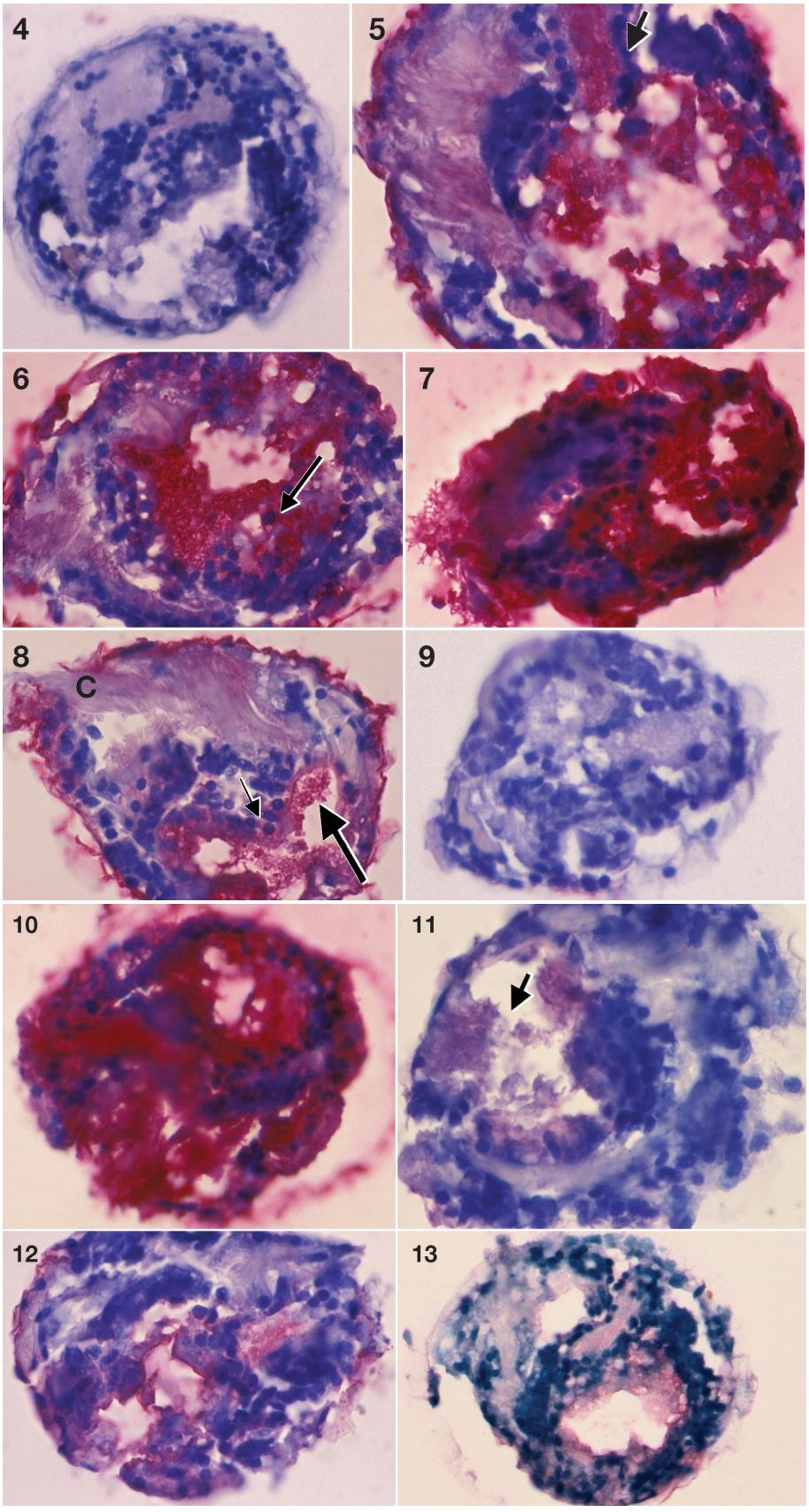
In contrast to larvae challenged with *Vibrio pectenicida*, examination of larvae challenged with LT 06 revealed apparently intact bacterial cells inside the digestive mass and inside mucosal cells (Fig. 8, indicated with small arrow). Otherwise, the challenge results with LT 06 apparently had similar impact on the scallop larvae as *V. pectenicida*. Infection was basically located in the gut and the digestive area, although spread to the surrounding area in some larvae. Although a few larvae were generally infected, larvae challenged for 48 h did not appear to be more severely affected by the infection than larvae challenged for 24 h. Despite challenge, 2 larvae, one in challenge Expt I (48 h) and one in challenge Expt III (48 h), displayed a total absence of specific immuno-staining (Fig. 9). Other larvae on the same sections, adjacent to these unstained larvae, were all positively stained (Fig. 10).

All the examined larvae examined from the challenge with LT 13 displayed positive immuno-staining. The infection was apparently less severe, as they were less positively stained than larvae challenged with *Vibrio pectenicida* and LT 06. Positive immuno-staining was limited to visual bacteria inside the digestive area and mucosal cells (Figs. 11 & 12). Surrounding tissues were not infected and appeared normal. Intracellular bacteria could not be observed.

DISCUSSION

Scallop larva challenged with *Vibrio pectenicida* and the *V. splendidus*-like strain LT 06 showed many similarities regarding pathology, immuno-staining and mortality. Apparently our study is the first attempt to characterise such infections in bivalves by immunohistochemistry. This method enabled us to visualise affected tissue in the digestive mass and gut area of larvae challenged with these bacteria. Infection seemed to spread from these areas to the surrounding tissues. Together with the mortality results, these observations demonstrate that LT 06, like *V. pectenicida*, is pathogenic to scallop larvae. This supports the results of Nicolas et al. (1996) who described a strain resembling *V. splendidus* associated with mortality in scallop *Pecten maximus* larval cultures.

Immunohistochemical examinations of larval groups challenged with *Vibrio pectenicida* revealed no bacterial cells, despite a high degree of positive immuno-staining. A general experience at our laboratory in studies of fish larvae with identical immuno-staining protocols (Ø. Bergh unpubl.) is that tissue samples exposed to pathogenic *Vibrio* spp. will be positively stained in areas around the stained bacteria. This is probably due to staining of partially dissolved extracellular bacterial products. Lambert et al. (1998, 2001)



Figs. 4 to 13. *Pecten maximus*. Immunohistochemical staining of paraffin sections from larvae. Avidine-biotin-alkaline phosphatase method, rabbit anti-A496, -LT 06 and -LT 13 serum and Shandon haematoxylin counterstained. Positive immunohistochemistry is visualised by red colour. Counterstaining gives tissues different tones of blue. Larval diameters range between 90 and 150 µm. Fig. 4. Unchallenged, healthy larva stained with anti-LT 06. Figs. 5, 6 & 7. Larva challenged with *Vibrio pectenicida* for 24 h (Fig. 6) and 48 h (Figs. 5 & 7) stained with anti-A496. In Fig. 5 note intracellular staining in mucous cells (arrow). Red area inside digestive mass is probably a cluster of bacteria. Figs. 6 & 7 show variation in infectivity between 24 and 48 h observed in Expt III. In Fig. 6 the esophagus (arrow) and digestive areas, in particular, are clearly positively stained together with the shell surface. Fig. 7. Totally infected larvae 48 h post challenge. Figs. 8, 9 & 10. Larvae challenged with LT 06 for 48 h, stained with anti LT 06. Fig. 8. Mantle surface, shell matrix and stomach wall are positively stained, and bacteria are visible and positively stained in the stomach (large arrow). Intracellular bacteria are verified in mucous cells (small arrow). The cilia (C) are also positively stained. Fig. 9. Note absence of immuno-staining in spite of bacterial exposure for 48 h. Larvae show no signs of infection. Fig. 10. Larvae situated next to larvae in Fig. 8. Note the generally positive immuno-staining. Figs. 11 & 12. Larvae challenged with LT 13 for 24 and 48 h, respectively, stained with anti-LT 13. Fig. 11. Bacteria are seen in stomach and digestive area (arrow). Positive staining is restricted to digestive mass, showing no signs of infiltrating the surrounding area. Fig. 12. Positive immunostaining is seen on shell surface, on stomach wall and in esophagus. Fig. 13. Unchallenged control larvae stained with anti-LT 13. Positive immuno-staining is seen in esophagus, stomach lumen and endothelial cells

demonstrated that *V. pectenicida* is highly toxic to *Pecten maximus* larvae due to an intracellular release of toxins that inhibit the respiratory burst activity of the haemocytes. These toxins may infiltrate the larval tissue. It is likely that polyclonal antiserum anti-A496 labelled some of these released toxins and possibly other compounds, and thereby positively stained the larval tissue, even though no bacterial cells were found. Verification of the presence of bacterial cells was difficult in all challenged larvae, partly due to clustering of bacterial cells. The large red area inside the digestive mass in some larvae (see Fig. 6) could be clusters of bacteria. The size of the scallop larvae (approximately 100 to 150 µm) and the low number of cells made observation of tissue degeneration and necrosis difficult. Most challenged larvae displayed positive immuno-staining on the shell surface and mantle. This could be due to bacterial adhesion to the surface of the larvae, but could also be due to lack of washing of the larvae prior to fixation.

Few papers describe scallop larval anatomy, and the majority are based on electron microscopy of veliger larvae (Cragg & Crisp 1991). However these examinations have limited relevance to immunohistochemical examinations. The development of the immunohistochemical protocol described herein was optimised for the visualisation of the presence and modes of action of certain bacteria. However, the method is clearly suboptimal for purposes of precise morphological studies.

The challenge experiments showed that all challenged larval groups suffered higher average mortality than the unchallenged control groups. In comparison with the larval groups challenged with *Vibrio pectenicida*, all challenged groups, except those challenged with LT 13, experienced average higher mortality. From the mortality results, we consider that the bacterial strains PMV 18, PMV 19, LT 06, LT 21 and LT 73 are pathogenic to scallop larvae.

In contrast, larval groups challenged with LT 13 suffered relatively low average mortality and the immunohistochemical examinations revealed a lesser degree of infection compared with larvae challenged with *Vibrio pectenicida* and LT 06. However, Denaturating Gradient Gel Electrophoresis (DGGE) showed bacteria apparently identical to LT 13 to be frequently present in larval cultures suffering from high mortality (R. A. Sandaa unpubl.). Thus this bacterium may act as a secondary opportunistic pathogen that causes disease and mortality in already weakened larvae. Many other factors, such as broodstock condition, egg quality, larval condition and feed affect larval growth, development and survival in mussels (Phillips 2002) and scallops (Seguineau et al. 1996, 2001, Soudant et al. 1998).

Some of the unchallenged control larvae were positively stained with the antiserum anti-LT 13 in the digestive mass and gut area. Compared with larvae challenged with LT 13, this positive staining was weaker. Although neither bacterial samples nor larval tissue samples revealed cross-reaction, cross-reaction with other bacteria present inside the larval lumen is possible. The scallop larvae in our experiment were not axenic, and were not kept in a sterile environment prior to the challenge experiments. When specific primers for the bacterial strains LT 06 and LT 13 were used, no other bacteria present in the wells were detected. Recently, characterisations of bacterial flora associated with *Pecten maximus* larvae have been performed by DGGE of PCR-amplified 16S rDNA (Sandaa et al. 2003). This method provides an overview of the bacterial community, including both culturable and non-culturable components. Full sequencing of the 16S rDNA and Restriction Fragment Length Polymorphism (RFLP) for genotyping 16S rRNA are 2 methods for characterising unknown cultured bacteria. The latter was used by Jensen et al. (2002) to characterise bacteria cultured from halibut *Hippoglossus hippoglossus* fry. LT 13 has been isolated from the microalgae *Chaetoceros calcitrans*, which is used as feed for scallop larvae (Torkildsen et al. 2005). The bacterium could be inside the larvae as a consequence of feeding. Bacteria accumulating in the algal cultures may influence the bacterial flora associated with filter-feeders such as bivalve larvae. Skjermo & Vadstein (1993) found that adding algae to the rearing water of halibut larvae increased the bacterial concentration by 45% and that the bacterial flora associated with fish larvae was related to the flora in the water. The DGGE profile of the bacterial community in a mixed algal culture resembled the DGGE profile of the algae present in the highest concentration (Sandaa et al. 2003). This suggests that additions of algae may influence the bacterial community. However, a clear difference in the DGGE profile of bacteria associated with the scallop larvae and the DGGE profile of the water samples was also found.

The bacterial strains PMV 18, PMV 19, LT 06, LT 21 and LT 73 all resemble *Vibrio splendidus* (Torkildsen et al. 2005). *V. splendidus* strains are widely spread in the marine ecosystem and cause disease in various aquatic organisms including the Pacific oyster (Lacoste et al. 2001, Waechter et al. 2002), great scallop (Nicolas et al. 1996), turbot *Scophthalmus maximus* larvae (Gatesoupe et al. 1999), gilt-head sea bream *Sparus aurata* (Balebona et al. 1998) and juvenile giant tiger shrimps *Penaeus monodon* (Leaño et al. 1998). The *V. splendidus* biovar II-related strain TNEMF6, caused a cumulative mortality of 80% in Pacific oyster spat (Waechter et al. 2002). *V. splendidus* infect a wide

range of animals. Not all *V. splendidus*-related strains are pathogenic (Waechter et al. 2002). This could explain the differences in mortality among the strains used in this challenge experiment. The number of larvae inside the wells likely affected larval survival. Wells containing large amounts of larvae may provide better growth for the bacteria and an increased in infection rates.

Three of the bacterial suspensions were at a concentration of 10^7 cells ml^{-1} instead of 10^8 when they were added to the wells. In Expt II the concentration of bacteria in the suspension of *Vibrio pectenicida* was 4.0×10^7 CFU ml^{-1}, and in Expts I and III the bacterial concentrations were 3.0×10^8 and 5.5×10^8 CFU ml^{-1}, respectively. The low mortality in Expt II compared to the mortality in Expt I may have been related to the differences in cell concentration of the bacterial suspensions. However, concentration differences between the suspensions used in Expts II and III did not affect the mortality. Cell concentrations of the PMV 18 suspensions added in Expts I and III were 8.0×10^7 and 5.0×10^7 CFU ml^{-1}, respectively. In Expt II the concentration was 2.0×10^8 CFU ml^{-1}. PMV 18 caused similar mortality rates in all 3 challenge experiments. Thus it cannot be concluded if differences in bacterial concentrations may have affected larval mortality. In a similar multidish challenge experiment with *Tenacibaculum ovolyticum* (previously *Flexibacter ovolyticus*), *V. anguillarum* and *Aeromonas salmonicida* subsp. *salmonicida*, on eggs and larvae of Atlantic halibut *Hippoglossus hippoglossus* and Atlantic cod *Gadus morhua*, mortality increased with increasing challenge dose (Bergh 2000).

In conclusion, immunohistochemistry can be a powerful tool for studies of diseases of larval bivalves. Our results indicate that the *Vibrio splendidus*-like strains tested are pathogenic to scallop larvae. The pathology resembles infections with *V. pectenicida.* The *Pseudoalteromonas*-like strain LT13 is probably not a primary pathogen, but could act as a secondary opportunistic bacterium.

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Paper II

Sandlund N & Bergh Ø (in press) Screening and characterisation of potentially pathogenic bacteria associated with Atlantic cod *Gadus morhua* larvae: Bath challenge trials using a multidish system. Dis Aqua Org

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Screening and characterisation of potentially pathogenic bacteria associated with Atlantic cod *Gadus morhua* larvae: Bath challenge trials using a multidish system.

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ABSTRACT

In intensive aquaculture systems, high concentrations of nutrients and high densities of fish larvae provide favorable conditions for opportunistic pathogenic bacteria to flourish. The purpose of this study was to screen potentially pathogenic bacterial strains isolated from moribund Atlantic cod *Gadus morhua* larvae, pollack *Pollachius pollachius*, coalfish *Pollachius virens*, Atlantic halibut *Hippoglossus hippoglossus*, rotifers, algae and water samples from different hatcheries. Three identical challenge experiments were carried out and a total of 53 strains were tested. A multidish system was used, with one cod egg placed in each well, together with 2 ml sterile seawater and exposed to the bacterial cultures. Final bacterial concentrations in the wells were 10^6 and 10^4 CFU ml^{-1}, respectively. Eggs and larvae not exposed to bacteria were used as unchallenged controls. Each experiment also included a challenged control group, larvae exposed to the known pathogenic strain, *Vibrio anguillarum* strain 610. The eggs were challenged approximately 48 hours prior to hatching and readings of mortality were taken every day throughout the yolk-sac period. In spite of the high challenge dose of 10^6 CFU ml^{-1}, only five of the bacterial strains tested caused higher mortality than the unchallenged control group. Four of these strains were identified by 16S rDNA and *GyrB* sequencing as resembling *V. anguillarum* and one strain resembled *Carnobacterium* sp. Most of the larvae exposed to these strains died within 10 days of challenge. Serotyping, by specific antisera and Mono-Va agglutination kits, of the strains

resembling *V. anguillarum* gave inconclusive results. This indicates differences in serology compared to the serotypes O1, O2 and O3, associated with disease (Toranzo et al. 2005). Three bacterial strains seemed to have a slower infection rate, indicating a longer incubation period than the ones found to be highly pathogenic. The remaining 45 strains did not seem to have a negative effect on larval survival, suggesting that these are not primary pathogens.

Key words: Screening, Cod larvae, Bath challenge, Opportunistic bacteria, Vibriosis, *Vibrio anguillarum*

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INTRODUCTION

Atlantic cod (*Gadhus morhua* L.) aquaculture has been expanding rapidly in Norway since 2000 (Svåsand et al. 2004). In 2006, about 10384 tonnes of farmed cod (full life cycle) were slaughtered and 213 licenses for cod farming to 103 companies were operational (Directorate of Fisheries, Bergen, Norway). Of these, 60-80 ongrowth farms and 15-20 hatcheries were in operation (Kongsvik 2007).

The production of juveniles has been a bottleneck in cod farming. Cod larvae have a relatively short yolk-sac stage and they start to feed on live feed, i.e. rotifers and *Artemia* spp., around three to four days post-hatching. Rotifers and *Artemia* are filter-feeding, capable to concentrate bacteria, and thus a potential source of pathogenic bacteria in the larval rearing system. Larvae also ingest bacteria by drinking water. High larval densities and suboptimal rearing conditions may provide good conditions for opportunistic bacterial pathogens to

flourish (Vadstein et al. 2004), and high rates of mortality are still common. Commercial enrichment diets for rotifers may enhance the growth of pathogens, including *Vibrio anguillarum* (Korsnes et al. 2006). Adult cod are frequently subject to classical vibriosis caused by *V. anguillarum* (reviewed by Samuelsen et al. 2006). *V. anguillarum* is also known to cause high mortality in a variety of fish species (reviewed by Thompson et al. 2004b).

The epiflora of cod eggs seems to be dominated by members of the *Cytophaga/Flavobacterium/Flexibacter* group, while *Vibrio* spp. are not frequent (Hansen & Olafsen 1989). In the past overgrowth of eggs were regarded a problem. However, disinfection has reduced this problem (Reviewed by Olafsen 2001).

While the composition of the intestinal bacterial flora associated with yolk-sac larvae of fish generally resembles the egg epibiota, a shift in the intestinal microbiota from a generally nonfermentative to a fermentative flora dominated by the *Vibrio/Aeromonas* group coincides with the onset of exogenous feeding (Vadstein et al. 2004). During first feeding, yolk-sac larvae in general are subject to a massive inflow of bacteria from the live feed organisms *Brachionus plicatilis* and *Artemia* spp., resulting in a shift in intestinal bacterial flora (Brunvold et al. 2007), and suspected pathogens such as *V. anguillarum* may accumulate in the live feed (Korsnes et al. 2006). The mucosal surfaces of fish serve as a substrate for bacterial adhesion (Spangaard et al. 2000) and thereby a potential portal of entry. Uptake of intact antigens from bacteria in the intestine of four- to six-day-old yolk-sac larvae of cod has been demonstrated, and it has been suggested that this uptake may play a role in immune development, or in nutrition (Olafsen & Hansen 1992).

Whether the bacteria are primary causes of mortality or secondary pathogens take advantage of weakened larvae is disputed. An important step would be to test whether any of the bacterial strains found has the ability to cause disease. Knowledge concerning sources and species of bacteria most commonly associated with cod larvae at different life stages is essential. It is also important to know the amount of bacteria needed to induce infection. The objectives of this study were to screen a large number of bacterial strains associated with diseased cod larvae, other marine cold water species and live feed cultures, and to test the virulence of the strains in a challenge model using cod yolk-sac larvae.

MATERIALS AND METHODS

Broodstock, eggs and larvae. Eggs were collected at the Sagafjord commercial cod hatchery, in the county of Hordaland, Norway (59° 45’ N, 5° 29’ E). The broodstock originated from the Bømlo and Halsnøy area in the same county.

All eggs were taken from the same group. They were disinfected immediately after fertilization in glutardialdehyde 300 ppm for 10 min, and kept in black conical 150 l tanks at 6.5 to 7.0°C. Eggs were transported from the hatchery to the Institute of Marine Research in boxes filled with ice, with the eggs stored in plastic bags. The transfer took about two to three hours.

Bacteria. A total of 117 bacterial isolates were collected, mostly from dead or moribund cod *Gadus morhua* larvae, while some were isolated from Pollack *Pollachius pollachius,* Coalfish *Pollachius virens* and Atlantic halibut *Hippoglossus hippoglossus* fry, water samples, rotifer and algal cultures. In order to reduce the number of strains used, the growth patterns of all

strains were compared on different growth media, such as marine agar (MA), Thiosulphate Citrate Bile Sucrose Agar (TCBS) (Merck KGaA, Darmstradt, Germany), Cytophaga medium (CA) (Whitman 2004) and blood agar (nutrient blood agar (Oxoid) supplemented with 5% sheep blood and 1.5% NaCl). 16S rDNA sequencing was also performed in order to compare the 16S rDNA sequence and look for similar DNA sequences. All strains with similar growth patterns and similarities in 16S rDNA sequences greater than 97% (Hagström et al. 2000, 2002) were eliminated from the challenge trails (data not shown). A total of 53 different bacterial strains were selected for further studies, which were performed in three identical challenge experiments (Table 1).

The challenge experiment included an unchallenged control (eggs and larvae not exposed to bacteria) and two challenged control groups (eggs and larvae challenged with *Vibrio anguillarum* strain 610, challenge dose 10^6 and 10^4 CFU ml $^{-1}$ respectively). *V. anguillarum* strain 610 is known to cause high mortality in fish species such as Atlantic cod *Gadus morhua* (Samuelsen & Bergh 2004, Vik-Mo et al. 2005, Seljestokken et al. 2006) and Atlantic halibut, *Hippoglossus hippoglossus* (Samuelsen et al. 1997) and is commonly used in our laboratory during challenge experiments. All bacteria were stored at –80ºC in a 20% glycerol/marine broth (Difco 2216, Difco, Detroit, MI, USA) stock. They were incubated at 15ºC and grown on petri dishes with Difco 2216 marine agar (MA) for 48 h. Colonies of the bacteria were transferred to Erlenmeyer flasks with 50 ml of marine broth (MB) (Difco 2216) and shaken at 80 rpm in a shaking incubator (INFORS AG CH-4103 Bottmingen, Switzerland) for 48 h at 7°C. The bacterial cultures, 30 ml, were harvested by centrifugation (Heraeus Sepathec Megafuge 1.0 R) at 2772 G for 10 min at 4ºC, washed twice in 30 ml phosphate-buffered saline (PBS) and suspended in 30 ml PBS. The cell concentration was determined by counting, using a Hawksley counting chamber.

The *V. anguillarum* strain 610 was originally isolated from cod suffering from vibriosis in the Parisvatnet research facility of the Institute of Marine Research (Øygarden municipality, Western Norway). *Moritella viscosa* was provided by Helene Mikkelsen at the Norwegian Institute of Fisheries and Aquaculture Research in Tromsø, and the strains F95B/98 and F95C/98 were provided by Anne Berit Olsen from the National Veterinary Institute in Bergen. Strains HI 21030 to HI 21069 were provided by Egil Karlsbakk at the Institute of Marine Research. These strains were all characterised by these researchers respectively, except for the API results and the growth studies on TCBS and CA medium for HI 21030- HI 21069.

Challenge experiments. Three identical challenge experiments were preformed. All eggs were exposed to bacteria approximately 48 h before hatching on day 10 or day 11 after fertilization.

Eggs were randomly taken and transferred to 24-well polystyrene dishes (Nunc, Roskilde, Denmark) by autoclaved Pasteur pipettes. The protocol was modified from Bergh et al. (1992, 1997). All eggs hatched within a time range of 10 h, and 7841 of 7848 eggs hatched successfully. The eggs were put separately into each well containing 2.0 ml of sterile 80% (28‰) seawater. The seawater was aerated just before use in order to minimize the possibility of contamination. Exactly 100 µl of bacterial suspension was added to each well. Final bacterial concentrations in the wells were 10^6 and 10^4 cells ml^{-1}, respectively. Final volume in each well was 2.1 ml. Three plates (72 wells) were used for each bacterial concentration, so that six plates (144 wells) were used for each bacterial strain. Three plates with unchallenged larvae were used as negative control. The eggs and larvae were incubated in darkness in a climate-controlled room at 7°C. All eggs and larvae were inspected each day and mortality was registered.

API 20E. API 20E tests (Biomérieux, Marcy l'Etoile, France) were performed according to the manufacturer's manual, with the following modifications: Bacterial cultures were dissolved in sterile physiological saline (9 g NaCl dissolved in 1 l distilled water). The McFarland 2 standard was used as reference to culture density. The API strips were incubated at 15°C for 48h.

Gram staining was performed with the Dagnostica MERCK, (Darmstadt, Germany) Gram staining set.

Immunostaining of bacterial smears. In order to test some of the *Vibrio anguillarum* strains used in these experiments for serotype, bacterial smears of HI 21412, HI 21413, HI 21414 and HI 21429 were stained with specific absorbed polyclonal antiserum against serotypes O2α, O2β and O1. All antisera were produced according to the method of Oeding (1957) and absorbed by the method of Knappskog et al. 1993. The antiserum against serotype O2α was absorbed against O2β and O1, the antiserum against O2β was absorbed against O1 and the antiserum against O1 was absorbed against O2β. All three antisera were diluted in tris-hydroxymethyl-aminomethane (TRIS)-buffered saline (TBS) with 2.5% bovine serum albumin (BSA). In order to prevent non-specific antibody binding, sections were blocked by using 5% BSA in TRIS-buffered formaldehyde for 20 min. Avidin-biotin-alkaline phosphatase complex (ABComplex/AP) reaction kit (Dako A/S, Denmark) and New Fuchsin Substrate system (Dako A/S) were used according to the manufacturer's manual. During the staining procedures, the different bacterial strains were kept separate to prevent cross-contamination. During staining both positive and negative controls were used. Known O2α (strain HI 610), O2β (strain HI 618) and O1 (strain HI 644) serotypes isolates were used as positive controls. The O2α and O2β were used as negative controls during staining procedures

with the O1 antiserum, O2β and O1 isolates were used when staining with O2α antiserum, and O2α and O1 isolates were used as negative controls when the O2β antiserum was used. All incubations were performed at room temperature (20°C) in a humidity chamber.

Mono-Va agglutination Kit against *Vibrio anguillarum*. Mono-Va tests (Bionor AS, Skien, Norway) were used to identify the isolates *Vibrio anguillarum* 610, HI 21412, HI 21413, HI 21414 and HI 21429. The test was done according to the manufacturer's manual.

DNA isolation. Genomic bacterial DNA was isolated from 1 ml of a liquid culture harvested at the end of the exponential growth phase, using the purification kit DNeasy® 96 tissue kit (Qiagen, Hilden, Germany). The protocol for gram negative bacteria was used.

PCR (Polymerase Chain Reaction) amplification of 16S rDNA genes.
Universal primers, 27f and 1492r (*Escherichia coli* numbering), were used for 16S rDNA analyses. The mix contained 2.0 μl PCR buffer (10x), 1.2 μl $MgCl_2$ (25 mM), 3.2 μl dNTP (1.25 mM/each, Promega, Madison, Wisconsin), 1.0 μl 27f Forward primer (10 μm), 1.0 μl 1492r Revers primer (10 μm), 0.2 μl Taq polymerise (5 U/μl, Promega) 7.4 μl Nuclease-free water (Eppendorf, Hamburg, Germany) and 4 μl template (approx. 50 ng/μl). The amplification was performed in an automated thermal cycler (Perkin Elmer, Gene Amp, PCR system 9700) and the cycles were as follows: initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 11 min. All PCR reactions were performed in 50μl reaction tubes.

PCR (Polymerase Chain Reaction) amplification of *GyrB* gene. Primers used for amplification of the *GyrB* gene were GyrB-1 (forward) and GyrB-2 (reverse) (Yamamoto & Harayama 1995). The mix contained 2.0 μl PCR buffer (10x), 1.5 μl $MgCl_2$ (25 mM), 4.0 μl

dNTP (1.25 mM/each, Promega), 1.0 μl GyrB-1 primer (10 μM), 1.0 μl GyrB-2 primer (10 μM), 0.5 μl Taq polymerise (5 U/μl) (Promega), 6 μl nuclease-free water (Eppendorf) and 4 μl template (approx. 50 ng/μl). The amplification cycle was as follows: initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min and extension/hold for 7 min. The amplification was performed in an automated thermal cycler (Perkin Elmer, Gene Amp, PCR system 9700).

Sequencing of 16S rDNA and *GyrB* genes. All PCR products were prepared for sequencing by using a Pre-Sequencing Kit (USB Corporation, Cleveland, Ohio). One cycle of 37°C for 15 minutes and in addition, another 15 minutes at 80°C were run. Primers used for sequencing 16S rDNA genes were the same as for the PCR amplification described in the paragraph above. The pre-sequencing mix contained, 1μl Big Dye mix(2.5x) (Big Dye version 3.1, Applied Biosystems, Foster City, California), 1.5 μl sequence buffer (5.0x), 2.0 μl primer (10μM) and 2.5 μl RNAse free water (Eppendorf, Germany) and 3 μl of template were used. The amplification cycles were as follows: initial denaturation at 96°C for one minute then 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min.

Primers used for sequencing *GyrB* genes were different from the ones used for PCR amplification, GyrB-1s (forward) and GyrB-2s (reverse) (Yamamoto & Harayama 1995). The same pre-sequencing mix and amplification cycles as used for 16S rDNA genes were used on the sequencing of the *GyrB* genes as well. The amplification was performed in an automated thermal cycler (Perkin Elmer, Gene Amp, PCR system 9700). The sequence analysis was performed by the sequence laboratory at the University of Bergen, using an ABI 3700 sequencing analyser (Applied Biosystems). The 16S rRNA and *GyrB* encoding gene

sequences were searched for nucleotide-nucleotide matches in the BLAST database at the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) to establish tentative strain identity (Altschul et al. 1990).

Statistical analyses. Since the survival and mortality data are not normally distributed, non-parametric tests were used. A 2 x 2 contingency table ($p < 0.00094$ Bonferroni correction for multiple independent tests), performed in Statistica v 7.0 (StatSoft, Tulsa, USA), was used to test for mortality differences among the treatment and control groups. Since multiple independent tests were used to test differences in mortality rate among all challenged larval groups and the three larval control groups, a Bonferroni correction was applied (to minimize the possibility of doing a type II error) (Rice 1989). We thus tested for 53 bacterial strains, and the p value was corrected by 53 ($p = 0.05/53 = 0.000094$): see Rice (1989). Yates correction was used since there was only one degree of freedom (df).

RESULTS

Bacterial characterisation. Further characterisation of the bacterial strains used in these challenge experiments showed that all bacterial strains were Gram-negative. Most of the strains were short rod-shaped bacteria except for F95B/98 and F95/C98, which were long filamentous bacteria. These two strains were also the only non-motile strains. On the other hand, HI 21050, HI 21402 and HI 21407 proved to be weakly motile. The majority of the strains were found to be oxidase-positive, except for the strains *Marinomonas* sp., HI 21050, HI 21059, HI 21068, HI 21069, HI 21017 and HI 22002 (See Table 1 for an overview of all details). In certain cases, the API results were inconclusive. In Table 2 these results are marked as +/-. HI 21050, which resembled *Carnobacterium* sp., did not produce any positive

results in the API tests, in spite of three attempts. API tests of the four strains HI 21412, HI 21413, HI 21414 and HI 21429 revealed similar phenotypic characterization as is known for other *Vibrio anguillarum* strains, referring to the ALO test (A: arginine decarboxylase, L: lysine decarboxylase, O: ornithine decarboxylase,): A+/L-/O- (Alsina & Blanch 1994) (see Table 2 for more details). This was confirmed by the 16S rDNA and *Gyr*B sequence analysis, which showed 98-99% similarity to various *V. anguillarum* (Table 1) gene sequences. Some of the sequencing analyses produced inconclusive results when the two genes were compared. HI 21404's 16S rDNA sequence showed similarities to *Vibrio* sp. while the *GyrB* sequence was 100% similar to *Aeromonas salmonicida* subsp. *salmonicida*. The 16S rDNA sequence for strain HI 21408 was 100% similar to *V. anguillarum*, while the *GyrB* sequence was 94% similar to *V. logei*. HI 22022 was also similar to *V. anguillarum* the 16S rDNA sequence was BLASTed, but the *GyrB* sequence showed most similarity to *Pseudoalteromonas rubra* (98%). HI 22019's 16s rDNA and the *GyrB* sequence were similar to *Pseudoalteromonas nigrificans* and *Vibrio splendidus*, respectively. The last strain to show differences was HI 22025. The16S rDNA sequence was 96% similar to *Tenacibaculum ovolyticum* while the *GyrB* gene sequence was almost identical (99%) to *V. splendidus*. The bacterial strain HI 22022 came out as 96% similar to the gram positive bacteria *Bacillus herbersteinensis* isolated from a medieval wall painting in the chapel of Castle Herberstein, Styria (Austria) (Wieser et al. 2005). Unfortunately no *GyrB* gene sequence was obtained for this strain. No *GyrB* sequences were obtained for HI 22019, HI 22015 and 22054 either, in spite of three attempts being made.

Cumulative mortality and statistical analysis. In these three challenge experiments, each experiment contained three control groups, an unchallenged control group and two challenged controls. Large differences in survival were found between the unchallenged and the

challenged control groups (Figs. 1-6). The differences in mortality rates among the control groups were significantly different throughout all three experiments, except for the lowest challenge dose,10^4, in experiment II (Table 3). Observations among the unchallenged control groups were not significantly different from each other except at the end of the experiments on day 15 ($p < 0.00094$, Bonferonni correction, data not shown).

Only a few of the strains tested caused high mortality rates early in the experiments that were significantly different from the negative control groups. The increase in mortality observed at the end of the experiments, was due to the lack of feeding.

The challenged controls groups displayed some differences in terms of when the larvae started to die. In the first experiment the larva tended to die about four days earlier, between days three and four, than larvae in experiments II and III (see Figs. 1-6). In the second experiment the cumulative mortality among the challenged control group was lower and significantly different from that of the challenged control groups in experiments I and III (Figs. 1-6 and Table 3, $p < 0.00094$, Bonferonni correction). On the other hand, the cumulative mortalities for all other bacterial strains tested in experiment II were in the same range as for the negative control group (Figs. 3 and 4) and found to not be significantly different from each other. However, the statistical analysis confirmed that mortality rates of the high-dose challenged control group and the unchallenged control group were significantly different from each other (Table 5). The mortality rates of the positive controls in experiments I and III were not found to be significantly different (data not shown).

As Figures 1, 2, 5 and 6 show, only the five strains HI 21412, HI 21413, HI 21414, HI 21429 and HI 21050 caused high mortality. HI 21412 appeared to be the most virulent strain of the

five. Mortality caused by this strain was significantly different from the mortality rate found in the negative control group on day three post-challenge (challenge dose 10^6 CFU ml^{-1}) and on day six (challenge dose 10^4 CFU ml^{-1}) (Table 4). The mortality rate among the remaining four strains was found to be significantly different from that of the unchallenged control groups from day six and onwards for the highest challenge dose and in low-challenged dose groups from day nine ($p < 0.00094$, Bonferonni correction details (Table 4). The lowest challenge dose did not cause any increase in mortality rate in larval groups challenged with HI 21050. Compared to the positive control, these strains did not produce significantly different results. Strains HI 21052, HI 22001 and HI 22027, resembling *Vibrio logei*, *Vibrio anguillarum* and *Vibrio splendidus*, respectively (see Table 1) led to cumulative mortality rates that were different from both control groups (Figs. 1, 2, 5 and 6). In spite of this, the mortality rates for these strains were significantly different from the unchallenged control ($p < 0.00094$, Bonferonni correction, Table 4) only at the end of the experiment (i.e. from day 12 and onwards), indicating that they had a longer incubation period. The same strains were also significantly different from the positive control, indicating they are less virulent (data not shown). It should be noted that the low challenge dose of these three strains did not appear to cause the same increase in mortality as was found in the high challenge dose groups.

Cumulative mortality rates in most challenge groups were similar to the negative control groups (Figs. 1-6) and mortality rates among these 45 remaining groups did not differ significantly from the negative control groups ($p > 0.00094$, Bonferonni correction, data not shown). The mortality rates for the same groups were also found to be significantly different from the positive control group ($p < 0.00094$, Bonferonni correction (data not shown). This indicates that these 45 strains had no harmful effect.

Immunostaining of bacterial smears and Mono-Va testing. The four pathogenic strains HI 21412, HI 21413, HI 21414 and HI 21429, which were found to have similar 16rDNA and *GyrB* sequence as *V. anguillarum*, were tested for positive immunostaining with three different antisera against the *V. anguillarum* serotypes O2α, O2β and O1. Positive immunostaining was only found when they were stained with the antiserum against the O2α serotype. On the other hand, when tested with the Mono-Va agglutination kit for *V. anguillarum* strains, no positive reaction was found in any of the four strains.

DISCUSSION

The aim of this study was to evaluate the virulence of candidate pathogenic bacteria among bacterial isolates associated with diseased cod larvae, other marine cold water fish and live feed cultures. Out of 53 bacterial strains tested, only the five strains HI 21412, HI 21413, HI 21414, HI 21429 and HI 21050 could be classified as primary pathogens, i.e. had a negative effect on cod larva survival. This indicated that most of the bacteria associated with and isolated from moribund cod *Gadus morhua* larvae, halibut *Hippoglossus hippoglossus*, coalfish *Pollachius virens* and pollack *Pollachius pollachius*, are not primary pathogens, i.e. they are probably not primary causes of disease. Similar results were found by Verner-Jeffreys et al. (2003) testing virulence among bacterial strains isolated from halibut hatcheries.

Four strains in this high mortality group were shown to resemble *Vibrio anguillarum* by 16S rDNA and *GyrB* analysis. This confirms that vibriosis may also be a problem in the aquaculture of early life stages of cod. However, serotyping of these four strains, HI 21412, HI 21413, HI 21414 and HI 21429, did not provide any clear results. Serological testing with

specific antisera against the *V. anguillarum* serotypes, O1, O2α and O2β, produced positive results only against the O2α serum. At the same time, no positive results were found by using a Mono-Va agglutination kit, which should have produced positive results for the O1, O2 and O3 serotypes. This can be explained by that these *V. anguilllarum* strains may differ from serotypes known today, for which commercially produced antisera exist. A recent study by Mikkelsen et al. (2007) showed that bacteria isolated from diseased cod differ from O2α and O2β isolates serologically, biochemically and genotypically. These authors further indicate that these *V. anguillarum* isolates belong to a new sero-subtype. However, the four isolates used in the present study are biochemically and genotypically consistent with *V. anguillarum*. The ALO test gave A+/L-/O- as a classification of the *V. anguillarum* strains (See Table 2). However, further studies should be carried out in order to compare already known isolates and serotypes with these findings. Studies of this kind are probably essential if efficient vaccines are to be developed. Vaccines developed for cod do not provide sufficient protection and vibriosis is still a problem in cod farming even though vaccines for cod have been on the market for more than 10 years (Samuelsen et al. 2006).

Strain HI 21050, resembling *Carnobacterium* sp., was isolated from a culture of the alga *Tetraselmis* sp. This alga is commonly used as a feed and enrichment in rotifer cultures (Muller-Feuga et al. 2003). Algal cultures are associated with bacterial populations. Population studies of algal cultures used as feed for scallop larvae have identified a variety of bacterial strains associated with the algal cultures (Sandaa et al. 2003, Nicolas et al. 2004), probably including opportunistic pathogens as well as commensal or mutualistic bacteria. In intensive aquaculture, cod larvae are offered rotifers, usually *Brachionus plicatilis*, and brine shrimp, mostly *Artemia franciscana,* as live feed (Svåsand et al. 2004, Reitan 2005). Recently, Korsnes et al. (2006) demonstrated the presence of bacteria with high sequence

similarity to *Vibrio anguillarum* in rotifer cultures and the gut of cod larvae fed rotifers. Both rotifers and *Artemia* sp. are filter feeders capable of concentrating large amounts of bacteria, and live feed is a major source of bacterial influx to the gastrointestinal tract of fish (Nicolas et al. 1989, Skjermo and Vadstein 1993, Makridis et al. 2000a, b). The present results support the view that the composition of this influx influences larval survival. However, as most strains did not induce mortality, they could be viewed as secondary pathogens, i.e. opportunists that invade larvae that are already stressed or weakened.

Three strains, HI 21052, HI 22001 and HI 22027, resembling *Vibrio logei*, *Vibrio anguillarum* and *Vibrio splendidus*, respectively, caused mortality rates significantly different from the negative control from day nine post hatching. These strains appeared to have a slower infection rate. The could be caused by non-optimal growth conditions for the specific bacterium, such as temperature, salinity and nutrients. Therefore, it cannot be out ruled that these bacterial strains might act like primary pathogens under different growth conditions. During laboratory studies fish are kept under controlled optimal conditions, which might make them more capable of dealing with an infection compared to fish kept in commercial farms. The bacterial strain HI 22001 is probably a less virulent strain of *V. anguillarum*. Differences in virulence among O1 isolates of *V. anguillarum* were reported by (Pedersen et al. 1997), and it is likely that differences in virulence will occur among all *V. anguillarum* serotypes. Less virulent O2 strains of *V. anguillarum* isolates, isolated from cod, have also been reported (Mikkelsen et al. 2007).

None of the three different *Vibrio logei* strains tested had any negative effect on larval survival. This was not expected prior to the challenge experiments. *V. logei* is frequently isolated from moribund and dead larvae and it has been assumed to play a significant role in

bacterial problems experienced in hatcheries. There are several possible explanations to why bath challenge did not have any effect. A challenged dose of 10^6 CFU ml^{-1} may not be sufficient to cause disease. It is possible to grow *V. logei* in cultures up to 10^8 CFU ml^{-1}, so a higher challenge dose should be tested in repeated experiments. Cod larvae drink water from hatching onwards (Mangor-Jensen & Addof 1987), thus bacteria will enter the gastrointestinal tract. Consequently the intestine as a route of entry for pathogenic bacteria cannot be ruled out. On the other hand the results lead to the hypothesis that a different route of entry than bath challenge or that a combination of both bath and oral exposure is needed. Experiments that deliver challenges via live feed have been performed on turbot *Scophthalmus maximus* larvae (Grisez et al. 1996, Planas et al. 2005) but to the best of our knowledge, no such experiments have been performed on cod larvae. Another explanation for the lack of pathogenicity is the possibility of quorum sensing. *V. logei* strains were first described as symbiotic with *Vibrio fisheri* in squids (*Sepiola robusta* and *Sepiola affinis*) light organs (Fidopiastis et al. 1998). The two luminous bacteria are closely related, and with *V. logei* being symbiotic with a bacterium capable of quorum sensing (Dunlap 1999, Milton 2006), it is reasonable to believe that *V. logei* might possess some of the same abilities as its fellow organism. This leaves room for speculation about whether or not *V. logei* is an opportunist taking advantage of other bacteria, perhaps through mechanisms of quorum sensing. The work done by Fidopiastis et al. (1998) also confirms the difficulty of distinguishing two closely related coexisting bacteria by growth and genetic analysis. During a disease outbreak, finding and isolating the primary pathogen could thus be difficult if other agents are present at high densities, as *V. logei* often is. The role of *V. logei* in disease outbreaks in cod hatcheries still needs to be elucidated.

Moritella viscosa has been isolated form cultivated cod that display skin lesions similar to the ones seen on salmonids (Colquhoun et al. 2004), but the bacteria showed some phenotypical differences from to the NCIMB 13584^T strain. The *M. viscosa* isolate used in these experiments did not have any negative effect on the cod larvae. In a study performed by Gudmunsdóttir et al. (2006), a bath challenge with *M. viscosa* resulted in mortality only when the challenge dose was as high as 10^7 CFU ml^{-1}. Similar results were obtained by Björnsdóttir et al. (2004), in challenge experiments on turbot juveniles (50 g). No clinical signs were found on fish challenged with the lowest dose (10^6 CFU ml^{-1}) in either of these two experiments. On this basis, future experiments on cod larvae should contain a higher challenge dose. However, in the present experiments we chose to use the same challenge dose for all strains tested. On the other hand, a challenge dose of 10^6 CFU ml^{-1} is frequently used in challenge experiments and is generally considered to be a high challenge dose (Bergh et al. 1992, Vik-Mo et al. 2005, Schrøder et al. 2006, Sandlund et al. 2006).

When comparing growth temperatures, Tunsjø et al. (2007) found that *M. viscosa* grew denser and had better motility at 4^{o}C and with the addition of 3 to 4% NaCl, than at 15^{o}C with 1% NaCl. Conditions for growth in these experiments should be within the range of optimal growth for this bacterium.

The reason for the differences seen between the challenged control groups, especially in experiment II, is not known. It could be caused by inaccuracy when the dilutions of these particular bacterial suspensions were made. It is known that loss of flagella, for example during the washing procedure, makes the bacterium less pathogenic, as the flagella are very important as a source of virulence (Milton et al. 1996, O'Toole et al.1996). Before challenge, all bacterial suspensions were examined in order to verify that the motile bacteria were still

intact and motile after the washing procedure. Hence it is unlikely that the loss of flagella was the cause of differences in mortality. Reduction or loss of virulence induced by washing of bacterial suspensions should also be considered. Given that all bacterial suspensions were grown under the same conditions, and that this washing procedure is routinely used in our laboratory, this is unlikely to have affected the results.

The same temperature was used both to grow the bacterial cultures and as the incubation temperature inside the air-conditioned room. This was done in order to keep conditions as close to the natural environment as possible, where the bacteria grow under the same conditions as the larvae. Changes in characteristics when bacterial strains have been grown at different temperatures had been observed, hence the decision to use the same temperatures.

To keep unfed cod larvae alive for up to 14 days post-hatch proves that the multi-dish system is well adapted for studying bath challenge for cod larvae as well as for halibut (Bergh et al. 1992, 1997), turbot (Bergh et al. 1997, Hjelm et al. 2004) and great scallop larvae (Sandlund et al. 2006). Only at the end of the experiments, 15 days post-hatching were significant differences between the unchallenged control groups in the three experiments found. These individual differences are most likely caused by the lack of feeding.

The larvae were kept alive until starvation point due to the possibility of losing significant data. To the best of our knowledge this is the first time this kind of experiment has been carried out on cod larvae, and we needed all three experiments to be performed identically to be able to rely on the results. Similar future experiments done under similar conditions can be brought to an end at an earlier stage.

None of the bacterial strains were re-isolated from the larvae. Larvae possess a sterile digestive system until hatching, when it is colonized by the egg flora (Reviewed by Vine et al. 2006). Reisolation of bacteria form larvae is difficult, particularly due to the small size of the larvae, the need for exterior washing or disinfection resulting in decreasing number of viable bacteria. Concerning this matter we chose by means of immunochemistry to verify the presence of *Vibrio anguillarum* (Engelsen et al. in press). Given that sterile water and a high concentration of bacteria were added to each well, it is reasonable to believe that the dominant bacteria inside the wells were the bacteria used for challenge. Furthermore, the eggs were selected at random and most of the larvae lived until the point of starvation, indicating that the larvae did not die of other factors than the bacterial strains added.

The use of API 20E as a diagnostic tool for aquatic bacteria has been debated for decades, as reviewed by Popovic et al. (2007). According to Alsina & Blanch (1994), this could be a useful tool when adjustments or modifications to the manufactory's manual are being made and as a supplement to other methods of identification. In our laboratory we have modified the API protocol to suit cold-water bacterial strains on the basis of previous experience. In spite of this we observed some inconclusive results among some of the bacterial strains tested, especially concerning the fermentation of sugars. This has occasionally been observed in our laboratory (authors' observations)

Analyses of 16S rDNA and *GyrB* gene sequences were primarily used as a preliminary stage of identification in order to limit the number of bacteria used in these challenge trials and for further characterisation, respectively. When the 16S rDNA and *GyrB* sequences were compared, some identifications were inconclusive (Table 1). This may have been due to polymorphism and heterogeneity in the 16S rDNA gene (Dahllöf et al. 2000, Moreno et al.

2002), which would make it difficult to identify strains based on one gene only. Several other genes have been suggested as additional sources of information for identifying bacterial strains; for instance *recA* (Thompson et al 2004a), *rpoB* (Dahllöf et al. 2000), G*yrB*, *fusA*, *nifD* (Holmes et al. 2004). Another aspect is the limited number of *GyrB* sequences available in the GeneBank, compared to 16S rDNA sequences. This is probably the cause of the low frequency of matches with *GyrB* found in the database. The average length of the fragments used in this study is in the range of 1250 – 1350 nucleotides for the 16S rDNA gene and 1100-1200 nucleotides for the *GyrB* gene, which is normally sufficient to obtain a match.

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Table. 1 Characterization of the bacterial strains used in this experiment. V. ang 610 = *Vibrio anguillarum* strain 610, Source = the bacterial strain's origin of isolation, Salmon *Salmo salar*, Cod *Gadus morhua*, Pollack *Pollachius pollachius*, Coalfish *Pollachius virens*, Halibut *Hippoglossus hippoglossus*, Tetra. sp = *Tetraselmis* sp. culture, Rot. Culture = rotifer culture, Long fil. = long filamentous, Cocc rod = coccoid rod shape, + = positive, - = negative, w = weak, gr = growth, Yel colon = yellow colonies, Gr colon = green colonies, TCBS = Thiosulphate citrate bile sucrose agar, CA = cytophaga agar, Hemolytic = haemolytic growth on blood agar 1.5% NaCl, N.D = not determined, no match = no match during BLAST search, No seq. obtained = no sequences were obtained for these strains

Bacterial strain	Source	Gram	Shape	Motility	Okxidase	Hemolytic	TCBS	CA	16S rDNA	GyrB
V. ang. 610	Control	-	Cocc rod	+	+	+	Yel colon.	+	*V. anguillarum*	*V. anguillarum*
Marinomonas sp.	Cod	-	rod	+	-	-	-	+	*Marinomonas* sp.	*Marinomonas* sp.
M. viscosa	Salmon	-	rod	+	+	+	N.D	N.D	*Moritella viscosa*	
F95B/98	Salmon	-	Long fil.	-	+	No gr	N.D	N.D	*Tenacibaculum* sp.	*Tenacibaculum* sp.
F95C/98	Salmon	-	Long fil.	-	+	No gr	N.D	N.D	*Tenacibaculum* sp.	*Tenacibaculum* sp.
HI 21030	Cod larvae	-	rod	+	+	-	w gr	+ (w)	*Marinobacter* sp.	
HI 21031	Cod larvae	-	rod	+	+	-	-	+	*Pseudoalteromonas* sp.	
HI 21037	Cod larvae	-	rod	+	+	-	-	+	*Pseudoalteromonas* sp.	
HI 21039	Cod larvae	-	rod	+	+	-	Gr colon.	+	*Vibrio logei*	
HI 21040	Cod larvae	-	rod	+	+	-	Gr colon.	-	*V. logei*	
HI 21041	Cod larvae	-	rod	+	+	-	w gr/ w Yel.	-	*Psychromonas* sp.	
HI 21047	Cod larvae	-	rod	+	+	-	Gr colon.	+	*Pseudoalteromonas* sp.	
HI 21050	Tetra. sp.	-	rod	w	-	+ (w)	w gr/Gr colon.	-	*Carnobacterium* sp.	
HI 21052	Cod larvae	-	rod	+	+	-	Gr colon.	+	*V. logei*	
HI 21056	Cod larvae	-	rod	+	+	-	Gr colon.	+	*Vibrio aff splendidus*	
HI 21059	Cod larvae	-	rod	+	-	-	-	+	*Marinomonas* sp.	
HI 21061	Pollack	-	rod	+	+	+	Gr colon.	+	*Marinomonas* sp.	
HI 21063	Pollack	-	rod	+	+ (w)	-	-	+	*Photobacterium cf.iliopiscarium*	
HI 21064	Pollack	-	rod	+	+ (w)	-	Gr colon.	+	*Vibrio wodanis*	
HI 21065	Cod larvae	-	Cocc rod	+	+	-	Yel. Colon.	+ (w)	*Shewanella-sairae/marinintestina*	
HI 21066	Coalfish	-	rod	+	+	+	Gr colon.	+	*Vibrio splendidus*	
HI 21068	Coalfish	-	Cocc rod	+	-	-	w gr	+	*Photobacterium cf.iliopiscarium*	
HI 21069	Cod larvae	-	rod	+	-	-	w gr	+	*Marinomonas* sp.	
HI 21400	Rot. culture	-	rod	+	+	-	w gr	+	*Vibrio* sp.	*Vibrio parahaemolyticus* (86%)
HI 21402	Cod larvae	-	rod	w	+	-	Gr colon.	+	*Vibrio fisheri* or *Vibrio logei* (99%)	*V. fisheri* (88%)
HI 21404	Cod larvae	-	rod	+	+	+	-	+ (w)	*Vibrio* sp.	*Aeromonas salmonicida* subsp. *salmonicida* (100%)
HI 21405	Cod larvae	-	rod	+	+	-	-	+ (w)	*Marinomonas* sp. (96%)	*Marinomonas vaga* (80%)

HI 21407	Cod larvae	-	rod	w	+	-	Yel. Colon.	+ (w)	*Vibrio* sp.	*V. parahaemolyticus* (86%)
HI 21408	Cod larvae	-	rod	+	+	-	Yel. Colon.	+ (w)	*V. anguillarum* strain 010610-3 (100%)	*V. logei* (94%)
HI 21410	Cod larvae	-	rod	+	+	++	Gr colon.	+	*V. splendidus*	*V. splendidus* (100%)
HI 21412	Cod larvae	-	rod	+	+	+ (w)	Yel. Colon.	+	*V. anguillarum* O2a (99%)	*V. anguillarum* (99%)
HI 21413	Cod larvae	-	rod	+	+	+ (w)	Yel. Colon.	+	*V. anguillarum* O2a (99%)	*V. anguillarum* NCMB 6 (98%)
HI 21414	Cod larvae	-	rod	+	+	+ (w)	Yel. Colon.	+	*V. anguillarum* (99%)	*V. anguillarum* (98%)
HI 21417	Cod larvae	-	rod	+	-	-	-	+	*Rhodococcus* sp. (99%)	*Rhodococcus erythropolis* (99%)
HI 21424	Cod larvae	-	Cocc rod	+	+	+	Yel/White colon	+	*V. wodanis* (99%)	*V. splendidus* (91%)
HI 21427	Cod larvae	-	rod	+	+	-	-	+	*Vibrio gallicus* (99%)	*V. parahaemolyticus* (86%)
HI 21429	Cod larvae	-	rod	+	+	+ (w)	Yel. Colon./Yel agar	+	*V. anguillarum* (99%)	*V. anguillarum* NCMB 6 (98%)
HI 21430	Cod larvae	-	rod	+	+	-	-	+	*V. gallicus* (97%)	*V. anguillarum* (98%)
HI 21433	Cod larvae	-	rod	+	+	-	-	+	*V. gallicus* (99%)	*V. parahaemolyticus* (86%)
HI 22001	Cod larvae	-	rod	+	+	-	-	+	*V. anguillarum*	*Pseudoalteromonas rubra* (98%)
HI22002	Cod larvae	-	rod	+	-	-	w gr/Gr	+	*Vibrio* sp.	No seq. obtained
HI 22019	Hallibut	-	rod	+	+	+	w Yel.	+	*Pseudoalteromonas nigrifaceis* (99%)	*V. splendidus* LT 06 or LP1 (99%)
HI 22022	Hallibut	-	rod	+	+	-	-	+	*Bacillus herbersteinensis* type strain D-1.5a (96%)	No seq. obtained
HI 22025	Hallibut	-	rod	+	+	No gr	Yel. Colon./Yel agar	+	*Tenacibaculum ovolyticum* (96%)	*V. splendidus* LT 06 or LP1 (99%)
HI 22027	Hallibut	-	rod	+	+	+	Gr colon.	+	*Vibrio* sp. Da2 or PMV19 (98%)	*V. splendidus* LP1 or LT 06 (99%)
HI 22029	Hallibut	-	rod	+	+	+	-	+	*Pseudoalteromonas haloplanktis* (98%)	*Pseudoalteromonas carrageenovora* (90%)
HI 22032	Hallibut	-	rod	+	+	+	Yel. Colon./Yel agar	+	*V. splendidus* (98%)	*V. splendidus* LP1 or LT 06 (98%)
HI 22034	Hallibut	-	rod	+	+	-	-	+	*Pseudoalteromonas* sp. EH-2-1 (99%)	*P. haloplanktis* (99%)
HI 22042	Hallibut	-	rod	+	+	-	-	+	*Pseudoalteromonas* sp.	*P. carrageenovora* (91%)
HI 22044	Hallibut	-	rod	+	+	+	Gr colon.	+	*Vibrio* sp.	*V. splendidus* LT06 or PMV18 (99%)
HI 22051	Hallibut	-	rod	+	+	No gr	-	+ (w)	*Tenacibaculum* sp.	No seq. obtained
HI 22054	Hallibut	-	rod	+	+	No gr	-	-	*Pseudoalteromonas* sp. (99%)	No seq. obtained
HI 22077	Rot. culture	-	rod	+	+	+	-	+	*Pseudoalteromonas* sp. (99%)	*P. haloplanktis* (98%)

Table 2. API 20E results for all strains used in these challenge experiments. ONPG = β-galactosidase, ADH = arginine dihydrolase, LDC = lysine decarboxylase, ODC = ornithine decarboxylase, CIT = citrate utilization, H_2S = H_2S production, URE = urease, TDA = tryptophane deaminase, IND = indole production, VP = acetoin production, GEL = galatinase, GLU = glucose, MAN = mannitol, INO = inositol, SOR = sorbitol, RHA = rhamnose, SAC = saccharose, MEL = melibiose, AMY = amygdalin, ARA = arabinose, V. ang 610 = *Vibrio anguillarum* strain 610, +/- = are used when results have differed between API runs, N.D = Not determined

Bacterial strain	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
V. ang. 610	+	+	-	-	-/+	-	-	-	-	-	+	+	-/+	-	+	-	+/-	-	-	-
Marinomonas sp.	+	-	-	+	-	-	-	-	-	-	-	+/-	+/-	+	+	-	+	+/-	+/-	+/-
M. viscosa	N.D	-	+	-	N.D	N.D	N.D	N.D	N.D	N.D	+	+	-	N.D	N.D	N.D	-	N.D	N.D	N.D
F95B/98	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
F95C/98	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
HI 21030	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-
HI 21031	-	-	-	-	+/-	-	+/-	-		+	+	-	+	-	-	-	-	-	-	-
HI 21037	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-/+	-	+	-	+
HI 21039	-	-	+	-	-	-	-	-	-	+/-	-	+/-	+	-	-	-	-	-	+/-	-
HI 21040	-	-	+	-	+/-	-	-	-	-	+/-	-	+/-	+	-	-	-	-	-	+/-	-
HI 21041	+	-	+	-	-	-	-	-	-	+/-	-	-	+	-	-	-	-	-	+/-	-
HI 21047	-	-	+	-	-	-	+	-	-	+/-	-	+/-	+/-	-	-	-	-	-	+	-
HI 21050	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HI 21052	-	-	+	-	-	-	+	-	-	+/-	-	+/-	+	-	-	-	-	-	+/-	-
HI 21056	+	-	-	-	-	-	-	-	-	+/-	+	+	+	-	-	-	-	-	+	-
HI 21059	+	-	-	-	+	-	-	-	-	+	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
HI 21061	+	-	-	-	-	-	-	-	-	+/-	+	+	+	-	-	-	-	-	+	-
HI 21063	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
HI 21064	+	-	+	-	-	-	-	-	-	+/-	-	+	-	-	-	-	-	-	+	-
HI 21065	-	-	+	-	-	-	+	-	+	-	-	+	+	-	-	-	+	-	+	-
HI 21066	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	+/-	-	-
HI 21068	+	+	-	-	+	-	-	-	-	+	-	+	+/-	+/-	+/-	-	+/-	+	+	+/-
HI 21069	+	-	-	-	+	-	-	-	-	+	-	+/-	+/-	+/-	+/-	+/-	-	+/-	+/-	+/-
HI 21400	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	-	-	+
HI 21402	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
HI 21404	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-
HI 21405	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

HI 21407	-	-	-	-	-	-	-	-	-	-	-	+/-	+	-	-	-	+	-	-	+
HI 21408	+	-	+	-	-	-	-	-	-	-	+/-	+	-	-	-	+	-	-	-	-
HI 21410	+	-	-	-	-	-	-	-	-	-	+	+/-	+	-	-	-	-	+/-	+	-
HI 21412	+	+	-	-	+/-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	+
HI 21413	+	+	-	-	+/-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	-
HI 21414	-	+	-	-	+/-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	-
HI 21417	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
HI 21424	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	+	-
HI 21427	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+/-	+
HI 21429	+	+	-	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	-
HI 21430	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	+
HI 21433	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+/-	+/-
HI 22001	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
HI22002	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
HI 22019	+/-	-	-	-	-	-	-	-	-	+/-	+	+	+	-	-	-	-	+	-	-
HI 22022	+	-	-	-	-	-	-	-	-	+/-	+	-	-	-	-	-	-	-	-	-
HI 22025	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	+	-
HI 22027	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	+	+	-
HI 22029	+	-	-	-	-	-	-	-	-	+/-	+	+	+	-	-	-	+	-	-	+
HI 22032	+	-	-	-	-	-	-	-	-	+/-	+	-	+	-	-	-	+	-	+	-
HI 22034	+	-	-	-	+/-	-	-	-	-	+/-	+	+	+	-	-	-	+	-	+	-
HI 22042	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	+/-
HI 22044	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	+	-
HI 22051	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
HI 22054	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HI 22077	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

Table 3. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.00094$, Bonferonni correction) for 2 x 2 contingency table negative control vs positive control in all three challenge experiments. 10^6 and 10^4 = Challenge dose 10^6 and 10^4. All significant p-values in bold. Exp = experiment , V. ang 610 = *Vibrio anguillarum* strain 610, Day = days post-hatch.

		Day 3		Day 6		Day 9		Day 12		Day 15	
Exp	Bacteria	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
1	V. ang 610 10^6	51,13	**.0000**	114.33	**.0000**	113.80	**.0000**	113.80	**.0000**	10.72	.0011
1	V. ang 610 10^4	16.78	**.0000**	95.68	**.0000**	97.37	**.0000**	113.80	**.0000**	8.51	**.0035**
2	V. ang 610 10^6	4.27	.0388	41.89	**.0000**	37.53	**.0000**	33.57	**.0000**	24.25	**.0000**
2	V. ang 610 10^4	.82	.3657	1.35	.2448	1.45	.2283	.42	.5157	6.37	**.0116**
3	V. ang 610 10^6	.60	.4383	33.27	**.0000**	103.44	**.0000**	114.33	**.0000**	38.61	**.0000**
3	V. ang 610 10^4	5.41	.0200	47.53	**.0000**	93.90	**.0000**	100.02	**.0000**	38.61	**.0000**

Table 4. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.00094$, Bonferonni correction) for 2 x 2 contingency table of all isolates significantly different from the negative control group. 10^6 and 10^4 = Challenge doses 10^6 and 10^4. All significant p-values in bold. Exp. = Experiment, Day = days post-hatch.

		Day 3		Day 6		Day 9		Day 12		Day 15	
Exp.	Isolate	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
1	HI 21412 10^6	20.70	**.0000**	101.59	**.0000**	121.02	**.0000**	125.08	**.0000**	13.37	**.0003**
1	HI 21412 10^4	.26	.6121	40.33	**.0000**	97.37	**.0000**	113.80	**.0000**	8.51	.0035
1	HI 21413 10^6	3.31	.0689	87.33	**.0000**	124.79	**.0000**	125.08	**.0000**	13.37	**.0003**
1	HI 21413 10^4	2.40	.1212	7.36	.0067	19.55	**.0000**	25.52	**.0000**	3.88	.0489
1	HI 21414 10^6	4.27	.0389	104.65	**.0000**	128.67	**.0000**	125.08	**.0000**	13.37	**.0003**
1	HI 21414 10^4	.26	.6121	1.57	.2109	23.68	.0000	45.61	.0000	8.51	.0035
1	HI 21047 10^6	0.00	1.0000	.83	.3626	3.04	.0810	21.25	**.0000**	6.67	.0098
1	HI 21047 10^4	0.00	1.0000	0.00	1.0000	0.00	1.0000	.13	.7160	.05	.8285
1	HI 22001 10^6	1.57	.2109	1.57	.2095	7.68	.0056	24.06	**.0000**	0.00	.0000
1	HI 22001 10^4	3.31	.0689	5.27	.0218	9.81	.0017	21.25	**.0000**	.36	.5476
3	HI 21429 10^6	2.69	.1012	49.50	**.0000**	121.21	**.0000**	114.33	**.0000**	38.61	**.0000**
3	HI 21429 10^4	.26	.6121	0.00	1.0000	1.35	.2448	25.23	**.0000**	22.34	**.0000**
3	HI 21050 10^6	6.41	.0114	16.00	**.0001**	34.92	**.0000**	40.09	**.0000**	9.23	.0024
3	HI 21050 10^4	0.00	1.0000	0.00	1.0000	6.06	.0138	11.05	.0009	8.09	.0045
3	HI 21052 10^6	.60	.4383	4.27	.0388	7.03	.0080	28.27	**.0000**	20.25	**.0000**
3	HI 21052 10^4	1.19	.2751	.39	.5304	.39	.5304	9.98	.0016	11.81	.0006
3	HI 22027 10^6	2.69	.1012	7.03	.0080	10.14	.0015	18.27	**.0000**	2.40	.1213
3	HI 22027 10^4	.51	.4764	.83	.3626	.17	.6767	.08	.7785	.11	.7383

Table 5. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.00094$, Bonferonni correction) for 2 x 2 contingency table of all isolates in challenge experiment II, not significantly different from the negative control Only challenge dose 10^6 is presented in this table. All significant p-values in bold. Exp. = experiment, Day = days post-hatch, V. ang 610 = *Vibrio anguillarum* strain 610.

		Day 3		Day 6		Day 9		Day 12		Day 15	
Exp.	Isolate	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
2	V.ang 610	4.27	.0388	41.89	**.0000**	37.53	**.0000**	**33.57**	**.0000**	24.25	**.0000**
2	*M. viscoca*	.11	.7431	.39	.5304	.08	.7785	.05	.8168	2.37	.1239
2	F95b/98	.00	1.0000	.00	1.0000	.08	.7785	.19	.6599	1.00	.3173
2	F95c/98	.17	.6767	.17	.6767	.97	.3254	.00	1.0000	.44	.5049
2	HI 21056	.00	1.0000	.00	1.0000	.08	.7785	.00	1.0000	4.75	.0293
2	HI 21059	.17	.6767	.00	1.0000	.00	1.0000	.00	1.0000	3.38	.0658
2	HI 21061	2.31	.1282	.83	.3626	.00	1.0000	4.66	.0309	2.79	.0948
2	HI 21064	.82	.3657	.82	.3657	.00	1.0000	.19	.6599	6.37	.0116
2	HI 21066	.82	.3657	.82	.3657	.00	1.0000	.19	.6599	6.37	.0116
2	HI 21069	2.31	.1282	.83	.3626	1.90	.1685	.06	.8096	1.36	.2432
2	HI 21408	2.31	.1282	.00	1.0000	.39	.5304	.00	1.0000	.25	.6169
2	HI 21410	2.31	.1282	2.31	.1282	.00	1.0000	.00	1.0000	5.53	.0187
2	HI 21417	.17	.6767	.00	1.0000	.39	.5304	.24	.6224	7.28	.0070
2	HI 21427	.17	.6767	.17	.6767	.97	.3254	1.07	.3016	1.33	.2482
2	HI 21430	.82	.3657	1.35	.2448	.27	.6055	1.11	.2924	7.28	.0070
2	HI 21433	.17	.6767	.00	1.0000	.97	.3254	1.76	.1849	2.79	.0948
2	HI 22029	.00	1.0000	.00	1.0000	.27	.6055	.42	.5157	6.37	.0116
2	HI 22042	.00	1.0000	.39	.5304	.00	1.0000	.42	.5157	4.04	.0445
2	HI 22044	.13	.7160	.13	.7160	.00	1.0000	.24	.6224	4.04	.0445
2	HI 22054	.00	1.0000	.13	.7160	.09	.7630	.57	.4497	2.26	.1330

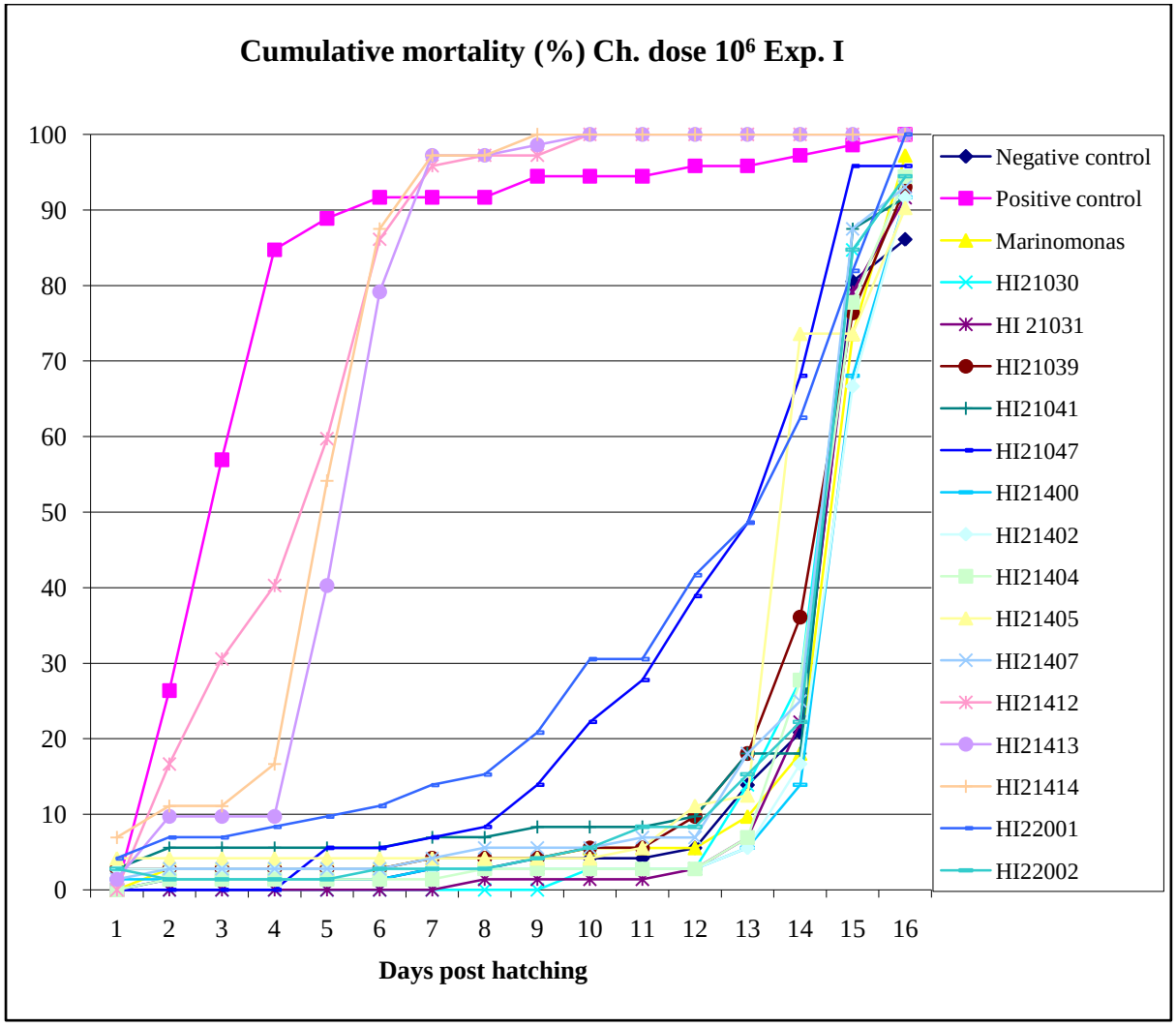

Fig. 1. *Gadus morha*. Cumulative mortality in percentage in Challenge experiment I, challenged dose 10^6. Negative control is larvae not challenged with bacteria. Positive control is the larval group challenged with *Vibrio anguillarum* strain 610. The other numbers refers to strain number.

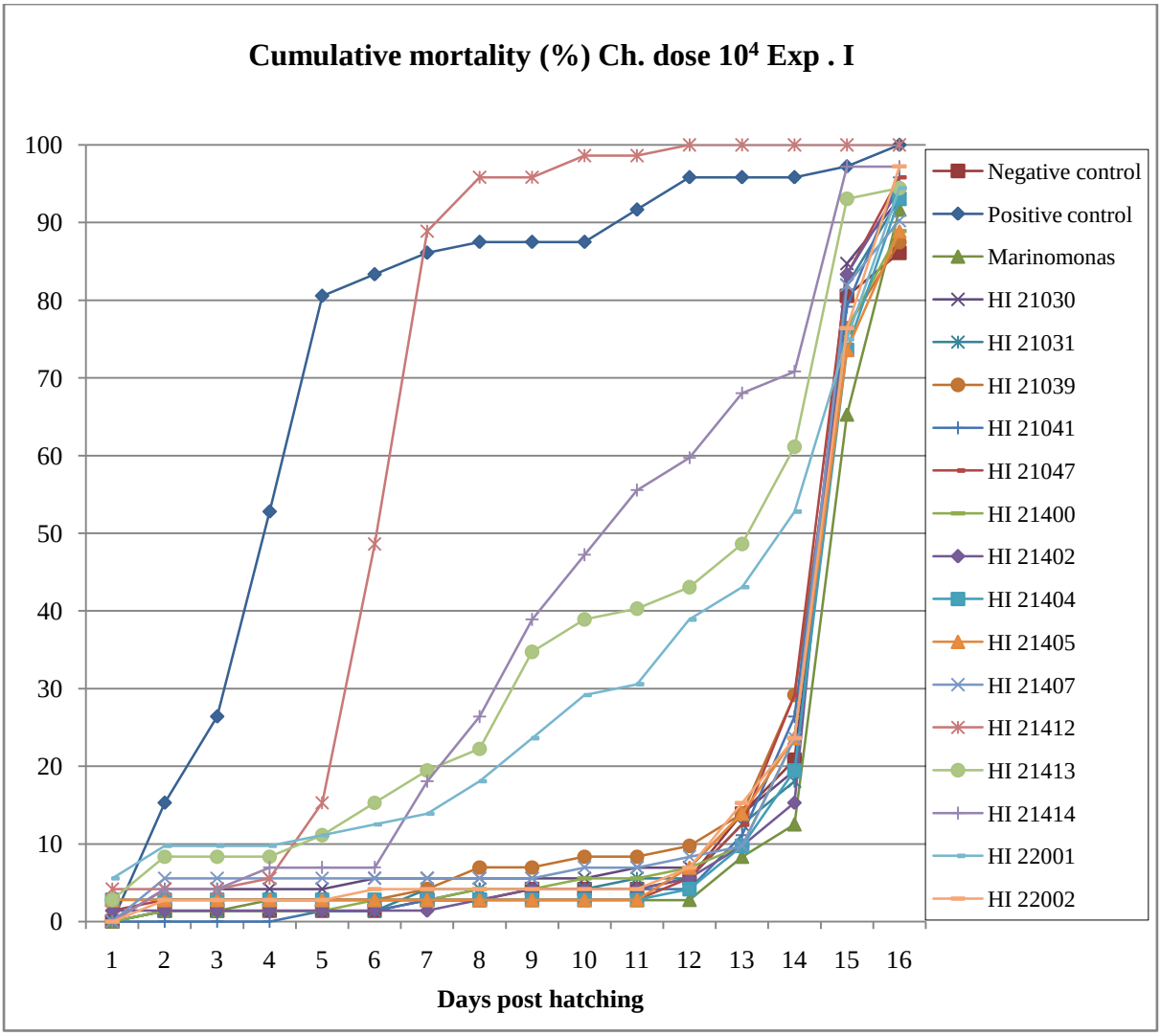

Fig. 2. *Gadus morha.* Cumulative mortality in percentage in Challenge experiment I, challenged dose 10^4. Negative control is larvae not challenged with bacteria. Positive control is the larval group challenged with *Vibrio anguillarum* strain 610. The other numbers refers to strain number.

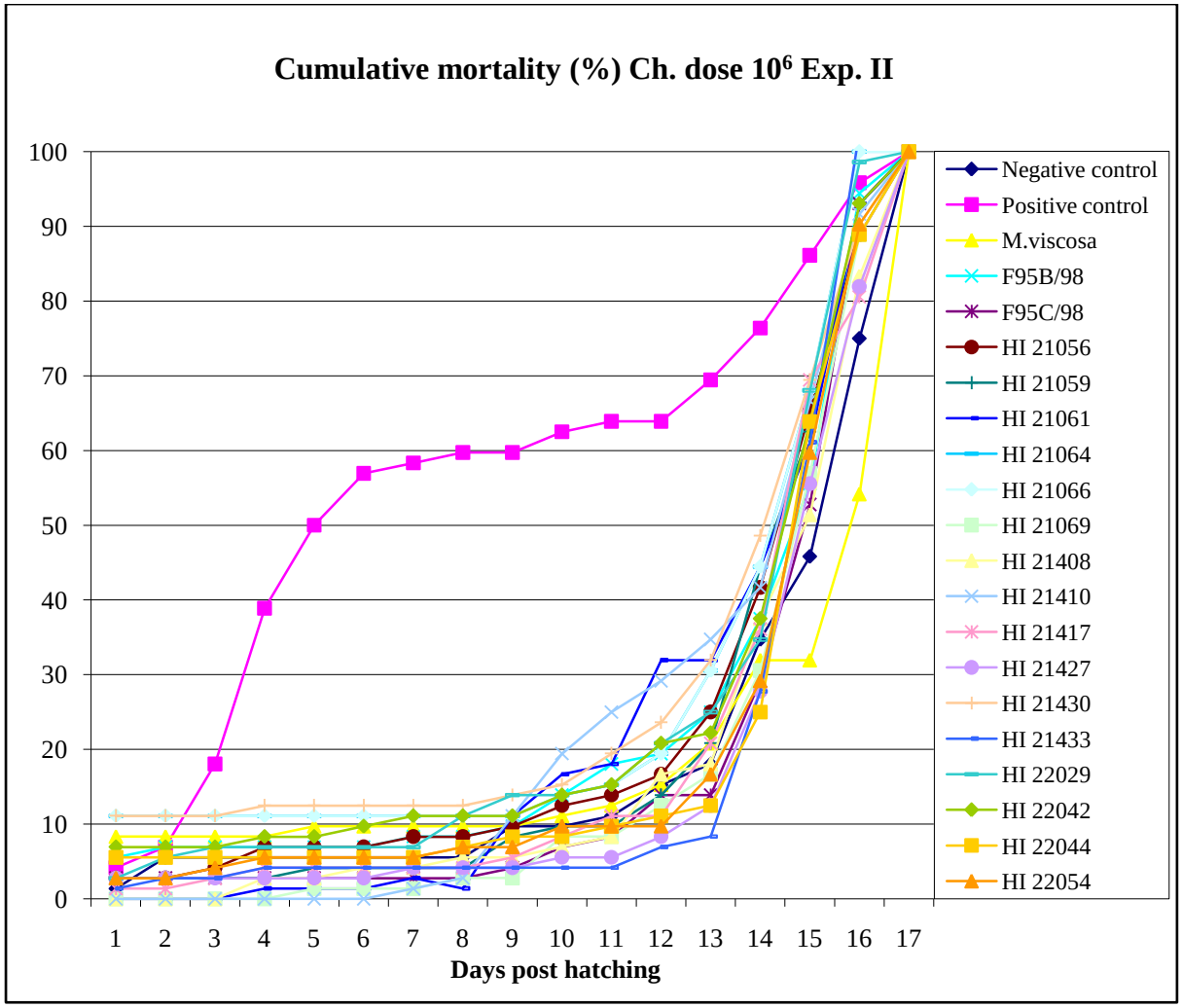

Fig. 3. *Gadus morha.* Cumulative mortality in percentage in Challenge experiment II, challenged dose 10^6. Negative control is larvae not challenged with bacteria. Positive control is the larval group challenged with *Vibrio anguillarum* strain 610. The other numbers refers to strain number.

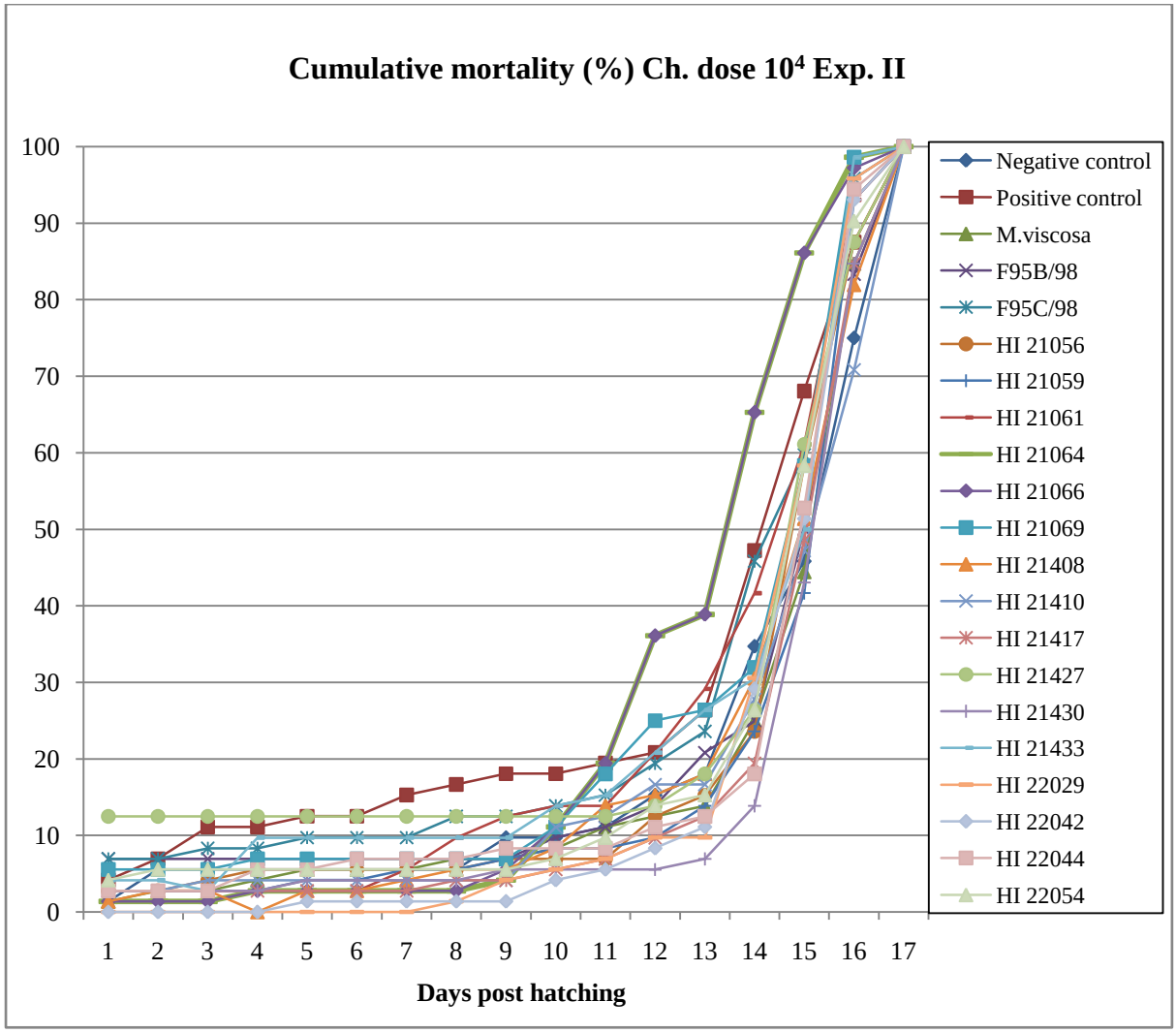

Fig. 4. *Gadus morha.* Cumulative mortality in percentage in Challenge experiment II, challenged dose 10^4. Negative control is larvae not challenged with bacteria. Positive control is the larval group challenged with *Vibrio anguillarum* strain 610. The other numbers refers to strain number.

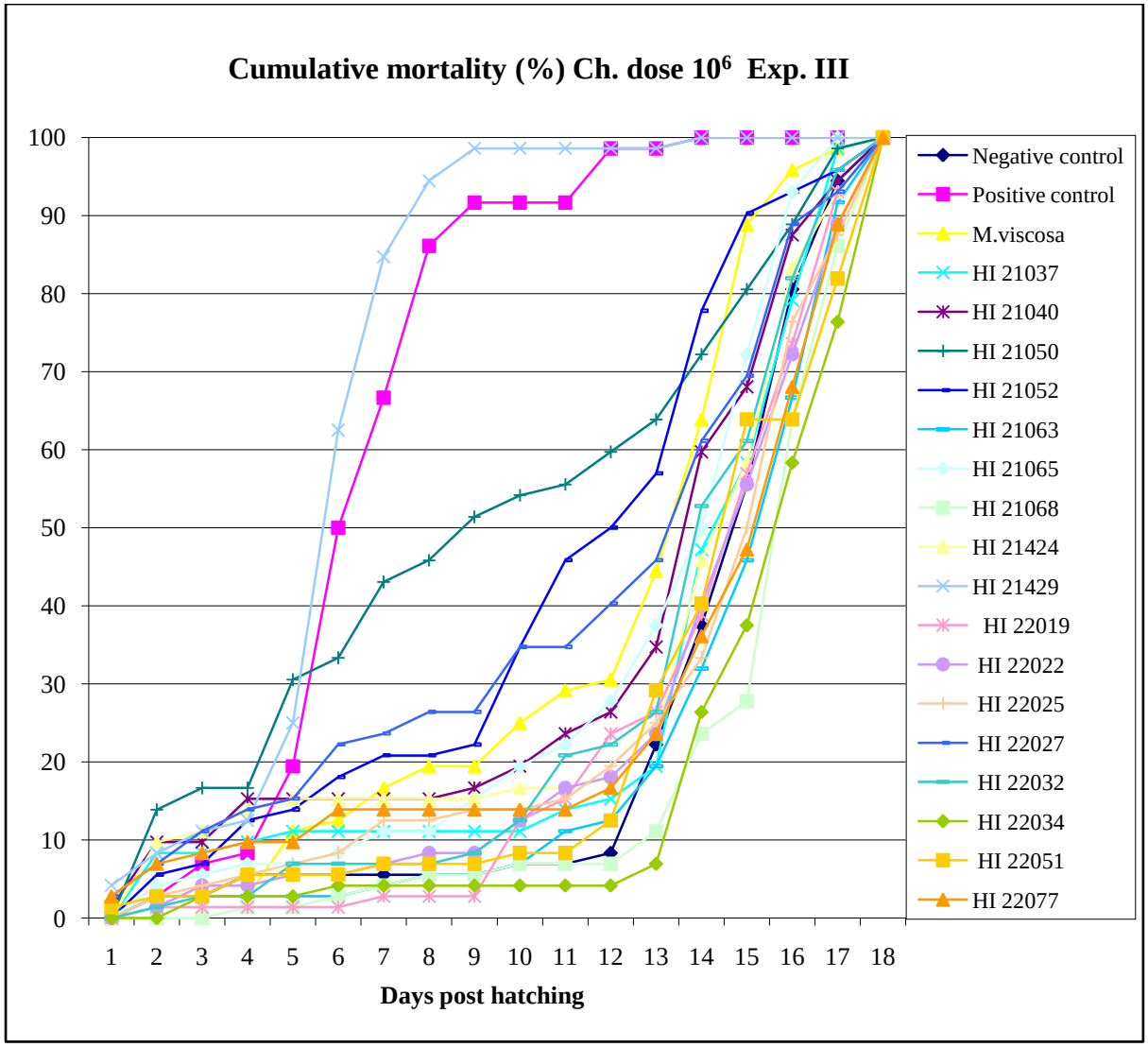

Fig. 5. *Gadus morha.* Cumulative mortality in percentage in Challenge experiment III, challenged dose 10^6. Negative control is larvae not challenged with bacteria. Positive control is the larval group challenged with *Vibrio anguillarum* strain 610. The other numbers refers to strain number.

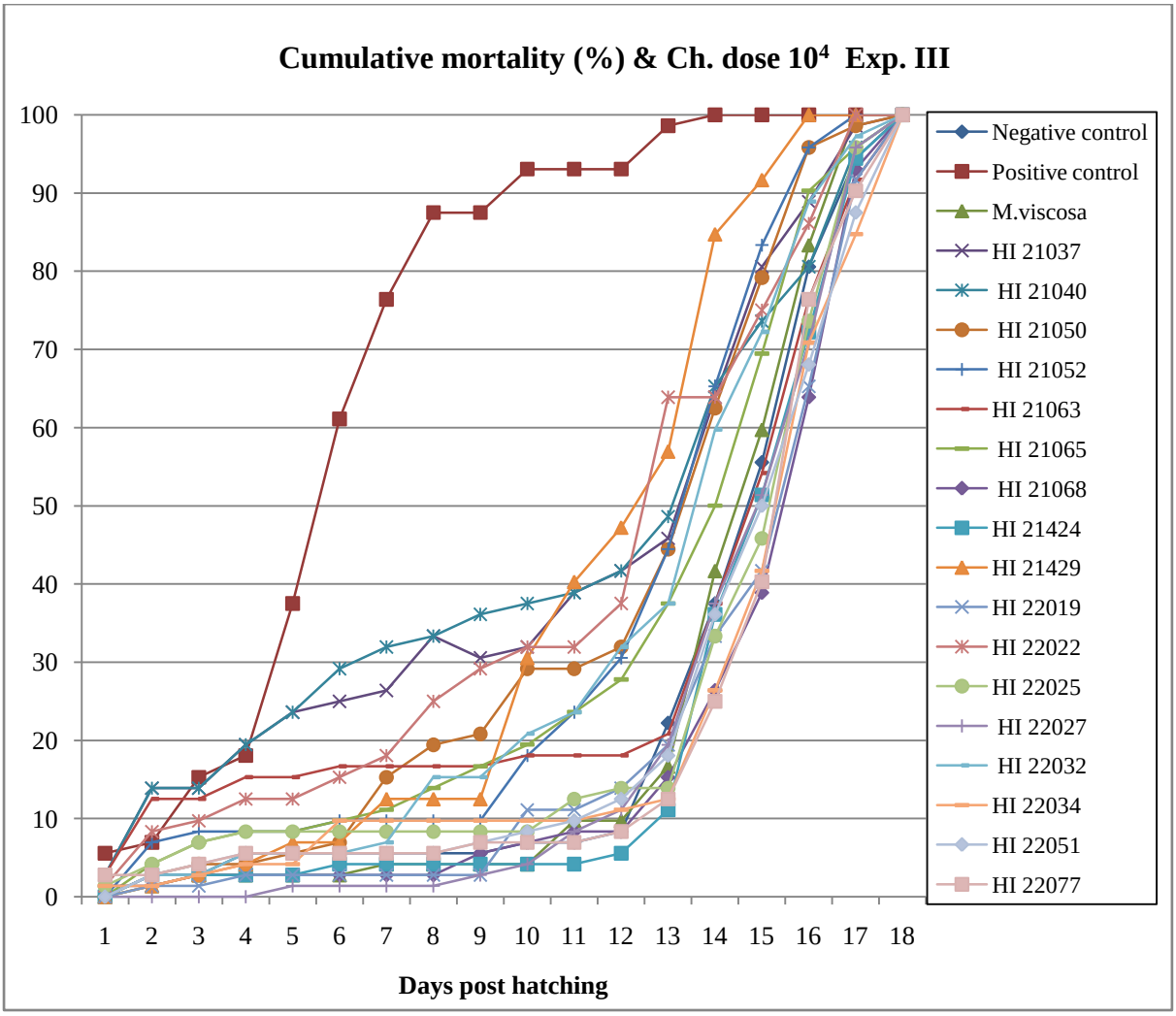

Fig. 6. *Gadus morha.* Cumulative mortality in percentage in Challenge experiment III, challenged dose 10^4. Negative control is larvae not challenged with bacteria. Positive control is the larval group challenged with *Vibrio anguillarum* strain 610. The other numbers refers to strain number.

Paper III

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Immunohistochemistry of Atlantic cod larvae *Gadus morhua* experimentally challenged with *Vibrio anguillarum*

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ABSTRACT

Farming of Atlantic cod *Gadus morhua* is one of the most rapidly growing sectors of Norwegian aquaculture. Classical vibriosis caused by *Vibrio anguillarum* is a problem in cod aquaculture. In order to prevent disease outbreaks, a thorough understanding of the infection route and the impact of the bacteria on the host is important. The intestinal tract, skin and gills have all been proposed as routes of entry for bacterial infection such as vibriosis. The aim of this study is to further develop our understanding of *V. anguillarum* serotype O2α infections in cod larvae, including possible route of entry, pattern of infection and histopathology. Cod eggs were transferred to a 24-well polystyrene multi-dish with 2 ml of sterile aerated 80% (28‰ salinity) seawater. Challenge doses were 10^4 and 10^6 CFU ml^{-1}. Unchallenged larvae were used as controls. Larvae for immunohistochemical examination were sampled from each group every day. In most of the larvae, wither no or very few bacteria were observed. Typical findings were clusters of bacteria in the space between the primary gill lamellae. None of these bacteria seemed to have adhered to the gills. Intestines of three out of 161 larvae examined showed positively immunostained bacteria in the intestine. Some of the bacteria appeared to be attached to the microvilli, but none were observed inside epithelial cells. Only two larvae from the low-challenge dose group showed clear signs of histopathology, which

was found in the intestine. It is not possible to draw any conclusions regarding the portal of entry.

KEY WORDS: *Vibrio anguillarum*, Cod larvae, Immunohistochemical studies, Challenge experiment

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INTRODUCTION

The Atlantic cod *Gadus morhua* is an economically important species on the Norwegian coast, and cod fisheries have long traditions (Svåsand et al. 2004). Declining wild cod populations, reduced catch quotas and thus rising prices have turned Atlantic cod farming into one of the most rapidly growing sectors of Norwegian aquaculture. A most important challenges to the creation of profitable cod farms has been the lack of large-scale fry production. It is not merely the lack of fry available, but also the instability of production and quality of the larvae produced that causes problems. The industry lacks a standardized production method, which may be one reason for the wide difference among larval groups (Svåsand et al. 2004). Infectious bacterial diseases have always been an important aspect in aquaculture. The high mortality of the larval stages is still a major bottleneck, partly because of infectious diseases (Vadstein 1997, Bricknell & Dalmo 2005). Compared to salmonids, Atlantic cod is poorly developed at hatching and undergo a long larval period before metamorphosis (Kjørsvik et al. 1991, Pedersen & Falk-Petersen 1992). During these stages

the fry are vulnerable to bacterial infections (Bricknell et al. 2006). Several important bacterial diseases affect Atlantic cod (reviewed by Samuelsen et al. 2006). Classical vibriosis, caused by *Vibrio anguillarum*, may be a problem for both adult fish and larvae. *V. anguillarum* and closely related bacterial species have been identified in many different marine habitats (reviewed by Thompson et al. 2004). This implies that the bacterium is native in these environments, and eradication of the disease is not possible. At least 23 O serotypes of *V. anguillarum* have been described (Pedersen et al. 1999). In salmonids, the main *V. anguillarum* serotypes that cause disease are O1 and O2α, while the serotypes O2α and O2β are most commonly associated with cod (Larsen et al. 1994, Pedersen et al. 1999). Both O2α and O2β have been isolated from diseased cod, but O2β seem to be the predominant serotype (Bricknell et al. 2006). A recent study published by Mikkelsen et al. (2007) indicates that some *V. anguillarum* isolates that cause disease in cod belong to a new sero-subtype because of their serological, biochemical and genotypical differences from other known serotypes.

Cod larvae have a fully competent immune system around 2 to 3 months after hatching (Schrøder et al. 1998). Hence, conventional vaccination prior to this stage will probably not have a positive effect.

Both exterior and internal surfaces like the skin, gills and intestinal tract have been proposed as entry portals for pathogenic bacteria (Baudin Laurencin & Germon 1987, Kanno et al. 1989, Smith et al. 2004). Studies involving intestinal mucus are frequently used to test growth, adherence and attachment of pathogenic bacteria (Garcia et al. 1997, Vine at al. 2004, Yan et al. 2007). The entry portal for *V. anguillarum* has been debated. The gut may be the initial site of infection in turbot larvae *Scophthalmus maximus* (Grisez et al. 1996), a proposition supported by experiments done by O'Toole et al. (1999) and Olsson et al. (1996).

V. anguillarum have been detected in cod larvae fed rotifers enriched with commercial products (Korsnes et al. 2006), and it is possible that live feed, such as rotifers and *Artemia* spp., may be vectors for opportunistic bacteria that infect cod larvae.

Spanggaard et al. (2000) found that the external surfaces (skin and fins) of rainbow trout were important sites for the attachment and proliferation of *V. anguillarum.* Bath challenge experiments on unfed cod yolk sac larvae have caused high mortalities (Sandlund & Bergh unpubl. data), suggesting that the skin is an important entry portal.

The gills have also been suggested as an entry portal for *V. anguillarum*. In an immersion challenge trial with rainbow trout, *Salmo gairdneri*, Baudin Laurencin & Germon (1987) suggested that the gills were the initial sites of infection. However, due to early observations of bacteria in the anterior and posterior intestine, they concluded that contamination by oral or anal routes was also a possibility. Inconclusive results were also found by Olsson et al. (1996). When they inoculated *V. anguillarum* directly on the gills of turbot they were unable to recover the pathogen from the spleen.

The aim of this study was to further develop our understanding of *V. anguillarum* serotype O2α infections in cod larvae by (1) using immunohistochemical methods to identify a possible portal entry route, (2) observing the pattern of infection and the tissues types infected, and (3) describing the histopathology.

MATERIALS AND METHODS

Broodstock, eggs and larvae. Cod *Gadhus morhua* eggs were provided by the commercial hatchery Sagafjord in Hordaland County, Western Norway (59° 45’ N, 5° 29’ E). The

broodstock originated from the area outside Bømlo/Halsnøy in the same county. The broodfish were kept in 40 m^3 tanks, each tank containing 60 to 80 fish that spawned naturally. The fertilized eggs were collected and disinfected with glutardialdehyde (300 ppm) for 10 minutes before incubating in black conical 150-liter tanks. The average temperature was between 6.5 to 7°C. A flow-through system provided 2 to 3 liters per minute of aerated water.

Bacterium. The bacterium used in this challenge experiment was *Vibrio anguillarum* strain HI-610 serotype O2α, originally isolated from cod suffering from vibriosis at the Parisvatnet research facility in Øygarden near Bergen, Hordaland county. This bacterium was previously serotyped according to Knappskog et al. (1993). Bacteria were stored at -80ºC in a 20% glycerol/marine broth (Difco 2216, Difco, Detroit, MI, USA) stock. They were incubated at 15º C and grown on petri dishes with Difco 2216 Marine Agar (MA) for 48 h. Colonies were transferred to Erlenmeyer flasks containing 50 ml of marine broth (MB) (Difco 2216) and shaken at 80 rpm in a shaking incubator (INFORS AG CH-4103 Bottmingen, Switzerland) for 48 h. 30 ml of each bacterial culture were harvested by centrifugation (Heraeus Sepatech Megafuge 1.0 R) at 2772 x *g* for 10 min at 4ºC, washed twice in 30 ml phosphate-buffered saline (PBS) and resuspended in 30 ml PBS. The cell concentration was determined by counting, using a Hawksley counting chamber. The bacterial suspension was examined in a microscope in order to verify that the bacterium were still viable prior to challenge.

Challenge experiment. Eggs were randomly selected, and individual eggs were transferred to separate wells in a 24-well polystyrene multi-dish (Nunc, Roskilde, Denmark) containing 2 ml of sterile aerated 80% (28‰ salinity) seawater. They were challenged with *Vibrio anguillarum* strain HI-610 on the same day by adding 100 µl of bacterial suspension to each well. All eggs hatched successfully after 48 hours. This experiment comprised three groups of larvae. The control group consisted of unchallenged larvae, groups I and II were challenged

with 10^4 colony-forming units (CFU) ml^{-1} and 10^6 CFU ml^{-1}, respectively. Three plates were used for each group, giving a total number of 72 larvae in each treatment group. Dead larvae were counted daily. To provide material for the immunohistological examinations, three extra plates with larvae for all three groups were prepared. The first samples were taken on day four after challenge and every following day until the experiment was terminated. Day 16 after hatching was the last sampling day in group I and the control group. Because all the larvae needed to be sampled alive, no samples were taken from group II after day 14 due to the high mortality in this group. Between two and five larvae were sampled from each group every day, and a total of 161 larvae were analyzed in this study. The experiment lasted 21 days.

Antisera. All antisera against *Vibrio anguillarum* HI 610 were produced according to the method of Oeding (1957) and absorbed by the method described by Knappskog et al. (1993). Due to availability limitations, two different batches of antiserum were used to perform the immunohistochemical analyses. The antisera were absorbed against serotype O2β strain HI-618 and serotype O1 strain HI-644, and tested for cross-reaction.

Immunohistochemistry. Larval samples were fixed in 4% phosphate-buffered formaldehyde, dehydrated in ethanol and embedded in paraffin. All the larvae were sectioned at 3 µm (Leica Jung Biocut 2035, Nussloch , Germany), incubated at 60°C for 30 min, dewaxed in xylene (Chemi-Teknik AS, Oslo, Norway), rehydrated in a series of ethanol baths and washed in running water. The absorbed polyclonal rabbit antiserum anti-HI-610 was diluted in Tris-hydroxymethyl-aminomethane (TRIS) – buffered saline (TBS) with 2.5% bovine serum albumin (BSA). In order to prevent non-specific antibody binding, sections were blocked by using 5% BSA in TRIS-buffered formaldehyde for 20 min. Avidine-biotin-alkaline phosphatase complex (ABComplex/AP) reaction kit (DAKO A/S, Denmark) and New Fuchsin Substrate system (Dako) were used to stain *Vibrio anguillarum*. A positive staining

was evident as red coloration. Shandon's hematoxylin was used for counterstaining, which gave the tissue a blue coloration. At each stage of staining, two controls were used. Unchallenged larvae were used as a negative control, and bacterial smears on microscope slides were used as a positive control. The same procedure was used to stain both larvae and bacteria. During the staining procedures, tissue sections and bacterial samples were kept apart to prevent cross-contamination. All incubations were performed at room temperature (20°C) in a humidity chamber. A Leica DMBE microscope equipped with a Leica Wild MPS52 phototube was used to photograph the tissue sections.

Hematoxylin-Erythrosine-Saffron (HES) staining. Heated, dewaxed and rehydrated larval sections were stained with hematoxylin, 1% erythrosine and saffron. These were added through a series of baths in order to stain nuclei, muscle and cytoplasm and connective tissue. This staining was performed in order to identify histopathology in the larvae.

Statistical analysis of mortality rates. The mortality data were not normally distributed and a non-parametric test was performed. A 2 x 2 contingency table analysis was performed in Statistica v 7.0 (StatSoft, Tulsa, USA) using a Bonferroni corrected p-value ($p < 0.00094$) for multiple independent tests. This was used to test for mortality differences among the treated groups and the control group. Multiple independent tests were used to test differences in mortality rates between the challenged larval groups and the larval control groups, and a Bonferroni correction was applied (to minimize the possibility of type II error) (Rice 1989). Because there was only one degree of freedom, Yates correction was applied.

This experiment formed part of a larger challenge experiment. The statistical analyses of mortality rates will therefore be published in a separate article by Sandlund & Bergh (in press). This article contains only data regarding the control and larval groups exposed to *Vibrio anguillarum* strain HI-610.

RESULTS

Cumulative mortality and statistical analysis. Mortality rates in the control group and group I were less than 0.15 during the first 12 days after hatching. At day 13, there was a rapid rise in group II mortality (Fig. 1). Cumulative mortality in this group increased between days 2 and 6 post hatching, stabilizing at approximately 0.6 for several days before increasing towards the end of the experiment (Fig. 1). The mortality rates of the control group and group II were significantly different ($p < 0.00094$, Bonferonni correction, data not shown), but rates in group I and the control group were not. Feed was not provided during this experiment; the rise in mortality at the end of the experiment was thus due to starvation.

Immunohistochemistry. The immunohistochemical studies provided no clear explanations for the portal entry route or for the pathology causing the high mortality. In most of the larvae studied, there were very few or no bacteria. Typical findings were clusters of bacteria in the space between the primary gill lamellae, but none seemed to have adhered to the gills. However, these clusters were only partially stained or not positively stained at all (Figs. 2 & 3). Two group II larvae (Figs. 4 & 5) and one group I larva (Figs. 6 & 7) contained stained bacteria in their intestines. Some of the bacteria appear attached to the microvilli, but although interstitial immunostaining was performed, no bacteria were observed inside the cells (Figs. 4 - 6). One group II larva had more bacteria in the intestine than any other larvae (Fig. 4). Except for the two group II larvae (Figs. 4 & 5), no histopathology was observed in any of the larval groups. Necrotic cells with some pycnotic nuclei (Fig. 5, arrow) and dissolved epithelial cell nuclear membranes occurred in the intestine. Note the rounding and dissociation of the epithelial cells in the brush border (Fig. 5, arrow). Three control group larvae had some bacteria in the space between the primary gill lamellas, but these were not specifically stained (data not shown).

DISCUSSION

To further understanding of *Vibrio anguillarum* infections in cod larvae *Gadus morhua* we investigated a possible entry portal, the pattern of infection, tissue types infected, and we described the histopathology. The results were inconclusive. In spite of the high mortality observed in the high challenge-dose group, little histopathology was observed in the course of the immunohistochemical examinations. The absence of histopathology also suggests that the disease develops acutely and that the larvae died before any pathological alterations became apparent.

Toxic components associated with and released from metabolized bacterial cells may cause disease. Supernatant from *Vibrio anguillarum* led to mortality in turbot *Scophthalmus maximus* larvae (Planas et al. 2005, 2006). Supernatants of bacterial cultures include extracellular products (ECP) produced by the bacteria. An experimental bath challenge (with typical *Aeromonas salmonicida* ssp. *Salmonicida*) to turbot and halibut yolk-sac larvae resulted in significant mortality (Bergh et al. 1997). Histological and immunohistochemical examinations of the larvae revealed no evidence of bacteria in affected tissues. Bergh et al. (1997) suggest that the mortality was a result of the production of toxic exudates by the bacteria, which may also explain our results with cod larvae. Supporting evidence is provided by Sandlund et al. (2006), who challenged great scallop *Pecten maximus* larvae with *Vibrio pectenicida*, but in this case there was histopathology in tissue samples. Furthermore, live and heat-killed *V. anguillarum* were equally lethal when injected into goldfish *Carassus auratus* (reviewed by Egidius 1987).

The immunohistochemical examinations revealed clusters of bacteria around the larval gills (Figs. 2 & 3). These cells appeared smaller than the bacteria found in the intestine. It has repeatedly been observed in our laboratory that this particular *Vibrio anguillarum* strain

changes its size and appearance when it is grown and transferred from an *in vitro* to an *in vivo* environment. The polyclonal antiserum used in this experiment attaches to the surface lipopolysaccharide (LPS) of the bacteria. When bacterial clusters are extremely compact, the form of the LPS may change or become unavailable to the antiserum, preventing positive staining. The bacteria may release LPS when entering the intestine and thus appear larger. However, in view of the near total absence of positive immunostaining of the gill-associated bacteria in all the larvae we examined, and the fact that fish larvae are not sterile (Verner-Jeffreys et al. 2003), it is likely that other bacteria are present in the larvae.

Suffocation caused by the bacteria that we observed around the gill lamellae is another possible explanation of larval death without apparent presence of bacteria. However, oxygen exchange through the body surface skin occur in larvae and juveniles of Atlantic salmon *Salmo salar* (Wells & Pinder 1996 a, b) and rainbow trout *Oncorhynchus mykiss* (Rombough 1998). It is likely that cod larvae are similarly capable of exchanging oxygen across the skin. Thus, bacteria clustered around the gills of cod larvae likely did not cause a lack of oxygen or influence mortality. In similar challenges to halibut larvae with *Vibrio fischeri*, large numbers of bacteria were observed between the gill arches, and there was necrosis in the gill epithelium (Bergh et al. 1992). None of the larvae examined in this experiment showed damaged gill tissue, supporting the suggestion that the bacteria observed in the gill area did not have any negative effects on the larvae. Moreover, clusters of bacteria also occurred in larval Group I and the control group, in which no bacteriogenic mortality occurred.

Bacterial epiflora may create lethal or sublethal conditions in the environment through excessive oxygen consumption (Hansen & Olafsen 1989) that affects fish eggs and larvae.

However, all of the eggs in this experiment hatched successfully within a period of 12 hours, indicating that they were healthy and unaffected by adverse environmental influences. Furthermore, all the eggs, and thus all the larvae, originated from the same egg bath and were randomly selected. If potentially unfavorable bacteria or other factors were present in the environment, all larval groups should have been affected in the same way.

As mentioned above, bacteria have three potential points of entry into the larvae: skin, gills and intestine. The mucosa serves as a substrate for bacterial adhesion in fish (Hansen & Olafsen 1989, Vine et al. 2004). *Vibrio anguillarum* may have a stronger tendency to adhere to intestinal mucosa than skin mucosa (O'Toole et al. 1999, Olsson et al. 1996). Conversely, other papers suggest that the skin is a major site of attachment and proliferation of *V. anguillarum* in ayu *Plecoglossus altivelis* and rainbow trout (Kanno et al. 1989, Spanggaard et al. 2000, respectively). Baudin Laurencin & Germon (1987) propose that the gills are the initial infection site in rainbow trout *Salmo gairdneri*. In their experiment, they inspected gills, anterior and posterior intestine and anterior kidney, but did not take skin samples. No cod larvae in the present challenge experiment had bacteria associated with the skin.

In our laboratories *Vibrio anguillarum* strain HI-610 is commonly used in challenge experiments, and a bath challenge dose of 10^4 CFU ml^{-1} usually causes high mortality (Sandlund & Bergh in press). However, in this present work, only the high challenge-dose group (10^6 CFU ml^{-1}) suffered significantly higher mortality than the control group. There are no obvious explanations of this result. The washing procedure prior to challenge may have stressed the bacteria, resulting in a temporary loss of virulence (Planas et al. 2005). Chemotactic motility mediated by the polar flagellum is essential for the virulence of *V. anguillarum* (Milton et al. 1996, O'Toole et al. 1996, Ormonde et al. 2000). However, examinations of the bacterial suspension prior to the challenge revealed no lack of motility.

The washing procedure used is a standardized procedure in our laboratory. In a similar challenge experiment with *V. anguillarum* and Atlantic halibut *Hippoglossus hippoglossus* yolk-sac larvae, temperature was suggested as an affective factor (Verner-Jeffreys et al. 2003). The authors suggested that low temperature (6°C) could cause the bacteria to multiply slowly inside the larvae, thus lowering fish mortality rates. This was also implied by Larsen (1984). However, cod larvae in this present study were reared at 7°C, the temperature used to grow the bacteria prior to challenge. We observed little difference in the growth rates at 7°C and 15°C for this *V. anguillarum* strain. Given our observations of motile bacteria prior to challenge and the fact that high mortality occurred in group II, we find it unlikely that the washing procedure or temperature are contributed significantly to the absence of mortality seen in the low challenge-dose group.

The decision to keep the larvae alive until the point of starvation, was due to the possibility of losing significant data. Thanks to the knowledge obtained in this experiment, future experiments done under similar conditions can be brought to an end at an earlier stage, i.e. until day 12 to 13 post hatching.

In order to obtain a correct impression of the histopathology and to avoid post mortem changes, all the larvae were sampled live. Sampling when larvae are moribund would be optimal, but practical considerations made it impossible to monitor the larvae so closely. It is probable that most of the larva in this experiment were sampled before any clear histopathology was present. This might explain the low correspondence between the histological findings and the mortality observed in the experiment.

Marine fish larvae drink water from day one post hatch (Mangor-Jensen & Adoff 1987) and will thereby ingest bacteria. Fish larvae have an undeveloped stomach at hatching (Kjørsvik et

al. 1991). The onset of the acid digestion is a gradual process and the lowering of the pH takes 2 to 3 months in sea bream *Sparus aurata* larvae (Yúfera et al. 2004). It is likely that the larval intestinal pH in this experiment was similar to that of seawater, which is not low enough to inhibit bacterial growth.

Apart from drinking water, infection of feed is another way of ensuring presence of bacteria in the intestine. To describe the infection route of *Vibrio anguillarum*, Grisez et al. (1996) orally fed infected *Artemia* sp. nauplii to turbot larvae. The bacteria were taken up endosomally from the brush border of the epithelium. They were transported to the lamina propria and onwards to the bloodstream and other organs. These findings contrast with our observations. The fact that we saw bacteria seemingly attached to the microvilli does not necessarily mean that they enter the larvae by this route. Olafsen & Hansen (1992) showed endocytosis of bacteria by epithelial cells in the hindgut of 4 to 6 day-old cod larvae and 10 to 12 day-old herring *Clupea harengus* larvae. They also found intact bacterial antigens in columnar epithelial cells in the foregut of 4 day-old cod yolk-sac larvae. It is possible that the findings of Grisez et al. (1996) detected merely the endocytosis of dissolved LPS rather than whole bacteria, since most antisera are designed to react on the LPS of bacteria. In our opinion, this does not provide conclusive evidence of the infection route in turbot.

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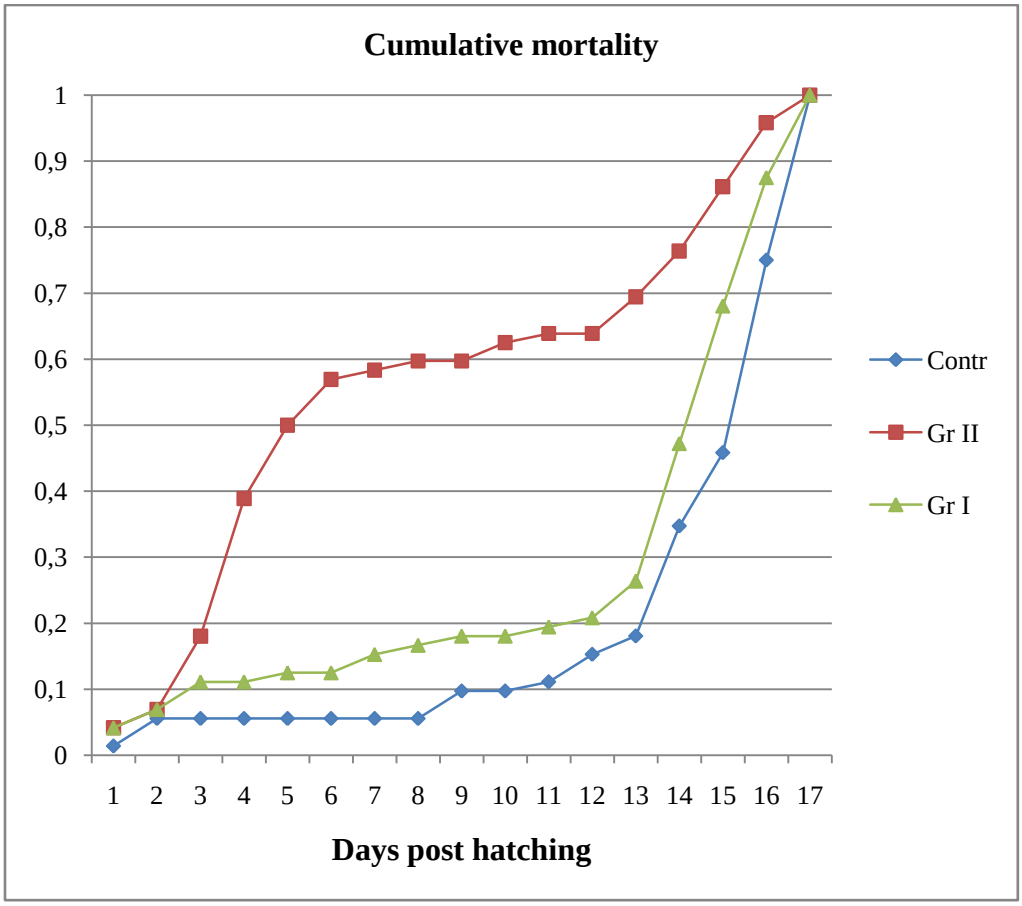
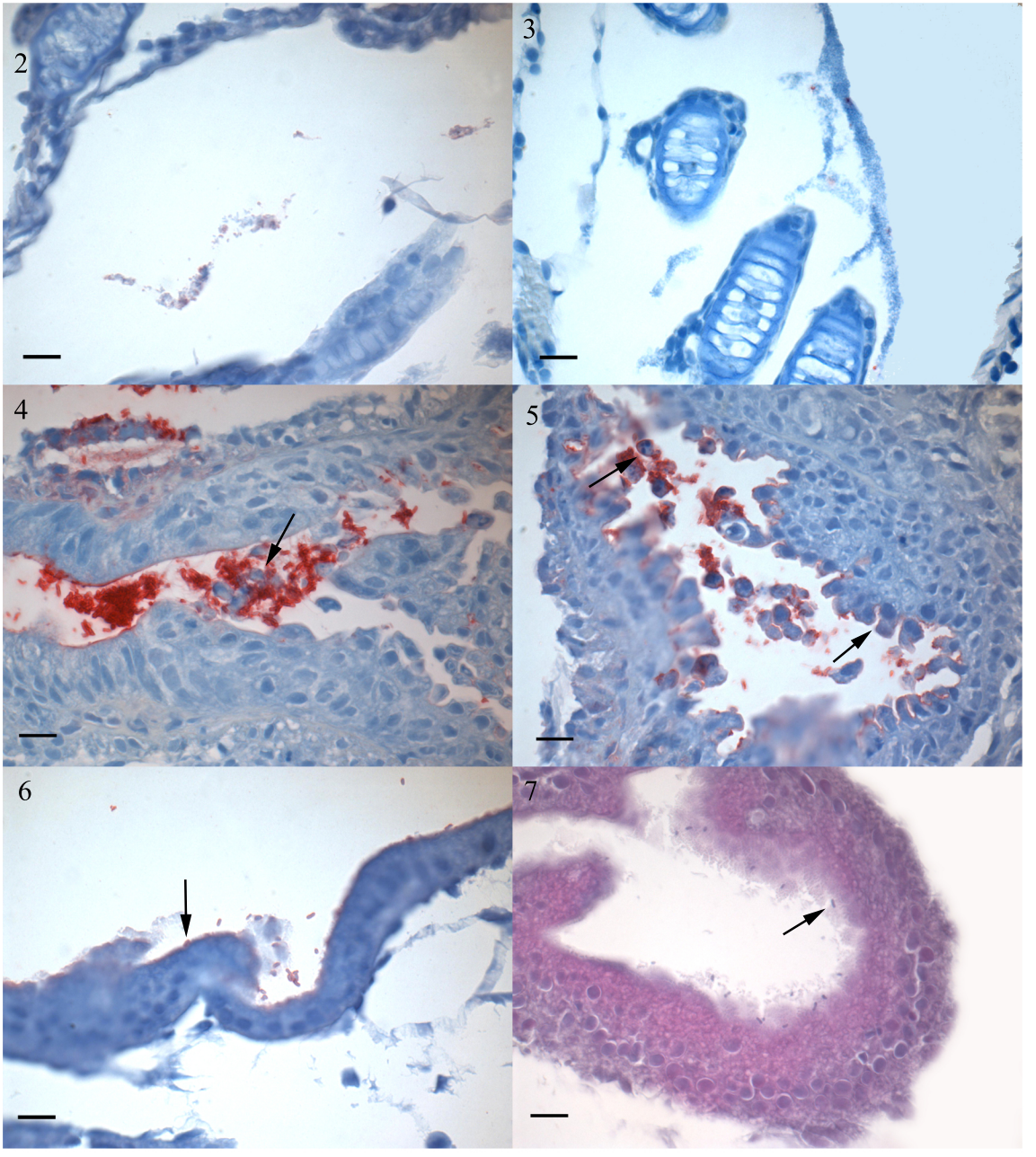


Fig. 1. *Gadus morhua*. Cumulative mortality for three larval groups. Contr: Control group containing larvae not exposed to bacteria. Gr I: Larval group I challenged with *Vibrio anguillarum* strain HI-610 dose 10^4 CFU ml^{-1}, Gr II: Larval group II challenged with *V. anguillarum* strain HI-610 dose 10^6 CFU ml^{-1}.

Figs. 2-7. *Gadus morhua*. Figs. 2-6: Immunohistochemical staining of larvae (paraffin sections). Avidine-biotin-alkaline phosphatase method, rabbit anti *Vibrio anguillarum* strain HI-610 serum and Shandon haematoxylin counterstained. Postive immunohistochemistry is visualized by red colour. Counterstaining gives tissue different tones of blue. Fig. 2. Gills of larvae from group I eight days post hatching. Bacteria are present in the space between the primary lamellae, some are clearly positively stained red while others show no positive colour. Fig. 3. Gills of group II larvae 15 days post hatching. Many bacteria are present, but little positive staining. Fig. 4. Intestine of group II larvae four days post hatching. Large numbers of bacteria are present. Necrotic cells are visible among the bacteria (arrow). Fig. 5. Intestine of group II larvae four days post hatching. Large numbers of bacteria are present. Necrotic cells are visible among the bacteria (arrow). Fig. 6. Intestine of group I larvae four days post hatching. Bacteria are present in the lumen and attached to the brush border of the intestine (arrow), but no necrotic cells are visable. Fig. 7. Intestine of same larvae as in Fig. 4 (group I four days post hatching) stained with hematoxyline-erythrosine-saffron. Bacteria are present (stained violet) in the brush border (arrow), but there is no necrosis. All Scale bars = 10 µm.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Paper IV

Sandlund N, Brunvold L, Patel S, Espedal PG, Olsen RH, Otterlei E, Sandlund L, Bergh Ø (Submitted) Immunohistochemical examinations of Atlantic cod larvae, *Gadus morhua*, exposed to *Vibrio* spp. through bioencapsulation in live feed. Aquaculture

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Correction: The cited article by P. Oeding was published in 1957, not in 1959

Immunohistochemical examinations of cod larvae, *Gadus morhua*, exposed to *Vibrio* spp. through bioencapsulation in live feed

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Abstract

Problems with rearing of cod larvae have been suggested to be related to opportunistic pathogenic bacteria, as the high densities of fish larvae and the live feed cultures may provide a source for opportunistic bacteria. The purpose of this experiment was to further investigate whether oral administration of the two bacteria *Vibrio anguillarum* and *Vibrio logei* through live feed can cause an outbreak of disease in cod larvae. Approximately 5000 eggs were transferred to six identical tanks. For oral administration *V. anguillarum* strain HI-610 and *V. logei* were bioencapsulated into rotifers and fed to the larvae. Two tanks were used for each treatment and two tanks served as controls. Samples for immunohistochemical analysis were taken every day. No mortality that could be related to the exposure to bacteria was observed in any of the challenged groups. Immunohistochemical analysis showed presence of *V. anguillarum* and *V. logei* inside the intestinal lumen, but no histopathology was observed. However, in one of the control tanks a mass mortality started on day 17 post-hatching, resulting in 99% mortality by day 21. Isolation of bacteria and immunohistochemical analysis demonstrated the presence of *Vibrio splendidus* in large quantities. Necrotic tissue inside lumen and gills were found. Denaturing gradient gel electrophoresis (DGGE) was employed as an additional method to investigate the outbreak. The DGGE results of bacteria associated with water samples from the two control tanks revealed different DGGE profiles. Although the DGGE results could not confirm presence of *V. splendidus,* a band in the same position as the *V. splendidus* marker was found in larval samples taken from the diseased tank.

Keywords: Cod, *Vibrio anguillarum*, *Vibrio logei*, *Vibrio splendidus*, bioencapsulation

1. Introduction

Over the past few years, cod, *Gadus morhua*, farming has been a growing industry in Norway, Scotland and Canada. The fall in the supply of wild cod has enabled cod farming to expand. Although a bright future had been predicted, problems with the larval stages have made it difficult to maintain stable production of juvenile cod. High rates of mortality are often experienced in hatcheries, especially around days 30 to 35 and 60 to 70 post-hatching (E. Otterlei, unpublished data). Abdominal swelling is often seen and larvae are found floating on the surface. Bacterial infections in these larvae have been hypothesized, but to date, no evidence of such infections has been published. Similar observations had been reported in farming of gilt-head sea bream, *Sparus aurata*, larvae (Sedano et al., 1996). Pathogenic bacteria may be present in the live feed cultures, such as rotifers and *Artemia* spp., and rearing systems for marine fish larvae (Brunvold et al., 2007, Korsnes et al., 2006, Mizuki et al., 2006, Sugita et al., 2005). Marine fish larvae are known to drink water (Olafsen and Hansen, 1998, Reitan et al., 1998, Mangor-Jensen and Adoff, 1987), allowing for colonization of the intestine even before active feeding commences (reviewed by Hansen and Olafsen, 1999). Changes in the bacterial flora associated with larvae typically occur at onset of feeding and with change of diet (Brunvold et al., 2007, Verner-Jeffreys et al., 2003). Live feed cultures as a source of opportunistic bacteria are an obvious assumption, as rotifers and *Artemia* spp. are believed to lay a heavy bacterial load on the larvae (Reitan et al., 1998). Both rotifers and *Artemia* spp. are filter-feeding organisms capable of consuming bacteria. Live feed production is a dynamic process, which makes it difficult to maintain a stable microflora. In such a changeable environment,

opportunistic bacteria may flourish, as pulses of organic matter may favour the growth of fast-growing, opportunistic bacteria (Skjermo and Vadstein, 1993). This leads to the suggestion that pathogenic and opportunistic bacteria play part in the high mortality in early life stages (Vadstein et al., 2004, Samuelsen et al., 2006).

Like most fish species, cod hatch at an ontogenetically relatively primitive stage. The larvae do not possess a fully competent immune system until 2-3 months after hatching (Schrøder et al., 1998), thus during this period the larvae are probably highly susceptible to opportunistic bacteria.

Vibrio logei has frequently been isolated from moribund and dead larvae and has therefore assumed by fish farmers to play a significant role in the bacterial problems encountered in hatcheries. However, in bath challenge experiments, various *V. logei* isolates have not produced mortality in cod yolk-sac larvae (Sandlund and Bergh, in press). This leads to the hypothesis that oral administration of *V. logei* is necessary to cause disease. Bioencapsulation of bacteria in live feed such as rotifers and *Artemia* nauplii (Makridis et al., 2000a, 2000b), and subsequent challenge of marine fish larvae via rotifers, has been successful (Planas et al., 2005). *Vibrio anguillarum* is a known pathogen that produces high rates of mortality in cod larvae (Sandlund and Bergh, in press) and cod fry (Samuelsen and Bergh 2004, Seljestokken et al., 2006, Vik-Mo et al., 2005) when administered via bath challenge. Experiments performed by Planas et al. (2005) and Grisez et al. (1996) showed *V. anguillarum* to be pathogenic to turbot, *Scophthalmus maximus*, larvae when administrated through live feed. The bacterium has been isolated from cod larvae fed rotifers enriched with commercial rotifer enrichment products (Korsnes et al., 2006).

The purpose of this study was to investigate infections, following oral administration of suspected pathogenic *Vibrio* spp. through rotifers. Uptake and processing were studied by means of immunohistochemistry, while changes in the bacterial flora associated with the cod larvae and water in the larval tanks were examined by PCR-amplified 16S rDNA and subsequent denaturing gradient gel electrophoresis (DGGE) analysis.

2. Materials and methods

2.1. Broodstock, eggs and larvae

Eggs were collected at the Sagafjord commercial cod hatchery, in the county of Hordaland, Norway (59° 45’ N, 5° 29’ E). The broodstock originated from the Bømlo and Halsnøy area in the same county.

All eggs were taken from the same group. They were disinfected immediately after fertilization in glutardialdehyde 300 ppm for 10 min, and kept in black conical 150 l tanks at 6.5 – 7.0°C. The eggs were transported from the hatchery to the Institute of Marine Research in boxes filled with ice, with the eggs stored in plastic bags. The journey (by car and ferry) took about two to three hours. At the Institute approximately 5000 randomly chosen eggs were placed in six identical 160 l tanks. The eggs were held at 34.8 ppt salinity seawater until hatching, three days after arrival. The eggshells were removed and the larvae kept in the same tanks. Temperature and oxygen were monitored daily. The temperature was 9.5 °C ± 0.4 °C throughout the experiment. The oxygen concentration levels were stable around 8.6

mg^{-l} ± 0.1 mg^{-l} (ppm) (OxyGuard® Handy MK III, Sterner AquaTech a.s., Langhus, Norway). The flow in the tanks was 0.85 l min^{-1}. The outlet pipes in each tank were covered with a 250 µm nylon mesh filter (Sefar Nytal, Heiden, Switzerland), to prevent larvae from escaping. The filters were aerated in order to prevent blocking the outlets.

2.2. Bacterial strains and challenge

The bacterial strains used were *Vibrio anguillarum* strain HI-610 serotype O2α and *Vibrio logei* HI 21039, Institute of Marine Research. *V. anguillarum* strain HI-610 were originally isolated form diseased cod at the Parisvatnet research facility and the *V. logei* strain was originally isolated from diseased cod larvae. Tanks 1 and 6 were control tanks, tanks 2 and 3 contained larvae challenged with *V. anguillarum,* while tanks 4 and 5 contained larvae challenged with *Vibrio logei*. Both bacteria were stored at –80 ºC in a 20% glycerol/marine broth (MB, Difco 2216, Difco, Detroit, MI, USA) stock. They were incubated at 15 ºC and grown on petri dishes with Difco 2216 marine agar (MA) for 48 h. Colonies of the bacteria were transferred to Erlenmeyer flasks with 250 ml of MB and shaken at 80 rpm in a shaking incubator (INFORS AG, CH-4103 Bottmingen, Switzerland) for 48 h at 9 °C. The same temperature was used both to grow the bacterial cultures and as the water temperature in the tanks during the challenge experiment. The bacterial cultures, 250 ml, were harvested by centrifugation (Sorvall RC-5B, Minnesota USA) at 4068 G for 10 min at 4 ºC, washed twice in 250 ml phosphate-buffered saline (PBS) and resuspended in 250 ml PBS.

2.3. Rotifer culture and bioencapsulation of bacteria

The rotifers (*Brachionus* sp.) were held in 70 l tanks at 30 ppt salinity, 24.5 °C ± 0.5 °C and cultured on Instant Algae® *Nannochloropsis* sp. (Reed Mariculture, Campbell, CA, USA) and Instant Algae® *Tetraselmis* sp. (Reed Mariculture) 10:1. Prior to feeding, the rotifers were enriched with *Pavlova* sp. (Reed Mariculture) every day throughout the whole experiment. The amount of algae used for feeding and enriching the rotifers depended on the daily growth and production of the rotifer cultures. It was ensured that the rotifers had access to food at all times. This was done visually by keeping the water coloured by the added algae. Rotifer production was expanded throughout the experiment as the larvae grew and the need for feed increased. The first day of feeding was on day 4 post-hatching. Every morning the rotifers needed for one day's feeding were harvested (60µm nylon mesh, Sefar Nytal, Heiden, Switzerland), washed in sea water and placed in a 45 l tank enriched with *Pavlova* sp. Larvae were fed four times a day at four-hour intervals. The first feeding of the day was given at 07:30 am and the last at 07:30 pm. All the larval tanks were fed at the same time with the same quantity of rotifers. The quantity of rotifers remaining in each tank at the end of each feeding was approximately 3000 rotifers ml^{-1}. The light in the lab went on at 07:00 am and off at 10:00 pm. The rotifer enrichment tank was emptied, washed and sterilized every morning before new rotifers were added.

Prior to the bioencapsulation of bacteria, the rotifers (200-250 rotifers ml^{-1}) were filtered (60µm nylon mesh, Sefar Nytal) washed, and transferred into buckets containing the bacterial suspension and seawater. The cell concentration was determined by counting, using a Hawksley counting chamber. Final concentration in buckets was 1.0-3.0 x 10^8 Colony Forming Units (CFU) ml^{-1}. Total volume in the buckets was 5 l. The rotifers were maintained in the bacterial suspension for 1 hour,

filtered (60μm nylon mesh), washed and fed to the cod larvae. Makridis et al. (2000b) showed that rotifers were much more efficient taking up bacteria when they grazed on a bacterial suspension containing 10^8 CFU ml^{-1} than one containing 10^7 CFU ml ml^{-1}. On average, the rotifers grazing on 1.8-3.5 x 10^8 CFU ml^{-1} contained 3.2-7.1 x 10^4 CFU ml^{-1} after 20-60 minutes of feeding.

The larvae were challenged a second time on day 15 post-hatch. The same procedures as described above were employed.

2.4. Bacterial sampling

Water samples were taken throughout the experiment in order to estimate numbers of bacteria, CFU, in each tank. Water samples were diluted in sterile seawater and plated on marine agar plates (MA, Difco 2216) and two parallel assays were run. The colonies were counted after 96 hours. To test for haemolysis, colonies were grown on blood agar (nutrient blood agar (Oxoid) supplemented with 5% sheep blood and 1.5% NaCl).

Live larvae were sampled at day 8, 20 and 22 post hatch from the tanks and sterilized with 0.1% benzalkonium chloride for 1 minute (Munro et al., 1994) and homogenized in 1 ml sterile sea water (Mixer mill 300, Retsch, Qiagen, Hilden, Germany) using Tungsten carbide beads (3mm, Qiagen), diluted in sterile seawater and plated on MA plates in duplicate. The colonies were counted after 96 hours. Colonies were then harvested and grown in marine broth (MB, Difco 2216) for 48 hours, and frozen at -80 °C in a 20% glycerol/MB stock.

2.5. Gram staining

Small amounts of bacterial culture together with a drop of distilled water were transferred to slides using an inoculation loop. The slides were air dried prior to heat fixing over open flame. The slides were cooled and stained according to the procedure listed in the Gram staining set (Diagnostica MERCK, Darmstadt, Germany).

2.6. Oxidase test

The bacterial strains for cytochrome oxidase were tested using the Bactident® Oxidase kit (MERCK), according to the manufacturer's manual.

2.7. DNA isolation Genomic bacterial DNA was isolated from 1 ml of a liquid culture harvested at the end of the exponential growth phase, using the purification kit DNeasy® 96 tissue kit (Qiagen). The protocol for gram-negative bacteria was used.

2.8. Identification of bacteria

2.8.1. PCR (Polymerase Chain Reaction) amplification of 16S rRNA genes

Universal primers 27f and 1492r (*Escherichia coli* numbering), were used for 16S rDNA analyses. The mix contained 2.0 μl PCR buffer (10x), 1.2 μl $MgCl_2$ (25 mM), 3.2 μl dNTP (1.25 mM/each, Promega, Madison, Wisconsin), 1.0 μl 27f Forward primer (10 μm), 1.0 μl 1492r Reverse primer (10 μm), 0.2 μl Taq polymerase (5 U/μl, Promega) 7.4 μl nuclease-free water (Eppendorf, Hamburg, Germany) and 4 μl template (approx. 50 ng/μl). The amplification was performed in an automated thermal cycler (Perkin Elmer, Gene Amp, PCR system 9700) and the cycles were as follows: initial denaturation at 95 °C for 5 min, then 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 11 min. All PCR reactions were performed in 50μl reaction tubes.

225

226 *2.8.2. Polymerase Chain Reaction (PCR) amplification of gyrB gene*

227 Primers used for amplification of the *gyrB* gene were gyrB-1 (forward) and gyrB-2

228 (reverse) (Yamamoto and Harayama, 1995). The mix contained 2.0 µl PCR buffer

229 (10x), 1.5 µl $MgCl_2$ (25 mM), 4.0 µl dNTP (1.25 mM/each, Promega), 1.0 µl gyrB-1

230 primer (10 µM), 1.0 µl gyrB-2 primer (10 µM), 0.5 µl Taq polymerase (5 U/µl)

231 (Promega), 6 µl nuclease-free water (Eppendorf) and 4 µl template (approx. 50 ng/µl).

232 The amplification cycle was as follows: initial denaturation at 95 °C for 5 min, then

233 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension

234 at 72 °C for 2 min and extension/hold for 7 min. The amplification was performed in

235 an automated thermal cycler (Perkin Elmer, Gene Amp, PCR system 9700).

236

237 *2.8.3. Sequencing of 16S rRNA and gyrB genes*

238 All PCR products were prepared for sequencing using a Pre-Sequencing Kit (USB

239 Corporation, Cleveland, Ohio). One cycle of 37 °C for 15 minutes and another 15

240 minutes at 80 °C were run. Primers used for sequencing 16S rRNA genes were the

241 same as for the PCR amplification described in the paragraph above. The pre-

242 sequencing mix contained, 1µl Big Dye mix(2.5x) (Big Dye version 3.1, Applied

243 Biosystems, Foster City, California), 1.5 µl sequence buffer (5.0x), 2.0 µl primer

244 (10µM) and 2.5 µl RNAse-free water (Eppendorf) and 3 µl of template were used.

245 The amplification cycles were as follows: initial denaturation at 96 °C for one minute

246 then 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and

247 extension at 60 °C for 4 min.

248

Primers used for sequencing *gyrB* genes were different from those used for PCR amplification, gyrB-1s (forward) and gyrB-2s (reverse) (Yamamoto and Harayama, 1995). The same pre-sequencing mix and amplification cycles as used for 16S rRNA genes were used to sequence the *gyrB* genes. The amplification was performed in an automated thermal cycler (Perkin Elmer, Gene Amp, PCR system 9700). The sequence analysis was performed by the sequence laboratory at the University of Bergen, using an ABI 3700 sequencing analyser (Applied Biosystems). The 16S rRNA and *gyrB* encoding gene sequences were searched for nucleotide-nucleotide matches in the BLAST database at the National Center for Biotechnology Information (NCBI) to establish tentative strain identity (Altschul et al., 1990).

2.9. Antisera

Antisera were made for *Vibrio anguillarum* strain HI-610 (anti-*V. anguillarum*), *Vibrio logei* strain HI 21039 (anti-*V. logei*) and *Vibrio splendidus* (anti- *V. splendidus*) according to the method described by Oeding (1959). Formaldehyde-killed washed bacteria were administrated by intravenous injection to the rabbits. The polyclonal antisera were absorbed as described by Knappskog et al. (1993), in order to minimize the possibility of cross-reaction. The anti-*V. anguillarum* serum was absorbed against *V. anguillarum* serotype O2β strain HI-618 and serotype O1 strain HI-644. The anti-*V. logei* serum was absorbed against *V. anguillarum* strain HI-610. Each of the absorbed antisera was tested for cross-reaction with bacterial strains and tissue samples prior to the immunohistochemistry. The anti-*V. splendidus* antiserum had been made prior to a different experiment (Sandlund et al., 2006) and was absorbed against *Vibrio pectenicida* and *Pseudoalteromonas* sp. The dilution used on

tissue samples was determined after a range of antiserum dilutions had been tested on bacterial and tissue samples.

2.10. Immunohistochemistry

To provide material for immunohistological examinations, 4-6 larvae were taken from each treatment group every day throughout the experiment. Larval samples were fixed in 4% phosphate-buffered formaldehyde, dehydrated in ethanol and embedded in paraffin. Larvae were sectioned at 3 μm (Leica Jung Biocut 2035, Leitz, Nussloch, Germany), incubated at 58 ºC for 30 min, dewaxed in xylene (Chemi-Teknik AS, Oslo, Norway), rehydrated in a series of ethanol baths and washed in running water. The absorbed polyclonal antisera, anti-*V. anguillarum*, anti-*V. logei* and anti-*V. splendidus* strain LT06 were diluted in Tris-hydroxymethyl-aminomethane (TRIS)-buffered saline (TBS) with 2.5% bovine serum albumin (BSA). In order to prevent non-specific antibody binding, sections were blocked by using 5% BSA in Tris-buffered formaldehyde for 20 min. Avidin-biotin-alkaline phosphatase complex (ABComplex/AP) reaction kit (DAKO A/S, Denmark) and New Fuchsin Substrate system (Dako) were used to stain both *V. anguillarum* and *V. logei*. A positive staining appeared with red coloration. Shandon's haematoxylin was used for counterstaining, which gave the tissue a blue coloration. At each staining, two controls were used. Unchallenged larvae were used as a negative control, and bacterial smears on microscope slides were used as a positive control. The same procedure was used to stain both larvae and bacteria. In order to avoid cross-contamination during the staining procedure, the tissue samples and the bacterial smears were processed separately. All incubations were performed at room

297 temperature (20 °C) in a humidity chamber. A Leica DMBE microscope equipped
298 with a Leica wild MPS52 phototube was used to photograph the sections.
299
300 During these immunohistological studies a total of 122 larvae were examined (see
301 Table 1 for details) and 281 larval tissue samples were immunostained with the three
302 antisera.
303
304 *2.11. DGGE (Denaturing Gradient Gel Electrophoresis) analysis*
305 Samples of 10 larvae were taken from each tank on days 19, 21, 25 and 27 post-hatch
306 and kept at –20 ^{o}C until analysis. On the same days twenty millilitres of water
307 samples from all tanks were filtered on 0.2 μm Dynagard hollow-fibre syringe filters
308 (Microgon InC. Laguna Hills, Ca.) and kept at –20 °C prior to analysis. DNA was
309 extracted from the homogenised cod larvae pellet and the syringe filters using the
310 commercial kit DNA isolation Wizard® Genomic DNA Purification (Promega,
311 Wisconsin, USA). The extracted genomic DNA was used as target DNA in the
312 polymerase chain reaction to amplify fragments suitable for subsequent DGGE
313 analysis using primer combinations EUBf (Giovannoni et al., 1990) and PRU517r
314 (Lane et al., 1985) as described by Sandaa et al. (2003). DGGE was performed using
315 a Dcode 16/16 cm gel system (BioRad, Herts, UK). PCR samples were loaded onto
316 8% (wt/vol) polyacrylamide gels in 0.5 x TAE (20 mM Tris, 10 mM acetate, 0.5 mM
317 Na_2 EDTA (pH 7.4)). The linear gradient of urea and formamide ranged from 35 to
318 60% denaturant. The electrophoresis was run at 60 ^{o}C for 20 h at 60 V. The gels were
319 stained for 1 h with a 1:10 000 dilution of SYBR Green II (Molecular Probes, OR,
320 USA) in distilled water before photography. Amplified 16S rDNA from the bacterial

isolates of *V. anguillarum*, *V. logei* and *V. splendidus* were used as markers in the DGGE profiles.

2.12. Screening for presence of nodavirus

Larvae from the various treatment groups were tested for the presence of nodavirus. Five larvae were pooled together to make a single sample, and homogenised using 3 mm tungsten carbide beads (Qiagen). Total RNA was purified using the RNeasy® Mini Kit according to the manufacturer's protocol, and 500 ng of total RNA was reverse transcribed using random hexanucleotides. cDNA was synthesised in a 15 µl reaction containing 1x RT-buffer, 5 mM $MgCL_2$, 2 mM dNTP, 2.5 µM random hexanucleotides, 6 U Rnase inhibitor and 20 U MuLV reverse transcriptase; the mixture was incubated at 25 ºC for 10 min, 42 ºC for 30 min followed by 95 ºC for 5 min. In order to analyse the samples for presence of nodavirus, using real time RT-PCR, the primers and probe were designed within the conserved region of the sequence in order to allow us to recognise virus variants. The samples were analyzed using 900 nM of forward primer AhRNA2tmUP3 (5'-GAGTTCGAAATTCAGCCAATGTG-3'), 900 nM of reverse primer AhRNA2tmLP3 (5'-GAAGCCAGCCACGTAACCA-3'), 250 nM TaqMan probe AhRNA2TaqM3 (6-FAM 5'-CCGCAAACACGGGC-3'-TAMRA) in addition to 12.5 µl 2x TaqMan Universal PCR Master Mix, and 2 µl cDNA (10-100 ng) in a final volume of 25 µl. PCR cycling was performed as follows: 50 ºC for 2 min, 95 ºC for 10 min, 40 cycles of 95 ºC for 15 sec followed by 60 ºC for 1 min. The primers employed in this assay generated a PCR fragment of 60 bp corresponding to the region 350 bp to 410 bp downstream of the start codon of RNA2, and the probe used

binds in the middle of this PCR fragment. The assay was performed on an Applied Biosystems 7500 Real-Time PCR System.

3. Results

3.1. Challenge experiment

During the first two weeks post start-feeding, mortality in the six tanks was low. Only 10 to15 larvae per day were observed floating on the surface of the water. These were removed throughout the day. All larvae in all six tanks grew well and had a good appetite. The larvae were equally distributed in the water column and showed normal swimming behaviour. Rotifers could be observed inside the intestinal lumen. A sudden change appeared in one of the control tanks on day 17 post-hatch. Observations revealed that most of the larvae had stopped feeding and appeared apathetic. Instead of showing normal swimming pattern they were drifting close to the surface. In the course of the next four days approximately 99 % of the larvae in this tank died. No technical problems occurred during the period leading to this incident and no changes in temperature, salinity or oxygen levels were seen. Nothing out of the ordinary could be observed the day prior to this sudden change. The larvae showed normal feeding and swimming patterns as seen in the other five tanks.

Due to difficulties with the daily collection of dead larvae inside the tanks, accurate daily mortality data could not be obtained. At the end of the experiment, however, all the larvae that remained in each tank were counted (Table 2). Given that approximately 5000 eggs were transferred into each tank at the beginning of the

experiment, the average mortality rates in the tanks with normal development were between 45 and 56% (Table 2). The low number of larvae remaining in tank 4 was due to technical problems at the end of the experiment, when the outlet pipe loosened and most of the larvae managed to escape. The remaining larvae in this tank developed normally.

3.2. Counting Colony Forming units (CFU) among the different experiment tanks

The concentration of culturable bacteria among the six tanks showed no differences in the course of the experiment. On day 0, a water sample taken before the eggs were added to the tanks showed the amount of bacteria to be 1.0×10^2 CFU ml^{-1}. On day 5 post-hatch, two days post start-feeding, this had increased to 7.0×10^2. Within the next two days, on day 7 post hatching, the CFU had increased to 1.1×10^4 CFU ml^{-1}. From then on, the CFU stabilized at between 1.2 and 4.4×10^4 CFU ml^{-1} in all tanks the rest of the experiment.

The number of CFU grown from the homogenized larvae rose towards the end of the experiment. Up to day 20 post-hatch the CFU had been stable at approximately 3×10^3 CFU $larva^{-1}$. Then, on day 22 post-hatch the CFU of homogenized larvae increased and stabilized again, at levels ranging from 2.1 to 3.1×10^5 CFU $larva^{-1}$ during the rest of the experiment.

3.3. Identification of bacterial strains

Ten strains isolated from homogenized larvae taken from each tank were identified using sequencing of the genes 16S rDNA and *gyrB*. All isolates were gram negative and oxidase positive. In one tank the dominant bacteria were found to differ from those in the other five tanks. Larvae sampled from the control tank that experienced

high mortality showed a clear dominance of *Vibrio splendidus* and all isolates were haemolytic on blood agar. The bacterial strains grown from larvae in the other five tanks were dominated by *Vibrio parahaemolyticus* and only four of these strains were haemolytic. The bacterial strain grown form water samples taken from the six experiment tanks did not reveal such differences and the isolates were identified as *V. parahaemolyticus* and *Pseudoalteromonas haloplanktis*. Only one of the *P. haloplanktis* strains was haemolytic. We were unable to reisolate either *Vibrio anguillarum* or *V. logei* from the challenged larvae.

3.4. Immunohistochemical examinations

No specific immunostaining were observed in unchallenged rotifers or larvae stained with anti-*V. anguillarum* 610 and anti-*V. logei* indicating that these bacteria were not present in the everyday feed (Fig. 1 and data not shown, respectively) Rotifers bioencaplulated with *V. anguillarum* strain HI 610 and *V. logei*, however, were positively stained (Figs. 2 and 3, respectively), confirming that the bacteria had been successfully administered to the larvae. No larvae sampled from the healthy control group were positively immunostained with anti-*V. splendidus* (Fig 4).

Larvae exposed to rotifers bioencapsulated with *V. anguillarum* showed positive immunostaining inside the lumen (Figs. 5 and 6), and it was possible to see free bacterial cells (large arrow, Fig. 6). Some bacteria seemed to be attached to the microvilli and mucus cells, but they did not seem to infect the larvae (Fig. 6, small arrow). No histopathology was observed in any of the larvae examined. Positive immunostaining of *V. anguillarum* cells in the gastrointestinal tract was found throughout the whole experiment.

Presence of *V. logei* was also confirmed by the immunohistochemical examinations (Figs. 7 and 8). The bacterium could be observed attached to the lumen brush border (arrow, Fig. 8), but no bacteria were observed interstitially. However, in contrast to larvae exposed to *V. anguillarum*, larvae challenged with *V. logei* seemed to have been able to rid themselves of the bacterium 72 hours post-challenge. At 48 hours post-challenge only a few larvae showed positive immunostaining inside the lumen cavity and at 72 hours none of them appeared to contain the bacterium (Fig. 9). No histopathological alterations were found.

Larval samples taken from the control tank that experienced high mortality revealed the presence of neither *V. anguillarum* nor *V. logei*. On the other hand, immunohistochemistry demonstrated that *Vibrio splendidus* cells were abundandt in the intestinal lumen (Fig. 10). These findings were consistent with the bacterial isolation performed on homogenized larvae. Shedding of necrotic epithelial cells (Fig. 10, arrow) and necrotic gill tissue (Fig. 11, arrow) was observed. Larval samples taken from the five other tanks together with the rotifer samples showed no positive immunostaining against *V. splendidus*.

3.5. Denaturing Gradient Gel Electrophoresis (DGGE)

From the DGGE profile shown in Figure 12, eight bands were cut out and sequenced and the results of the BLAST search are presented in Table 3. Samples were also taken from the other four tanks but these results are not included, as they provided no additional information of interest to this study.

3.5.1. Control tanks (1 and 6)

One band (band 2, Fig. 12) in the PCR-DGGE profile of bacteria in water from tank 1 at day 21 post hatch, appeared in the same position as a band from the *Vibrio splendidus* isolate used as a marker in the DGGE analysis. This band also appeared in the PCR-DGGE profiles of bacteria associated with both larvae and water from tanks 1 and 6, but with a weaker signal (Fig 12). Sequence results from the BLAST search showed 99% similarity to *Vibrio* sp. (Table 3).

The DGGE analysis of bacteria associated with water and larval samples from tank 1 resulted in different DGGE profiles, with six bands in the DGGE profile of water that were different from the two bands in the DGGE profile of larvae (Fig. 12). There was also a change in the bacterial community associated with larvae from day 19 to days 25 and 27 post-hatch (tank 1). Two dominant bands in the DGGE profile of larvae on day 19 were in different positions from two dominant bands associated with larvae on days 25 and 27 post-hatch. The DGGE profiles of the bacteria associated with larvae on days 25 and 27 post-hatch in tank 1 resembled the PCR-DGGE profiles associated with larvae sampled from tank 6 in the same time period, with two dominant bands 7 and 8 (Fig. 12). Sequencing of these bands showed 96 and 97% similarity respectively to *Alteromonas* sp. (Table 3). The DGGE analysis of bacteria associated with larval samples in tank 6 produced DGGE profiles with two dominant bands in the same position in the sampling period. Bands in similar positions were seen in the DGGE profile of bacteria associated with water in tank 6 on day 21 post-hatch. One band in the PCR-DGGE profile of larvae sampled on day 19 post-hatch in tank 6 was sequenced as *Vibrio* sp. with 99% sequence similarity (Table 3).

The DGGE profiles of the bacterial community in the water from tanks 1 and 6 on day 21 post-hatch were slightly different, in that six bands were in similar positions between the two tanks, and two bands appeared in the DGGE profile from tank 6 in different positions from the DGGE profile from tank 1 (Fig. 12). The signal intensity of the six bands in similar position in the DGGE-profiles also differed between the two tanks.

None of the larvae examined tested positive for nodavirus.

4. Discussion

The larval groups challenged with *Vibrio anguillarum* strain HI-610 and *Vibrio logei* did not suffer any increase in mortality compared to the unchallenged control groups. This was confirmed by the findings of the immunohistochemical examinations. No or little histopathology was found in the larvae examined. All the larvae demonstrated the presence of bacteria post-challenge, demonstrating that the transfer of bacteria had been successful (Fig. 5-8). In support of this, examinations of rotifers showed the presence of bacteria (Figs. 2 and 3).

The immunohistological analysis of larvae exposed to *V. anguillarum* revealed no signs of histopathology. This particular *V. anguillarum* strain is known to cause high mortalities in bath challenge experiments in both juvenile cod (Seljestokken et al., 2006, Vik-Mo et al., 2005) and cod yolk-sac larvae (Sandlund and Bergh, in press). Planas et al. (2005) used a *V. anguillarum* strain to test bioencapsulation of bacteria into live feed as an infection model for turbot, *Scophthalmus maximus*, with success. The strain used in this present study is known to change size depending on how it is

grown. When grown *in vitro*, either on agar or in a liquid medium, the shape of the bacterium is almost coccoid. Grown *in vivo* and becoming infective, the shape changes to rod shape (Sandlund, unpublished observations). The bacteria seen in the tissue samples were rod-shaped (Fig. 6).

Rotifers are found to be more efficient at grazing bacteria in a bacterial suspension of 10^8 CFU ml^{-1} than in one of 10^7 CFU ml^{-1}. The number of bacteria bioencapsulated within each rotifer was found to be between 3.2 x 10^4 and 7.1 10^4 CFU ml^{-1} (Makridis et al., 2000b). In the study by Makridis et al. (2000b) there was only a small difference between 20 minutes and 60 minutes of grazing in terms of uptake. Differences in uptake between the two strains tested were also found. On the basis of these results and estimates that cod larvae eat an average of 168 rotifers per day on day 5 and 480 on day 15 (Van der Meeren et al., 2005), each larva would consume an average of 10^6 bacteria per day of challenge, we consider the amount of bacteria administrated to the larvae to be sufficient to cause disease. Previous results showed that bath challenge of cod yolk sac larvae with *V. anguillarum* strain HI-610 caused high mortality at a challenge dose of 10^4 CFU ml^{-1} (Sandlund and Bergh, in press). It should also be in mind that the larvae were fed rotifers bioencapsulated with bacteria four times a day during the days of challenge. This ensured a stable supply of bacteria fed to the larvae.

The immunohistological examinations revealed the presence of *V. anguillarum* inside the lumen throughout the experiment post-challenge. However, no histopathology was found, which is consistent with the absence of mortality that can be directly related to the challenge. *V. anguillarum* was described as surviving a passage through the

gastrointestinal tract of turbot, *Scophthalmus maximus* (Olsson et al., 1998). The same study also demonstrated that *V. anguillarum* is able to colonize the intestine when administrated orally by coating pellets with a bacterial suspension. This is consistent with the findings of the assumed infective stages of *V. anguillarum* in our immunohistological examinations. However it does not explain the absence of mortality. Considering the origin of the bacterium and the fact that the same strain and cultivation protocols and environmental conditions were used in the experiment by (Sandlund and Bergh, in press), results indicate that the bacterium do not cause disease when administrated orally to cod larvae. In contrast to larvae challenged with *V. anguillarum*, *V. logei* was only found prior to 72 hours post-challenge. Most of the larvae examined showed little presence of *V. logei* at 48 hours post-challenge, and after 72 hours none of the larvae contained the bacterium, indicating that it had been eliminated from the gastrointestinal tract. Bioencapsulation of bacteria into live feed as a method to administer bacteria to marine larvae has been used successfully by Planas et al. (2005) and Makridis et al. (2000a, 2000b). This, together with the immunohistochemical findings, leaves room for speculation that *V. logei* is unable to infect healthy cod larvae. Supporting this hypothesis is the finding by Sandlund and Bergh (in press) where no increased mortality was observed when cod yolk sac larvae were bath challenged with the same *V. logei* strain (challenge dose 10^6 CFU ml^{-1}). In a similar experimental design with halibut, *Hippoglossus hippoglossus* fry, to which non-pathogenic *Vibrio* spp. via *Artemia* sp. had been added, Makridis et al. (2001) found that the bacteria did not become established in the intestine. Similarly, the probiotic *Roseobacter* sp. added to turbot larvae by Planas et al. (2006) did not become established in the larvae, and was only found in the intestinal lumen of the larvae when examined by immunohistochemistry.

During an outbreak of disease, bacteria are secreted into the environment or tank, raising the challenge pressure; hence, the larvae will be constantly exposed to bacteria. During this challenge experiment the bacteria were added on only two days, which might not have been sufficient in order to cause disease. It might be speculated that the challenge dose was too low, or that the bacterium should have been added over a longer period of time in order to cause disease. Our findings might also indicate that the larvae are capable of eliminating the bacterium from the intestine. This however, is in contrast to the findings in larval batches that have suffered high mortality rates in large-scale aquaculture, where *V. logei* is frequently isolated (E. Karlsbakk, Institute of Marine Research, pers. comm.). During this laboratory experiment environmental factors were tightly controlled, making it easier to maintain a stable environment. This again helped to avoid unnecessary stress, which might otherwise have made the larvae more susceptible to disease.

Two weeks post start-feeding, larvae in one of the two control tanks started to die and within four days 99% of the larvae in this tank were dead. Technical problems in that specific tank are unlikely to be due to the fact that all tanks used the same water-flow, water and food source. No shifts in temperature, oxygen levels, water-flow or feeding problems were identified. Water samples from all tanks displayed stabile CFU values, and by cultivation and sequencing similar bacterial isolates with presence of *Vibrio parahaemolyticus* and *Pseudoalteromonas haloplanktis* were identified. The DGGE analysis of bacteria associated with water samples from tank 1 and 6 on day 21 post-hatch also revealed different DGGE profiles, but the presence of *V. parahaemolyticus* and *P. haloplanktis* could not be verified by this method.

570

571 Identification of bacterial strains isolated and grown from larval homogenate revealed
572 that tank 1 differed from the other five tanks. In tank 1, *Vibrio splendidus* was the
573 dominant strain while in the other tanks *V. parahaemolyticus* was the most frequently
574 isolated strain. This may explain the high mortality in control tank 1, as *V. splendidus*
575 is often associated with mortality of a variety of fish species including corkwing
576 wrasse, *Symphodus melops* (Jensen et al., 2003), turbot larvae, *Scophthalmus*
577 *maximus* (Gatesoupe et al., 1999), and bivalves such as scallop larvae, *Pecten*
578 *maximus* (Sandlund et al., 2006, Torkildsen et al., 2005) and Japanese oyster
579 *Crassostrea gigas* (Gay et al., 2004, Sugumar et al., 1998) and gorgons such as
580 *Paramuricea clavata* and *Eunicella cavolinii* (Martin et al., 1999).
581

582 The immunohistochemical findings demonstrated the presence of *V. splendidus* in all
583 examined larvae from tank 1. All larvae showed extended necrotic tissue in the lumen
584 area and in the gills (Figs. 10 and 11). In order to substantiate this finding *V.*
585 *splendidus* was used as a marker in the DGGE analysis. In the DGGE profile of
586 bacteria in water on day 21 post-hatch in tank 1, one band appeared in the same
587 position as the *V. splendidus* marker (Fig. 12). This band showed 99% sequence
588 similarity to a *Vibrio* sp. originally isolated from crab (Table 3, Gudkovs et al.,
589 unpubl.). The same sequence was also found analysing bacteria in larvae on day 19
590 post-hatch in tank 6. It should be noted that similar unspecific species results are
591 commonly seen using the *16S rRNA* gene to discriminate between vibrios, as this
592 genus possess high similarity within this gene.
593

The DGGE analysis showed that the larval samples from the two control tanks 1 and 6 had different DGGE profiles on day 19 post-hatch, this might correspond to the different health status seen for the larvae (Fig. 12). Sequencing of bands from the DGGE profile associated with larvae on days 25 and 27 in tank 6 showed 96 and 97% sequence similarity to *Alteromonas* sp. (Table 3, Pinhassi et al., 1997). This sequence result was also seen by Brunvold et al. (2007), analysing apparently healthy larvae. Towards the end of the experiment on days 25 and 27 post-hatch, the DGGE profiles of the larvae became more similar, and the remaining larvae in tank 1 survived till the end of the experiment.

The DGGE method circumvents cultivation, and thus includes both cultivable and non-culturable bacteria. The method have been used to study bacterial communities associated with a wide variety of aquaculture purposes (Griffiths et al., 2001, Rombout et al., 2001, Sandaa et al., 2003, Jensen et al., 2004, Brunvold et al., 2007). In this present study the DGGE results were not consistent with the culturing, i.e. positive identification of *V. parahaemolyticus* and *P. haloplanktis* were not detected in the DGGE profile of water samples and only indications of the presence of *V. splendidus* were found in the DGGE profile of larvae. DGGE is based on PCR and several publications points to the biases introduced by this amplification (Farrelly et al., 1995, Wintzingerode et al., 1997, reviewed by Kanagawa 2003). Biases are also associated to the primer target *16S rRNA*, with the possibility of heteroduplex formation resulting from the multiple copies of the *16S rRNA* gene seen in bacteria (Moreno et al., 2002, Dahllöf et al., 2000). Despite this, we conclude that differences in water profile were found and the results indicate a more similar profile towards the end of the experiment.

Slight differences between the microbial communities in the individual tanks might explain how opportunistic bacteria like *V. splendidus* could infect only one of the larval groups. The larval group that experienced high mortality was screened for presence of nodavirus, but the results were negative, ruling out nodavirus infection as the cause of the massive mortality. We conclude that *V. splendidus* probably caused the disease outbreak in tank 1. This is the first time that *V. splendidus* has been associated with high mortality in cod larvae.

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Table 1

Number of larvae in the different treatment groups examined using immunohistochemical analysis. Control = control group, *V. anguillarum* = larvae sampled from groups challenged with *Vibrio anguillarum*, *V. logei* = larvae sampled from groups challenged with *Vibrio logei*, Dying control = larvae sampled from the control group suffering from high mortality

Treatment	Number of examined larvae
Control	47
V. anguillarum	28
V. logei	27
Dying control	20
Total	122

Table 2

Number of larvae remaining in each tank at the end of the experiment. Tanks 1 and 6 were control tanks, tanks 2 and 3 contained larvae challenged with *Vibrio anguillarum*, tanks 4 and 5 contained larvae challenged with *Vibrio logei*. Number of larvae = the number of remaining larvae in the tanks at the end of the experiment, % survival is an approximate value of the mortality in each experimental tank, based on 5000 eggs being added to each tank.

Tank	Number of larvae	% survival
1	89¤	2¤
2	2364	47
3	2168	44
4	278*	5*
5	2434	49
6	2755	55

¤ = Tank 1 suffered from high mortality from day 17 post hatch.

* = Tank 4 had problems with the outlet pipe, which allowed the larvae to escape the tank.

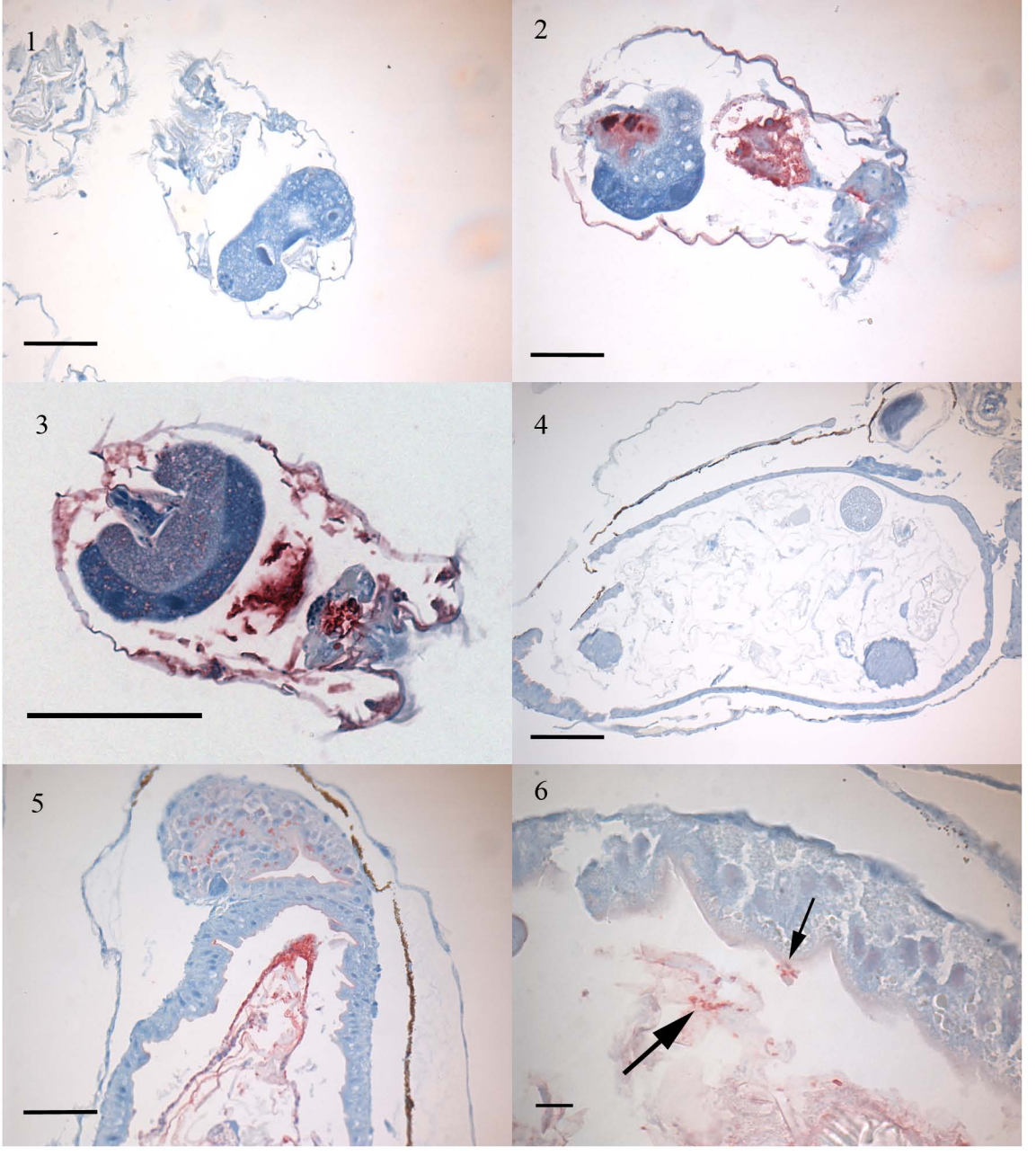
Table 3

Results from BLAST search of the bands cut out and sequenced from the DGGE profile of 16S rDNA isolated from water and larvae samples from tank 1 and 6 (Fig. 12).

DGGE bands	Closest relative	Similarity[a] (%)	GenBank accession number	Reference
1	Uncultured gamma proteobacterium	94	EF215819	Dang et al. 2008
2	*Vibrio* sp.	99	DQ146983	Gudkovs et al. Unpubl.
3	*Alteromonas* sp.	97	U64027	Pinhassi et al. 1997
4	*Vibrio* sp.	90	DQ146983	Gudkovs et al. Unpubl.
5	Uncultured bacterium	92	EF646105	Woebken et al. 2007
6	*Vibrio* sp.	99	DQ146983	Gudkovs et al. Unpubl.
7	*Alteromonas* sp.	96	U64027	Pinhassi et al. 1997
8	*Alteromonas* sp.	97	U64027	Pinhassi et al. 1997

[a] = sequences were aligned to the closest relative based upon BLAST search in the GenBank database. The similarity was calculated with gaps not taken into account.

Figs. 1 to 3. Immunohistochemical staining of paraffin sections of rotifers, *Brachionus* sp. Figs. 4 to 11. Immunohistochemical staining of paraffin sections of cod, *Gadus morhua*, larvae. All sections have been immunostained with Avidin-biotin-alkaline phosphatase method. Shandon haematoxylin counterstained. Positive immunohistostaining is visualized in red. Counterstaining gives tissue various tones of blue. Fig 1. Control rotifer immunostained with anti- *V. anguillarum*. No positive immunostaining was found. Fig. 2. Rotifer embedded with *Vibrio anguillarum* positively immunostained with anti-*V. anguillarum*. Positive immunostaining is seen on the surface and inside the lumen. Fig. 3. Rotifer embedded with *Vibrio logei* positively immunostained with anti-*V. logei*. Positive immunostaining is seen on the surface and inside the lumen. All three figs. with magnifications 200x, scale bars 100 µm. Fig. 4. Control larvae immunostained with anti-*V. splendidus* . No positive immunostaining was found. Magnification 400x, scale bar 50µm. Figs. 5 and 6. Intestine of larvae challenged with *V. anguillarum* positively immunostained with anti-*V. anguillarum*. Fig. 5. Magnification 400x, scale bar 50µm. Fig. 6. Bacteria are seen attached to the brush border (small arrow), but no bacteria were observed interstitially. Bacteria were also observed as free cells in the intestinal cavity (large arrow). Magnification 1000x, scale bar 10µm. Figs. 7 to 9. Intestine of larvae challenged with *V. logei* immunostained with anti-*V. logei*. Fig. 7. Magnification 400x, scale bar 50µm. Fig. 8. Bacteria attached to the brush border, but no infection of the epithelium was observed. Magnification 1000x, scale bar 10µm. Figs. 9. Larvae exposed to *V. logei* 72 hours post-challenge. No positive immunostaining was seen. Magnifications 200x, scale bars 100 µm. Figs. 10 and 11. Control larvae positively immunostained with anti- *V. splendidus*. Fig. 10. Intestine of larvae showing an extensive infection of *V. splendidus*. Pathology is observed as shedding of necrotic epithelial cells (arrow) Magnification 400x, scale bar 50µm. Fig. 11. Gill lamellas infected with *V. splendidus*. Early stages of necrotic cells can be seen (arrow). Magnification 1000x, scale bar 10µm.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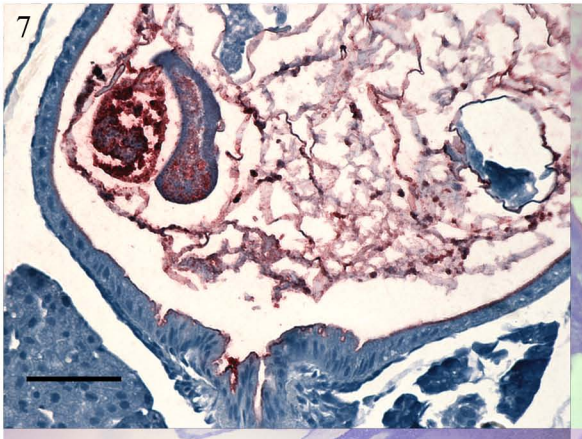
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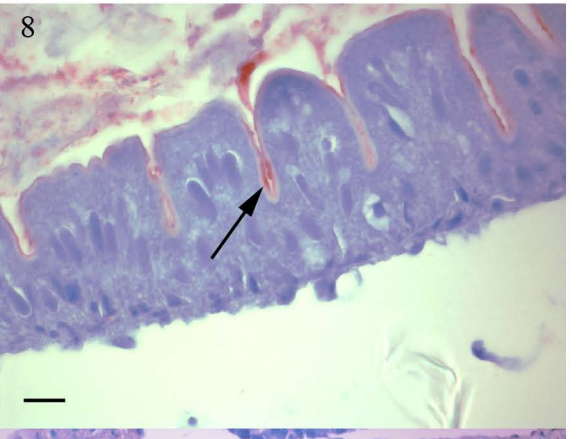
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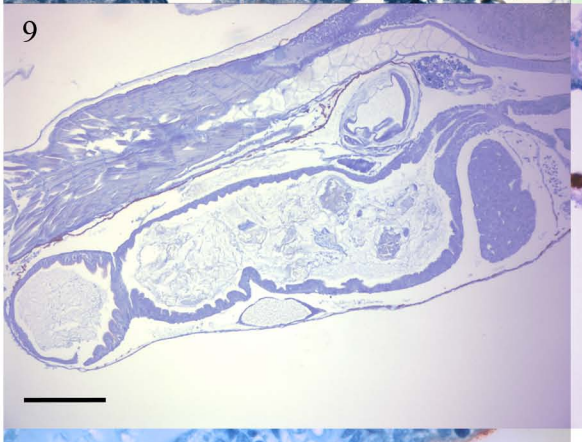
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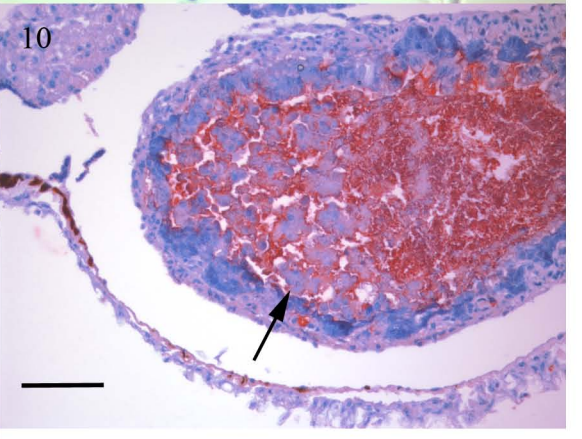
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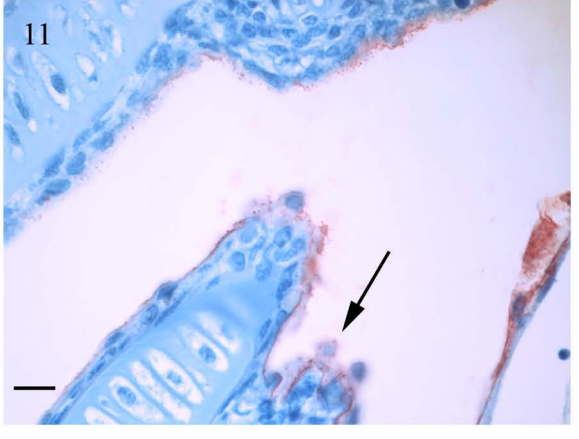
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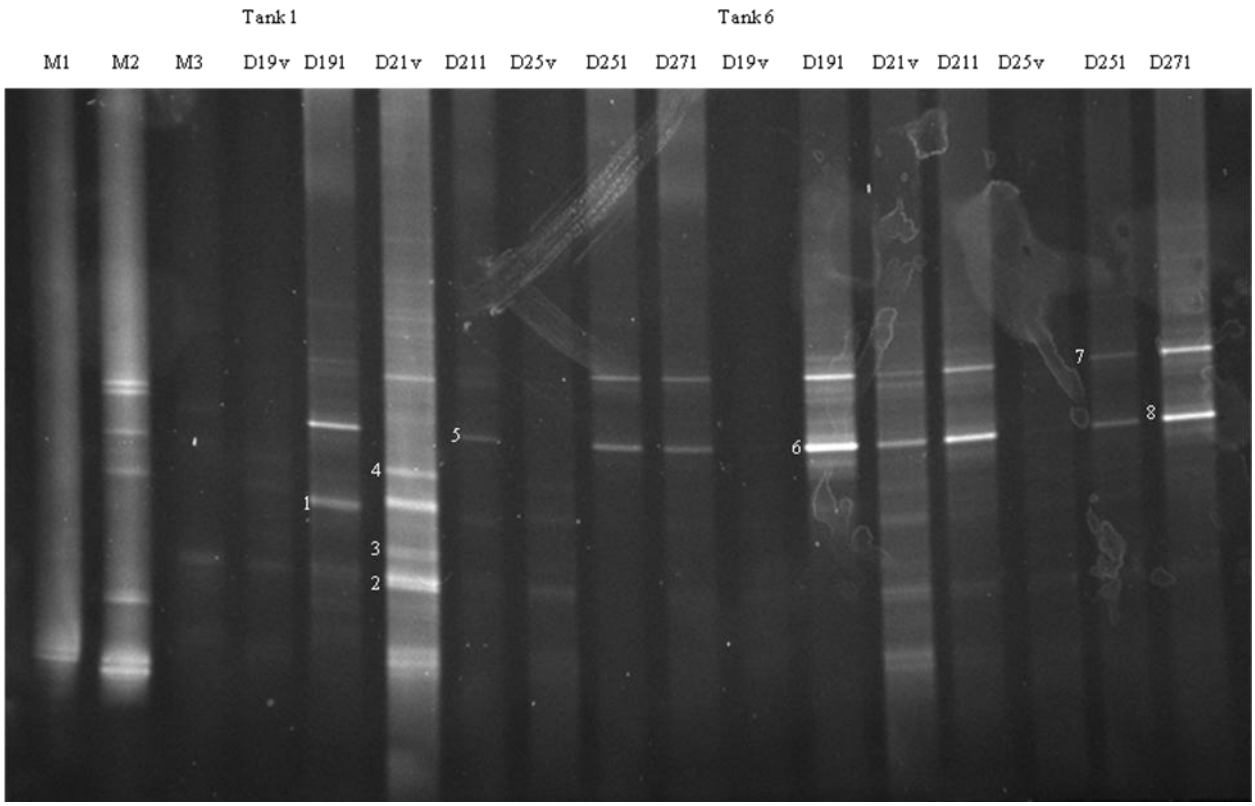
Figure 12. The DGGE profile display the DGGE profile of PCR amplified 16S rDNA isolated from larvae (l) and water (v) samples from tank 1 and 6 on days 19, 21, 25 and 27 post-hatch. The bacterial isolates *V. anguillarum* (M1), *V.logei* (M2) and *V.splendidus* (M3) were used as markers in the DGGE profile.



Paper V

Sandlund N, Rødseth OM, Knappskog D, Fiksdal IU, Bergh Ø Comparative susceptibility of turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus*, and cod, *Gadus morhua* yolk-sac larvae challenged with different serotypes of *Vibrio anguillarum* and *Vibrio* spp. Manuscript

Title: Comparative susceptibility of turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus*, and cod *Gadus morhua* yolk-sac larvae challenged with different serotypes of *Vibrio anguillarum* and *Vibrio* spp.

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ABSTRACT

In intensive aquaculture systems high mortalities are frequently observed during the early life stages of marine fish. The aim of this study was to investigate differences in susceptibility to various serotypes of *Vibrio anguillarum* O1, O2α and O2β, *Vibrio salmonidida* and *Vibrio splendidus* for turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus* and cod *Gadus morhua*. A multidish system was used, with one egg distributed to each well added 2 ml (turbot and cod) or 10 ml (halibut) of sterile seawater and bacterial cultures. Final concentrations in the wells were 10^6 and 10^4 CFU ml^{-1}, respectively. Unchallenged eggs and larvae were used as controls. Larvae in challenged groups suffering from high mortality were examined by immunohistochemistry, using absorbed polyclonal antisera. The O2α serotype was pathogenic to all three species whereas the O1 serotype was pathogenic to halibut and

cod. The immunohistochemical examinations revealed differences in histopathology. The O1 serotype caused a more severe and developed histopathology compared to O2α. In larvae exposed to O1 histopathology and bacterial cells were seen in dermis, gastrointestinal tract, brain and eye area while in larvae exposed to the O2α serotype pathology was scarce and limited to the gastrointestinal tract. These results could imply that there are unknown differences in the immunity among the species or that these pathogens are host specific even to early life stages of fish. The O2β strain did not cause a significant increase in mortality.

Key words: Turbot, halibut, cod, yolk-sac larvae, Bath challenge experiment, Immunohistochemistry, *Vibrio anguillarum*, Vibriosis

INTRODUCTION

Turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus* and cod *Gadus morhua* are increasingly important species in European aquaculture. The production has however been limited by unstable production of juveniles due to high mortalities during the larval stages. Vibriosis has been and still is one of the major disease problems of the aquaculture industry. *Vibrio anguillarum, Vibrio salmonicida*, *Vibrio ordalii* and *Vibrio vulnificus* are among the pathogens causing the greatest losses in aquaculture worldwide (Reviewed by Toranzo et al. 2005). Good vaccines developed in the late 1980-ties early 1990-ties has minimized the problem in salmonid farming, but vaccines developed for cod are not providing sufficient protection (Reviewed by Sommerset et al. 2005, Bricknell et al. 2006, Samuelsen et al. 2006), hence vibriosis in marine fish farming is still causing severe losses.

49 The pathogen *Vibrio salmonicida* is the causative agent of "cold water vibriosis", a disease
50 usually breaking out during winter or at temperatures below 15° C (Egidius et al. 1986). It is
51 known to be a problem in farming of salmonid fish, but little information is available
52 concerning *V. salmonicida* in marine fish species (Reviewed by Bricknell et al. 2006). *Vibrio*
53 *splendidus* has been described as pathogenic to fish (Thomson et al. 2005, Bergh &
54 Samuelsen 2007) and bivalves (Gómez-León et al. 2005, Sandlund et al. 2006).

55 The greatest losses to vibriosis in the aquaculture industry are caused by *V. anguillarum*, a
56 species consisting of 23 O known serotypes (Pedersen et al. 1999). The various serotypes are
57 often associated with certain species of fish and not all are considered pathogenic. Serotypes
58 associated with disease in farmed fish are O1, O2 and O3 (Reviewed by Toranzo et al. 2005).
59 The O2 serotype has been divided into two subserotypes O2α and O2β. Whilst O2α is isolated
60 from both salmonid and marine fish, O2β is mostly isolated from cod and other non-salmonid
61 fish (Mikkelsen et al. 2007). In turbot farming O1 is the dominant serotype (Larsen et al.
62 1994) while both serotypes O1 and O2 cause disease in halibut (Bergh et al. 1997, Bricknell
63 et al. 2000, Hoare et al. 2002). O3 serotype is rare compared to the other two and usually
64 isolated from eel (Reviewed by Toranzo et al. 2005).

65 In spite of all the published work on the bacteria listed above, little is known when it comes to
66 infections during the early life stages. In addition the aspects concerning host differences in
67 susceptibility to a pathogenic agent are of interest. As new species emerge in the aquaculture
68 industry it is likely that pathogens are transferred among the species. Although species may
69 not develop disease, they may serve as reservoirs of the pathogen. The purpose of this work
70 was to perform a comparative challenge study of turbot, halibut and cod larvae comparing
71 susceptibility to different serotypes of *V. anguillarum*, an atypical strain of *V. salmonicida*
72 and a *V. splendidus* strain.

MATERIALS AND METHODS

Broodstock, eggs and larvae. Fish eggs were provided from the broodstock at Institute of Marine Research, Austevoll Research Station, Storebø, Norway, and incubated as described by Bergh et al. (1997) and Bergh (2000).

Bacterial strains. The bacterial strains used were *Vibrio anguillarum* strain HI-610 serotype O2α, strain HI-618 serotype O2β, strain HI-644 serotype O1, *Vibrio salmonicida* strain HI-651 (Wiik et al. 1995) from the Institute of Marine Research and *Vibrio splendidus* strain HI-1576 originally provided by the National Veterinary Institute, Norway. Strain HI-610 and HI-618 were originally isolated from diseased cod, HI-644 was isolated from diseased turbot, HI-651 and HI-1576 were originally isolated form diseased halibut fry. All bacterial strains were stored at –80ºC in a 20 % glycerol/marine broth (MB, Difco 2216, Difco, Detroit, MI, USA) stock. They were incubated at 15ºC and grown on Petri dishes with Difco 2216 marine agar (MA) for 48 h. Colonies of the bacteria were transferred to Erlenmeyer flasks with MB (Difco 2216) and shaken at 80 rpm in a shaking incubator (INFORS AG CH-4103 Bottmingen, Switzerland) for 48 h at 10°C. The bacterial cultures were harvested by centrifugation (Heraeus Sepathec Megafuge 1.0 R) at 2772 G for 10 min at 4ºC, washed twice phosphate-buffered saline (PBS) and suspended in PBS.

It should be noted that bacterial suspensions used for challenging turbot larvae were grown at 15°C.

All three species were challenged with all five bacterial strains. Two different challenge doses were used, equivalent to a bath challenge concentration in wells of approximately 10^6 (high)

and 10^4 CFU ml^{-1} (low), respectively. Unchallenged larvae were used as control. Mortality of the larvae was recorded daily. The challenge protocols are modified from (Bergh et al. 1992, 1997).

The eggs are fragile and that resulted in some of the eggs being damaged during the handling of the eggs. This is the cause for the challenge groups containing different amount of larvae.

Challenge experiment with turbot, *Scophthalmus maximus*, larvae. The turbot, *Scophthalmus maximus*, eggs and larvae were incubated as described by Bergh et al. (1997) held in 24-wells multi dishes at 15°C in an air conditioned room. These challenge groups contained 72, 71, 70 or 69 larvae, as larvae damaged through treatment were killed and discarded from the experiment. The eggs were challenges one day prior to hatching. The experiments lasted for five days post hatch (d.p.h.).

Challenge experiment with halibut, *Hippoglossus hippoglossus*, larvae. Challenge experiment with halibut, *Hippoglossus hippoglossus*, larvae were performed in darkness in an air conditioned room at 6°C as described by Bergh et al. (1992, 1997). The eggs were challenged four days prior to hatching. One day after hatching, the wells were washed, as the water and remains of the eggshell was removed, and 10 ml of sterile seawater was added. These challenge groups contained either 60 or 58 larvae. The halibut larvae were only exposed to the highest challenge dose. Halibut larvae have a long yolk-sac stage and this experiment lasted for 23 d.p.h.

Challenge experiment with cod, *Gadus morhua*, larvae. Cod, *Gadus morhua*, eggs and larvae were incubated and challenged as described by Bergh (2000). In this experiment, 24-well multi dishes were used, with 2 ml sterile seawater and one egg per well The eggs were incubated at 6°C. These challenge groups contained 72 and 69 larvae. The eggs were

challenged seven days prior to hatching. The experiments lasted for 10 d.p.h. Two separate larval batches were challenged in order to verify the reproducibility of the experimental protocol.

Antisera. Antisera were made for *Vibrio anguillarum* strain HI-610 and HI-644 according to the method described by Oeding (1957). Formaldehyd-killed, washed bacteria were administrated by intravenous injection to the rabbits. The polyclonal antisera were absorbed as described by Knappskog et al. (1993), to minimize the possibility of cross-reaction. The anti-*V. anguillarum* serum was absorbed against the other serotypes used in this experiment, meaning the O2α serotype serum was absorbed against serotype O2β and serotype O1 strain HI-644. Each of the absorbed antisera was testes for cross-reaction with bacterial strains and tissue samples prior to the immunohistochemistry. The dilution used on tissue samples was determined after testing a range of antiserum dilutions on tissue samples.

Immunohistochemistry. To provide material for immunohistological examinations, additional multiwall dishes were set up within the different treatments. Two to three larvae were taken from each treatment group daily during periods of mortality throughout the experiment. Larval samples were fixed in 4% phosphate-buffered formaldehyde, dehydrated in ethanol and embedded in paraffin. Larvae were sectioned at 3 μm (Leica Jung Biocut 2035), incubated at 58ºC for 30 min, dewaxed in xylene (Chemi-Teknik AS, Oslo Norway), rehydrated in a series of ethanol baths and washed in running water. The absorbed polyclonal antisera, anti-*V. anguillarum* 610 and 644 were diluted in Tris-hydroxymethyl-aminomethane (TRIS) – buffered saline (TBS) with 2.5% bovine serum albumin (BSA). To prevent non-specific antibody binding, sections were blocked by using 5% BSA in Tris-buffered formaldehyde for 20 min. Avidin-biotin-alkaline phosphatase complex (ABComplex/AP) reaction kit (DAKO A/S, Denmark) and New Fuchsin Substrate system (Dako) were used to

stain all samples. A positive staining appeared with red coloration. Shandon's haematoxylin was used for counterstaining, which gave the tissue a blue coloration. Unchallenged larvae were used as a negative control, and bacterial smears on microscope slides were used as a positive control. All incubations were performed at room temperature (20°C) in a humidity chamber.

Statistical analyses. Since the survival and mortality data are not normally distributed, non-parametric tests were used. A 2 x 2 contingency table ($p < 0.01$) Bonferroni correction for multiple independent tests), performed in Statistica v 7.0 (StatSoft), was used to test for mortality differences among the treatment and control groups. Since multiple independent tests were used to test differences in mortality rate among all challenged larval groups and the three larval control groups, a Bonferroni correction was applied (to minimize the possibility of doing a type II error) (Rice 1989). We thus tested for 5 bacterial strains, and the p value was corrected by 5 ($p = 0.05/5 = 0.01$): see (Rice 1989). Yates correction was used since there was only one degree of freedom (df).

RESULTS

Challenge experiments

All undamaged eggs hatched normally and none of the control groups suffered from high mortality.

The cumulative mortality and statistical analysis showed that turbot, *Scophthalmus maximus*, larvae suffered the highest mortality when challenged with *Vibrio anguillarum* serotype O2α

(Fig. 1). Mortality was found to be significantly different from the control group two days post hatching (p.h.) in both challenges dose groups (table 1 & 2, $p < 0.01$ Bonferroni correction). Towards the end of the experiment, at day four and five p.h., the larval group challenge with the low dose of *Vibrio splendidus* and *Vibrio salmonicida* respectively, experienced mortality significantly different from the control group (table 2).

Halibut, *Hippoglossus hippoglossus*, larvae were most susceptible to *V. anguillarum* serotype O2α and serotype O1 (Fig. 2). The statistical analysis showed that mortality in these challenge groups was significantly different from the control group from day seven post hatch and onwards (table 3, $p < 0.01$ Bonferroni correction).

The cumulative mortality observed in the two separate groups of cod, *Gadus morhua*, larvae were similar (data not shown), hence cumulative mortality and statistical analysis are only shown for one of the groups (Fig. 3, table 4 & 5). Cod larvae showed equal susceptibility to the high challenge dose of *V. anguillarum* serotype O1 and O2α (Fig. 3). The statistical analysis showed that the mortality among these challenge groups were significantly different from the control group at day three and four p.h. and onwards, respectively (table 4, $p < 0.01$ Bonferroni correction). For the low challenge dose groups there were only observed significantly difference in mortality between the control and the group challenged with *V. anguillarum* serotype O2α at day seven p.h. and onwards (table 5). Although the cumulative mortality reached 35% in the high dose challenge group of *V. anguillarum* serotype O2β (Fig. 3), this was only significantly different from the control around day seven and eight p.h. (table 4, $p < 0.01$ Bonferroni correction).

Immunohistochemistry. Immunohistochemical examinations were used for verification instead of re-isolation of bacteria, hence performed on larval groups suffering the highest

188 mortality compared to the larval control group. No control larvae were positively
189 immunostained with either of the antisera (Fig. 4).

190 The immunohistochemical findings in turbot larvae challenged with *V. anguillarum* serotype
191 O2α showed bacterial cells in the gastrointestinal tract, the abdominal cavity, urine bladder
192 (Fig. 5, arrow) and kidney.

193 In halibut and cod however, the immunohistochemical findings were not always consistent
194 with the observed mortality. Differences in histopathology were observed in halibut larvae
195 challenged with *V. anguillarum* serotype O1 and O2α (Figs. 6 and 7, respectively). The most
196 severe tissue damages were found in larvae challenged with the O1 serotype. Large quantities
197 of bacteria were seen in the dermis and brain area (Fig. 6, arrow) and necrotic cells were seen
198 in the muscle tissue (data not shown). Necrotic tissue and bacterial cells were also observed in
199 the yolk-sac area, the gastrointestinal tract and in the area around the brain and eyes (data not
200 shown). In larvae challenged with the O2α serotype, histopathology was limited to some
201 necrotic cells in dermis and positive staining in the head area (Fig. 7). In addition bacterial
202 cells were observed in the gastrointestinal tract (data not shown).

203 In cod larvae, the histopathology was scarce in spite of high mortality (Figs. 8 and 9). Larvae
204 challenged with the O1 serotype had bacteria in dermis and head region (Fig. 8, arrow).
205 Bacterial cells were observed in the oesophagus and gastrointestinal tract in larvae challenged
206 with the O2α serotype (Fig. 9, arrow).

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208

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DISCUSSION

The results revealed differences in susceptibility among turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus* and cod *Gadus morhua* to various serotypes of *Vibrio anguillarum*. The high challenge dose of *V. anguillarum* serotypes O2α and O1 had a significantly negative effect on all three species, except for O1 in the turbot experiments. This could imply differences in the immune system among the three species or that the bacterium has a preference towards one specific host. The unspecific immune system has to some extent been studied in cod (Lange et al. 2004, reviewed by Falk-Petersen 2005) and halibut larvae (Lange et al. 2006, reviewed by Falk-Petersen 2005). In cod larvae the complement component C3 was detected one day post hatch (d.p.h.) and found in most organs at 15 d.p.h (Lange et al. 2004). I halibut the C3 factor was found 30 d.p.h. (Lange et al. 2006), however this was the earliest sampling in that particular study. In turbot the spleen and thymus appears at the end of the yolk-sac stage (reviewed by Falk-Pedersen 2005), indicating that the specific immune response can be expected after this stage. A recent study by Corripio-Miyar et al. (2007) studying immunoglobulin M (IgM) development in haddock *Melanogrammus aeglefinus*, suggest the immune system begins developing around 25-29 d.p.h. Considering the absence of a well developed specific immune system in cod and halibut at this stage (Schrøder et al. 1998, Lange et al. 2006), it will be interesting to pursue the results from this present study to understand the role of immune response to various antigens.

Preference towards a host has to some extent been found among *V. anguillarum* strains tested on different species of fish mucus (Larsen et al. 2001). The same study showed an overall preference towards rainbow trout mucus rather than mucus from cod, common bream *Abramis brama* and flounder *Platichtys flesus*. Differences in host susceptibility has also been shown for *Mycobacterium marinum* (Wolf & Smith 1999). The study showed great

differences in inflammatory response between striped bass, *Morone saxatilis*, and the tilapia hybrid (*Oreochromis niloticus* x *O. mossambicus* x *O. aureus*). The striped bass that experienced the highest mortality also displayed the most severe clinical signs (granulomas with necrosis) and inflammation. Difference in virulence has also been shown in experiments involving various species of bivalves exposed to different *Vibrio* strains (Nicolas et al. 1992, 1996, Luna-González et al. 2002).

As previously described *V. anguillarum* serotype O2β is most frequently associated with disease in cod and non-salmonid fish (Mikkelsen et al. 2007). The O2β isolate used in this present study did however not cause any negative effects on any of the three species. Difference in virulence (between the O2α and the O1 serotype) was also verified in terms of challenge dose. The low challenge dose (tested with cod and turbot only) of O2α serotype was pathogenic to turbot and cod larvae while the low challenge dose of the O1 serotype did not cause any increase in mortality. Variations in virulence among *V. anguillarum* strains have been reported on several occasions (Larsen et al. 1988, Reviewed by Toranzo & Barja 1990, Lemos et al. 1991, Pedersen et al. 1997).

The increase in mortality started at various days post hatch among the three species (Figs. 1-3). In the halibut group mortality increased at day seven post hatching, while in the turbot group it started at day two and in the cod group at day three-four. This can partly be related to rearing temperature. Halibut and cod were reared at 6°C, compared to 15°C for turbot, which may slow bacterial growth and delay infection. In addition, the ontogenetic development is faster in turbot than in halibut, whereas cod may be viewed as an intermediate in this respect.

The immunohistochemical examinations revealed differences in the infection caused by the *V. anguillarum* serotypes O1 and O2α. The overall histopathological observations in all three species challenged with the O1 serotype were more extensive and wide-spread (Figs. 6 & 8)

compared to the infection seen in larvae challenged with the O2α serotype (Figs. 5, 7 & 9). The infection was more widely spread throughout the larvae and larval tissues. These are similar findings to the study by Engelsen et al. (in press). In that study cod yolk-sac larvae challenged with the *V. anguillarum* O2 α strain experienced high mortality and the histopathological observations were scarce. These findings indicate the bacterial strains to use different infection mechanisms. It has been shown that *V. anguillarum* serotype O2 does not adhere to mucus as many other fish pathogenic bacteria (Knudsen et al. 1999). Engelsen et al. (in press) suggested excretion of toxins as a possible cause of death.

The *Vibrio splendidus* and *Vibrio salmonicida* strains tested in this present experiment are most likely avirulent strains. Only at the end of the turbot experiment *V. splendidus* challenged larvae experienced a significantly higher mortality compared to the control. This could be caused by the lack of feeding when the yolk-sac stage is coming to an end. No increased mortality was detected in the other larval groups challenge with the same bacterium. As reviewed by (Thompson et al. 2004) *V. splendidus* was originally described as a non-pathogenic strain isolated from the aquatic environment. Avirulent strains of *V. splendidus* have previously been reported in challenge experiments performed on halibut larvae (Verner-Jeffreys et al. 2003) and turbot (60g) (Farto et al. 1999). *V. salmonicida* is associated with mortality at low temperatures and regarded as non-pathogenic to salmon above 10°C (Enger et al. 1991). The temperatures used in the halibut and cod experiments should be optimal for virulence studies including this bacterium. In the turbot study performed on 15°C the temperature could have been too high to cause disease.

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Table 1. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.01$ Bonferonni correction) for 2 x 2 contingency table control vs bacterial strains used in challenge of turbot, *Scophthalmus maximus*. Challenge dose 10^6. All significant p-values in bold. V. ang 610 = *Vibrio anguillarum* strain 610 serotype O2α, V. ang 618 = *Vibrio anguillarum* strain 618 serotype O2β, V. ang 644 = *Vibrio anguillarum* strain 644 serotype O1, V. salm 651 = *Vibrio salmonicida* strain 651, V. spl 1576 = *Vibrio splendidus* strain 1576. D.p.h = days post-hatch. Day 0 = the day the eggs hatched.

	V. ang 610		V. ang 618		V. ang 644		V. salm 651		V. spl 1576	
D.p.h	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
0	1.51	.2192	.00	1.0000	.52	.4697	1.36	.2439	1.51	.2192
1	.00	.9678	.66	.4174	.21	.6492	1.22	.2694	.44	.5095
2	.52	**.0030**	2.42	.1201	.05	.8285	.71	.4005	.00	.9693
3	37.68	**.0000**	5.06	.0244	.66	.4174	.35	.5567	.00	.9678
4	48.24	**.0000**	5.85	.0156	1.00	.3162	.01	.9066	.21	.6469
5	50.55	**.0000**	5.85	.0156	1.42	.2342	.01	.9066	.21	.6469

Table 2. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.01$ Bonferonni correction) for 2 x 2 contingency table control vs bacterial strains used in challenge of turbot, *Scophthalmus maximus*. Challenge dose 10^4. All significant p-values in bold. V. ang 610 = *Vibrio anguillarum* strain 610 serotype O2α, V. ang 618 = *Vibrio anguillarum* strain 618 serotype O2β, V. ang 644 = *Vibrio anguillarum* strain 644 serotype O1, V. salm 651 = *Vibrio salmonicida* strain 651, V. spl 1576 = *Vibrio splendidus* strain 1576. D.p.h = days post-hatch. Day 0 = the day the eggs hatched.

	V. ang 610		V. ang 618		V. ang 644		V. salm 651		V. spl 1576	
D.p.h	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
0	.05	.8231	.07	.7978	1.43	.2312	.01	.9127	.00	.9708
1	1.42	.2342	.00	.9678	.77	.3812	.01	.9066	.03	.8592
2	20.18	**.0000**	.21	.6469	.25	.6161	.11	.7362	1.53	.2162
3	31.11	**.0000**	.43	.5097	2.16	.1413	.30	.5855	6.12	.0134
4	34.70	**.0000**	.43	.5097	2.16	.1413	.56	.4540	7.89	**.0050**
5	40.49	**.0000**	.73	.3921	7.61	**.0058**	.90	.3433	8.84	**.0030**

Table 3. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.01$ Bonferonni correction) for 2 x 2 contingency table control vs bacterial strains used in challenge of halibut, *Hioppoglossus hippoglossus*. Challenge dose 10^6. All significant p-values in bold. V. ang 610 = *Vibrio anguillarum* strain 610 serotype O2α, V. ang 618 = *Vibrio anguillarum* strain 618 serotype O2β, V. ang 644 = *Vibrio anguillarum* strain 644 serotype O1, V. salm 651 = *Vibrio salmonicida* strain 651, V. spl 1576 = *Vibrio splendidus* strain 1576. D.p.h = days post-hatch.

	V. ang 610		V. ang 618		V. ang 644		V. salm 651		V. spl 1576	
D.p.h	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
1	.26	.6111	.00	.9864	.30	.5870	.00	.9933	.00	1.0000
3	.18	.6753	.00	.9763	.21	.6443	.24	.6230	.00	1.0000
5	3.60	.0577	.22	.6353	.68	.4089	.19	.6599	.00	1.0000
7	7.62	**.0058**	.00	.9691	10.38	**.0013**	.19	.6599	.00	1.0000
9	12.26	**.0005**	2.07	.1498	24.23	**.0000**	.19	.6599	.18	.6753
11	23.13	**.0000**	3.84	.0501	34.32	**.0000**	.19	.6599	.18	.6753
13	38.13	**.0000**	3.84	.0501	43.95	**.0000**	.64	.4225	.18	.6753
15	59.26	**.0000**	3.84	.0501	59.70	**.0000**	1.26	.2615	.18	.6753
17	72.16	**.0000**	3.84	.0501	62.21	**.0000**	2.82	.0930	.18	.6753
19	74.97	**.0000**	3.84	.0501	67.43	**.0000**	3.72	.0538	.18	.6753
21	71.51	**.0000**	3.35	.0674	66.74	**.0000**	2.41	.1205	.00	1.0000
23	64.70	**.0000**	4.05	.0443	62.79	**.0000**	.71	.3996	.11	.7412

Table 4. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.01$ Bonferonni correction) for 2 x 2 contingency table control vs bacterial strains used in challenge of cod, *Gadus morhua*. Challenge dose 10^6. All significant p-values in bold. V. ang 610 = *Vibrio anguillarum* strain 610 serotype O2α, V. ang 618 = *Vibrio anguillarum* strain 618 serotype O2β, V. ang 644 = *Vibrio anguillarum* strain 644 serotype O1, V. salm 651 = *Vibrio salmonicida* strain 651, V. spl 1576 = *Vibrio splendidus* strain 1576. D.p.h = days post-hatch. Day 0 = the day the eggs hatched.

	V. ang 610		V.ang 618		V. ang 644		V. salm 651		V. spl 1576	
D.p.h	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
0	.26	.6121	.17	.6767	.16	.6895	1.36	.2433	.00	1.0000
1	.00	1.0000	.00	1.0000	.16	.6895	1.36	.2433	.00	1.0000
2	1.57	.2095	.97	.3254	1.64	.2007	1.36	.2433	.97	.3254
3	3.88	.0490	2.27	.1317	7.87	**.0005**	1.57	.2095	2.27	.1317
4	14.50	**.0001**	2.18	.1396	7.19	**.0074**	3.57	.0589	1.07	.3016
5	20.25	**.0000**	2.01	.1566	9.76	**.0018**	4.05	.0442	.57	.4497
6	23.05	**.0000**	2.63	.1049	16.77	**.0000**	4.05	.0442	.97	.3247
7	29.09	**.0000**	7.49	**.0062**	19.40	**.0000**	4.05	.0442	.97	.3247
8	37.53	**.0000**	7.49	**.0062**	20.77	**.0000**	4.84	.0278	.97	.3247
9	33.57	**.0000**	3.93	.0474	14.91	**.0001**	1.55	.2125	.00	1.0000
10	36.57	**.0000**	4.33	.0375	13.10	**.0003**	1.00	.3162	.00	1.0000

Table 5. Yates-corrected Chi-square (χ^2) values and p-values ($p < 0.01$ Bonferonni correction) for 2 x 2 contingency table control vs bacterial strains used in challenge of cod. *Gadus morhua*. Challenge dose 10^4. All significant p-values in bold. V. ang 610 = *Vibrio anguillarum* strain 610 serotype O2α, V. ang 618 = *Vibrio anguillarum* strain 618 serotype O2β, V. ang 644 = *Vibrio anguillarum* strain 644 serotype O1, V. salm 651 = *Vibrio salmonicida* strain 651, V. spl 1576 = *Vibrio splendidus* strain 1576, D.p.h = days post-hatch. Day 0 = the day the eggs hatched.

	V. ang 610		V. ang 618		V. ang 644		V. salm 651		V. spl 1576	
D.p.h	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value	χ^2	p-value
0	.22	.6425	.24	.6220	1.36	.2433	.00	1.0000	.00	1.0000
1	.00	.9614	.24	.6220	1.36	.2433	.00	1.0000	1.36	.2433
2	.57	.4502	1.01	.3141	.00	1.0000	.97	.3254	.26	.6121
3	1.11	.2918	1.64	.2007	.00	1.0000	1.57	.2095	.26	.6121
4	.13	.7147	.10	.7574	.00	1.0000	.30	.5853	1.19	.2751
5	.73	.3932	.00	.9772	.09	.7630	.07	.7909	1.90	.1685
6	3.01	.0830	.09	.7680	.09	.7630	.27	.6055	1.90	.1685
7	17.68	**.0000**	.30	.5840	.00	1.0000	.97	.3247	1.90	.1685
8	21.86	**.0000**	.30	.5840	.08	.7785	.97	.3247	1.90	.1685
9	36.53	**.0000**	.00	.9443	.57	.4497	.00	1.0000	5.41	.0200
10	34.10	**.0000**	.18	.6755	.48	.4871	.00	1.0000	5.70	.0170

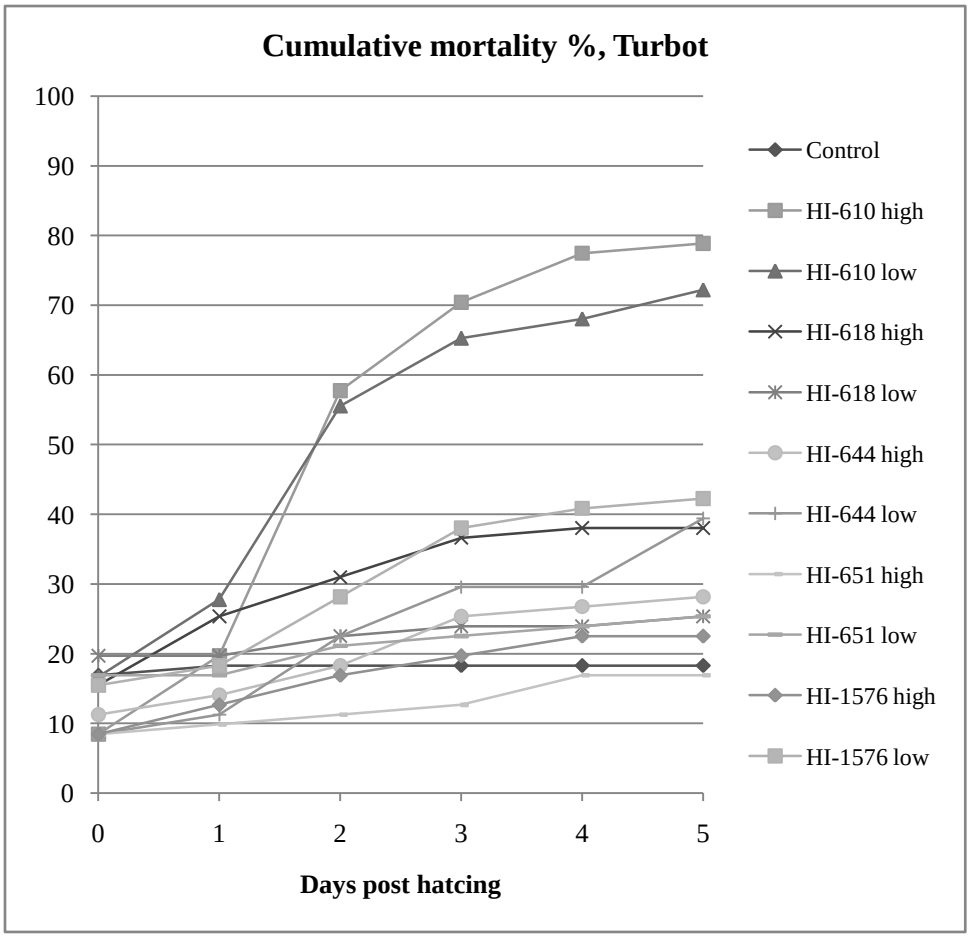

Figure 1. *Scophthalmus maximus*. Cumulative mortality percentage of turbot larvae challenged with the bacterial strais: HI-610 = *Vibrio anguillarum* strain 610 serotype O2α, HI-618= *Vibrio anguillarum* strain 618 serotype O2β, HI-644 = *Vibrio anguillarum* strain 644 serotype O1, HI-651 = *Vibrio salmonicida* strain 651 and HI-1576 = *Vibrio splendidus* strain 1576. high = challenge dose 10^6, low = challenge dose 10^4. Control = unchallenged larvae

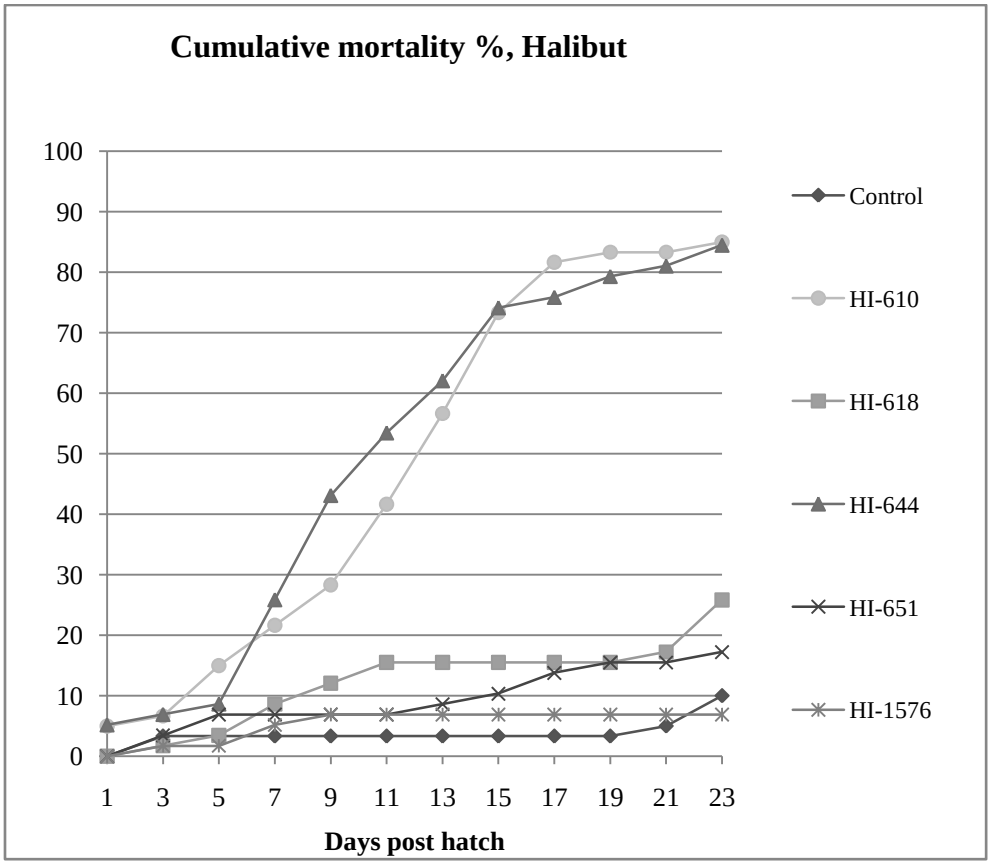

Figure 2. *Hippoglossus hippoglossus*. Cumulative mortality percentage of halibut larvae challenged with the bacterial strains: HI-610 = *Vibrio anguillarum* strain 610 serotype O2α, HI-618= *Vibrio anguillarum* strain 618 serotype O2β, HI-644 = *Vibrio anguillarum* strain 644 serotype O1, HI-651 = *Vibrio salmonicida* strain 651 and HI-1576 = *Vibrio splendidus* strain 1576. Challenge dose 10^6. Control = unchallenged larvae.

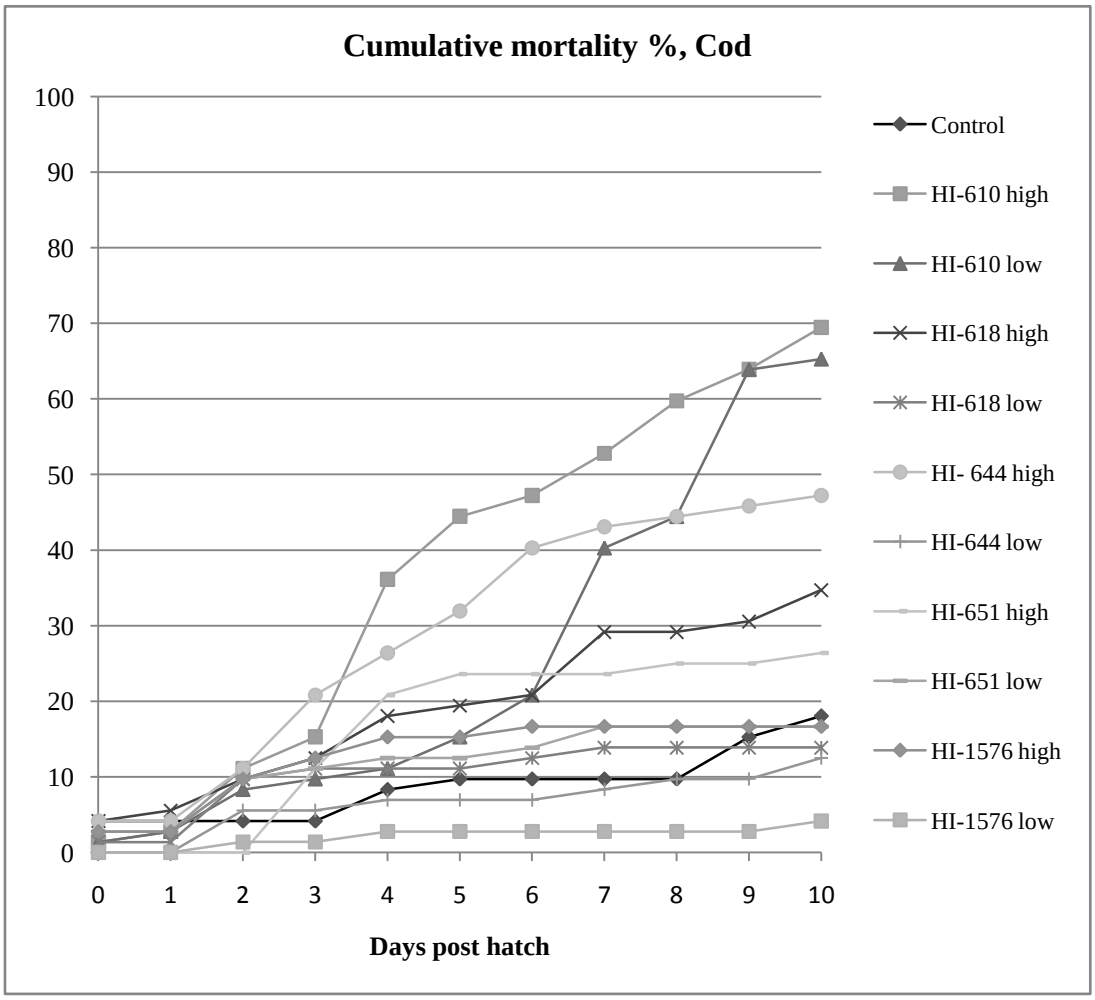

Figure 3. *Gadus morhua*. Cumulative mortality percentage of cod larvae challenged with the bacterial strais: HI-610 = *Vibrio anguillarum* strain 610 serotype O2α, HI-618= *Vibrio anguillarum* strain 618 serotype O2β, HI-644 =Vibrio *anguillarum* strain 644 serotype O1, HI-651 = *Vibrio salmonicida* strain 651 and HI-1576 = *Vibrio splendidus* strain 1576. High = challenge dose 10^6, low = challenge dose 10^4. Control = unchallenged larvae. Day 0 = day of hatching.

Figs. 4 to 9. *Scophthalmus maximus, Hippoglossus hippoglossus, Gadus morhua*. Immunohistochemical staining of paraffin sections from larvae. Avidin-biotin-alkaline phosphatase method, rabbit anti *Vibrio anguillarum* strain HI-610 and *Vibrio anguillarum* HI-644 serum and Shandon haematoxylin counterstained. Postive immunohistochemistry is visualized by red colour. Counterstaining gives tissue different tones of blue. Fig 4. Turbot, *Scophthalmus maximus* control larvae. No positive immunostaining observed, Magnification 100x, scale bar 100 µm. Fig 5. Turbot larvae challenged with *V. anguillarum* HI-610. Positive immunostaining of bacterial cells in urine bladder (arrow). Magnification 1000x, Scale bar 10 µm. Fig 6. Halibut, *Hippoglossus hippoglossus*, challenged with *V. anguillarum* HI-644, head and brain area. Bacterial cells are seen in between the brain and dermis (arrow). Magnification 1000x, scale bar 10µm. Fig. 7. Halibut challenged with *V. anguillarum* HI-610. Head area. Magnification 1000x, scale bar 10 µm. Fig. 8. Cod, *Gadus morhua*, challenged with *V. anguillarum* HI-644. Positive immunostaining in dermis and muscle tissue (arrow). Magnification 400x, scale bar 50 µm. Fig. 9. Cod challenged with *V. anguillarum* HI-610. Positive immunostaining of bacterial cells in intestine (arrow). Magnification 1000x, scale bar 10 µm.