Targeted approaches towards controlling the microbial gut flora in first feeding fish larvae

Final Report on the Seafish / BMFA project (April 2005 – February 2006)

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DETAILS OF THE PROJECT

Title : Targeted approaches towards controlling the microbial gut flora in the first feeding fish larvae

Project code : 20411
Contract Reference Number : *
Commencement Date : April 1st 2005
Completion Date: 28 February 2006
Scientific Objectives:

To deliver a report that will cover the following:

1. Specificity of *V. splendidus* DMC-1 infection
2. To determine whether hly- mutants of *V. splendidus* can act as model probiotic bacteria and protect larvae, and other susceptible larvae, from virulent wild-type *V. splendidus* DMC-1.
3. To determine whether naturally occuring hly- *V. splendidus* can act as probiotic bacteria
4. Effectiveness of egg disinfection methods against viruses
5. Live feed disinfection
6. Recolonisation of Artemia after disinfection
7. Testing potential probiotic organisms for their ability to protect larvae from *V. splendidus* DMC-1.

1. Specificity of infection of *Vibrio splendidus* DMC-1

We isolated *V. splendidus* DMC-1 from a larval rearing "crash" in a Spanish turbot hatchery and have shown that it is lethal to turbot larvae only when administered on live food. It will make an ideal organism for studying bacteria-larvae interactions, especially involving probiotics, as we have shown precisely how this organism kills turbot larvae – by secreting a cytolytic toxin (haemolysin) of the same family as a toxin involved in gastroenteritis in humans. Damage to cells lining the larval gut may either be lethal in itself or it may fatally impair nutrient uptake across the gut at a crucial stage of development.

In this work package we wish to know whether similar bacteria may be causing losses of other larval species and will test (a) whether *V. splendidus* DMC-1 kills halibut and cod larvae at the first feeding stage, and (b) if the collection of *V. splendidus* isolates we have made from cod [Reid, unpublished] and halibut larvae [Verner-Jeffreys et al. 2003b] are similar to *V. splendidus* DMC-1 and may provide some explanation for the gradual losses of larvae seen in some rearing batches.

2. To determine whether hly- mutants of *V. splendidus* can act as model probiotic bacteria

The hly- transposon mutants of *V. splendidus* DMC-1 grow at the same rate as DMC-1 in broth cultures and appear only to lack production of the haemolysin. Therefore, this mutant provides an ideal model to determine if a harmless organism with the same *in vitro* growth characteristics as the pathogen can limit growth of the pathogen in the larval gut to a level that is harmless.

To test this hypothesis, turbot larvae will be exposed for 5 days to rotifers or *Artemia* that have been incubated in a suspension of hly- *V. splendidus*. Control groups will be exposed to normal cultures of rotifers or *Artemia* and both groups will then be challenged by exposure to rotifers or *Artemia* containing virulent *V. splendidus* DMC-1. Mortalities will be compared, larvae fixed for histology to determine whether enteritis is evident, and the bacterial flora analysed to determine the proportions of hly+ (*V. splendidus* DMC-1) and hly- (*V. splendidus* hly- mutant) bacteria present.

3. To determine whether naturally occurring hly- isolates of *V. splendidus* can act as probiotic bacteria

The above transposon mutants provide an excellent model system but would not be considered suitable for use as probiotics themselves. Therefore, we will also test a range of hly- *V. splendidus* previously isolated from cod, halibut and turbot larval rearing experiments. Those from turbot have already been shown to be avirulent in larval turbot [Munro et al. 1995]. These will be tested in experiments conducted as above for their ability to protect larvae from pathology and mortalities caused by virulent *V. splendidus* DMC-1.

4. Effectiveness of egg disinfection methods against viruses

Egg disinfection has been primarily targeted against bacteria using agents such as glutaraldehyde, Kick-start and Pyceze. However, viruses are important pathogens of cod and halibut larvae and it is possible that these agents are transmitted vertically [Breuil et al., 2002]. However, it is not clear whether virus is adhering externally to eggs or has been internalised. Otter Ferry have expressed a specific interest in this area and it will be included as a sub-project funded by a summer studentship. In this work package we will : -

4a. test the above current egg disinfectants, and Virkon as a control, for their ability to inactivate IPN virus and nodavirus in free solution. This will be done by exposing suspensions of virus to these agents for different time intervals and determining residual virus by tissue culture assay. We currently hold stocks of IPN virus and have regularly assayed it in tissue culture. Similar assays will be developed for nodavirus [Aranguren et al. 2002].

4b. Determine whether IPN virus and nodavirus adhere to eggs by mixing suspensions of the viruses with eggs, after incubation, washing to remove loosely attached virus, homogenisation and assay on tissue cultures using TV-1 cells [Aranguren et al. 2002].

4c. If time and resources permit, set up established RT-PCR assays [Johansen et al. 2004] for nodavirus and IPN virus to assay for virus in egg homogenates.

5. Live feed disinfection

Reduction in overall bacterial load on live food organisms is important in achieving good survival of larvae by reducing the numbers of harmful organisms present. A number of methods have been devised for reducing the bacterial load of rotifers, e.g. UV irradiation [Munro et al. 1993; 1999] and *Artemia*, e.g. using Pyceze [Treasurer 2004] or phylatol [Birkbeck and Barbour, unpublished] but it is necessary to identify the most practical measures for routine use in the context of commercial hatcheries.

6. Recolonisation of *Artemia* after disinfection

One of the problems identified with general reduction of bacterial levels on live food is the rapid re-growth of organisms that occurs with the possibility that rapidly growing harmful organisms, e.g. vibrios may come to dominate the flora of *Artemia* or rotifers [Munro et al, 1999].

In this work package we will use the live food organisms and methods developed with the hatcheries to reduce bacterial loading of live food and recolonise with mixtures of selected bacteria isolated from larval rearing systems and shown to be harmless to halibut [Verner-Jeffreys et al. 2004], turbot [Munro et al. 1995; Thomson et al. 2005] or cod larvae [Reid and Birkbeck 2005a]. The stability over 24 hours of the flora of rotifers and *Artemia* recolonised in this way will be compared with the original flora. We have recently developed a Denaturing Gradient Gel Electrophoresis method to identify vibrios [Reid and Birkbeck 2005b] and with this it will be possible to monitor changes in vibrio populations rapidly. Those bacteria which can produce stable population changes will be evaluated in laboratory challenge experiments against cod or halibut larvae to determine (a) that they are not harmful to larvae and (b) whether they can protect larvae from challenge by pathogens such as *V. splendidus* DMC-1.

7. Testing potential probiotic organisms for their ability to protect larvae from *V. splendidus* DMC-1

Organisms identified as potential probiotics in Work Package 6 will be tested for their ability to protect larvae from challenge with *V. splendidus* DMC-1 in trials such as described above (sections 2 and 3, above).

PROJECT COSTS

Seafish	£15656
BMFA (co-funder)	£ 4000

TOTAL STAFF INPUT

Dr Helen Reid (post-doctoral scientist)	7 months x full time	= 0.58 person-years
Mrs. Irene Houghton (technician)	4 months x half time	= 0.16 person-years
Also working on the project but not funded by it:		
Mrs. B. Adam (technician)	7 months x 0.3	= 0.18 person-years
Damien Maura (placement student)	10 wks full time	= 0.2 person-years
Beatrice Darde (placement student)	10 wks full time	= 0.2 person-years
2 Honours project students	10 weeks x 0.6	= 0.24 person-years
T.H. Birkbeck x 11 months x 0.1		= 0.11 person-years
	Total	= 1.67 person-years

Notes.

1. Dr. Reid was funded for 6 months on this contract and 1 month from other sources. She went on maternity leave in August 2005.
2. After Dr. Reid's departure, and following transfer of Mrs. Adam to a different project, Mrs. Houghton was employed as a part-time technician for 4 months to replace Dr. Reid.
3. Mrs. Adam was not funded on this project and was available only for the first 7 months of the work.
4. Two placement students from the University of Brest made very significant contributions to the project during their 10 week visit.

IPR ARISING FROM THE PROJECT

No technology arising from the project is patentable although the work on disinfection of Artemia with bronopol is likely to be very beneficial in the industry.

EXECUTIVE SUMMARY

The Scottish aquaculture industry is keen to expand the rearing of cod, haddock and halibut as there is strong consumer demand for these species and Scottish waters provide ideal conditions for their culture. However, expansion of the industry requires a reliable and increasing supply of juvenile fish for ongrowing and this is currently a limiting factor in industry development. Bacteria are widely considered to be a major factor in larval losses and the aim of this work is to control the bacterial flora that develops in the digestive tract of the marine fish larvae. This requires a better understanding of the bacterial species present, identification of harmful species, and development of methods to control the composition and total level of bacterial populations. Prior to this project we had shown that a bacterial pathogen (*Vibrio splendidus*) from turbot larvae in Spain killed the larvae by producing an enterotoxin almost identical to an enterotoxin affecting humans. The specific aims of this project were to determine whether similar bacteria exist in British marine hatcheries, to characterise other pathogenic bacteria causing larval losses and to devise with industry effective disinfection methods to control bacterial populations in live feed such as *Artemia*.

Main findings

- *Vibrio splendidus* DMC-1, a pathogen of turbot larvae in Spain also kills cod larvae.
- A survey of *V. splendidus* from British hatcheries showed that isolates from cod (Ardtoe) and halibut larvae (Ardtoe, Otter Ferry and Mannin) contain the same enterotoxin gene as *V. splendidus* DMC-1 responsible for death in larvae, and the same regulatory gene.
- The enterotoxin genes from the UK isolates were closely related and distinguishable from that of the Spanish isolate.
- Disinfectants currently used to treat marine fish eggs in British hatcheries (1/625 glutaraldehyde or 1/250 Kick Start) are effective against IPN virus causing >99% inactivation within 1 minute.
- Pyceze (bronopol) kills bacteria such as the halibut egg pathogen *Tenacibacter ovolyticus* quite slowly. Bronopol at 100 µg ml^{-1} had no significant effect on *T. ovolyticus* within 1 hour. At 200 µg ml^{-1} 90% of bacteria were killed in 24 – 38 min, and > 99.99% killing was achieved within 2 hours.
- For controlling bacterial growth during *Artemia* decapsulation and enrichment, Pyceze (bronopol) is an extremely effective disinfectant, almost eliminating bacteria during decapsulation whilst retaining high viability of *Artemia* without inhibiting enrichment.
- Two bacterial isolates associated with losses of cod larvae in rearing trials at Ardtoe in 2003 were identified as an atypical strain of *Vibrio anguillarum* and *Vibrio logei*, respectively. This is the first report of *V. logei* as a fish pathogen.

Main implications

- It is likely that *V. splendidus* can cause mortalities in larval rearing in UK hatcheries and this would be best controlled by effective disinfection live feed.
- Bronopol is a very effective disinfectant controlling bacterial levels in *Artemia* live feed.
- For marine fish egg disinfection the currently used Kick Start or glutaraldehyde are effective against viruses as well as bacteria.

Action to follow

Bronopol disinfection of live feed should be tested more widely in hatcheries

SCIENTIFIC REPORT

Scientific objectives and primary milestones

The objectives of the work are given in detail in the Project Application. In brief they were:

1. To investigate the specificity of infection of *Vibrio splendidus* DMC-1

Vibrio splendidus was isolated from a number of larval rearing "crashes" in a Spanish turbot hatchery and strain DMC-1 was characterised in detail by Macpherson (2004) who showed that it is kills turbot larvae by secreting a lethal toxin of the same family as aerolysin, a toxin that causes gastroenteritis in humans. We wish to know whether similar bacteria may be causing losses of other larval species and will test:

(a) whether *V. splendidus* DMC-1 kills halibut and cod larvae at the first feeding stage, and

(b) if the collection of *V. splendidus* isolates we have from cod and halibut larvae are similar to *V. splendidus* DMC-1 and may provide some explanation for the gradual losses of larvae seen in some rearing batches.

2. To determine whether hly- mutants of *V. splendidus* can act as model probiotic bacteria

Transposon mutants of *V. splendidus* DMC-1 that do not kill larvae Macpherson (2004) provide an ideal model to determine if a harmless organism with the same *in vitro* growth characteristics as the pathogen can limit growth of the pathogen in the larval gut.

3. To determine whether naturally occurring hly- isolates of *V. splendidus* can act as probiotic bacteria

The above transposon mutants may provide an excellent model system but would not be considered suitable for use as probiotics themselves. Therefore, we will also test a range of hly- *V. splendidus* previously isolated from cod, halibut and turbot larvae.

4. Effectiveness of egg disinfection methods against viruses

Previously, egg disinfection has been primarily targeted against bacteria. However, viruses are important pathogens of cod and halibut larvae and we aim to : -

4a. test current egg disinfectants, for their ability to inactivate IPN virus and nodavirus.

4b. Determine whether IPN virus and nodavirus adhere to eggs.

4c. Set up established RT-PCR assays for nodavirus and IPN virus.

5. Live feed disinfection

To identify the most practical measures for routine live feed disinfection in hatcheries.

6. Recolonisation of *Artemia* after disinfection

To use the live food organisms and methods developed with the hatcheries to reduce bacterial loading of live food and recolonise with mixtures of selected bacteria isolated from larval rearing systems and shown to be harmless to halibut, turbot or cod larvae.

7. Testing potential probiotic organisms for their ability to protect larvae from *V. splendidus* DMC-1

Organisms identified as potential probiotics in Work Package 6 will be tested for their ability to protect larvae from challenge with *V. splendidus* DMC-1 in trials such as described above (sections 2 and 3, above).

RESULTS OF THE RESEARCH

Introduction

The purpose of this project was to provide basic scientific information to allow a better understanding of how the microbial flora of fish larvae develops and how it could be controlled so as to improve larval rearing success. The main effort was focused on the above sections 1, 4, 5 and 7. This was due in part to the earlier than expected departure of Dr. Reid on maternity leave and the need for a more stable aquarium challenge system to carry out sections 2 and 3 of the work. However, the success of bronopol in *Artemia* disinfection (section 5) in allowing virtually complete elimination of bacteria at the live food enrichment stage reduced the importance of the probiotic approach. The larval challenge trials carried out at the beginning of the project showed that we had identified two new pathogens of cod larvae and it was considered important to characterise these pathogens as part of the project.

1. Pathogens in marine larval fish rearing and specificity of infection of *Vibrio splendidus*

The normal microbial flora of turbot, halibut, cod

Several studies have described the digestive tract bacterial flora that develops from first feeding to weaning in turbot (Nicolas et al., 1989; Munro et al., 1993, 1995; halibut (Bergh et al. 1994; Verner-Jeffreys et al., 2003; Jensen et al., 2005) and cod (Reid et al., BBSRC report 2005). In all cases, bacterial numbers are low or absent prior to first feeding after which time bacterial numbers rise rapidly to $10^5 – 10^6$ bacteria per larva due to bacteria introduced on the rotifers or *Artemia*. When rotifers are the initial food the flora is diverse and contains many non-vibrios. However, once *Artemia* are given as food the flora quickly changes to become dominated by vibrios, a pattern that continues, albeit with changes in the vibrio species present, through to weaning on to dry food. The larval gut provides a selective environment (nutrients, pH, etc.) that favours growth of particular bacteria. Therefore, those bacteria that are dominant on the live feed, e.g. *Vibrio alginolyticus* in *Artemia* may be virtually absent in the larval gut in favour of *V. splendidus* (Thomson et al. 2005), an organism present in low numbers in *Artemia* cultures.

In early studies on mortalities in marine fish larval rearing, recognised pathogens of adult fish were not identified as the cause of poor larval rearing performance (Nicolas et al., 1989; Munro et al., 1993, 1995). However, within the last few years a number of marine vibrios have been identified as pathogens of marine fish larvae and these are noted below.

Pathogens in larval marine finfish rearing

Vibrio anguillarum

Vibrio anguillarum is a pathogen capable of causing high mortalities in a very wide range of fish (Austin and Austin, 1999; Inglis et al, 1993) and it has been associated with mortalities in juvenile and larval turbot, particularly in Northern Spain (Toranzo et al., 1997). More recently, losses of cod larvae have been attributed to *V. anguillarum* in Norway. As part of the current project we have characterised *V. anguillarum* strains causing losses in Norway (strain 270-04) and at Ardtoe (n8d10).

Characterisation of *Vibrio anguillarum* isolates from Atlantic cod larvae in this project

Isolates Norway 270-04 and Ardtoe n8d10 were identified as being *V. anguillarum* from partial sequences of the 16S rRNA gene and they were also compared with reference strains of *V. anguillarum*, ATCC 19264 (the type strain) and NCIMB 829, in approximately 50 standard biochemical tests. These tests confirmed that isolate 270-04 was a typical strain of *V. anguillarum* closely related to the culture collection reference strains and other virulent isolates we have characterised from juvenile cod (Jones et al., 2000) over the whole range of tests used. The Ardtoe isolate n8d10 from moribund cod larvae appears atypical and it is

distinctly different from the reference strains in several tests. It reacted conventionally in the key tests for *V. anguillarum* (e.g. fermentative metabolism of glucose, sensitive to the vibriostatic agent O/129, oxidase and catalase positive, nitrate reduction and being arginine dihydrolase positive). However, it was unusual in being unable to utilise sorbitol or sucrose as carbon sources and in its ability to grow at 4°C (270-04 also grew at 4°C whereas the reference strains did not). On TCBS agar isolate n8d10 produced green colonies (sucrose negative) whereas the other *V. anguillarum* strains fermented sucrose and gave yellow colonies. Neither the Ardtoe isolate n8d10 nor Norway isolate 270-04 belonged to the common serotypes O1 or O2 and they may be forwarded to Denmark to see typing is possible with the more extensive schemes available there (Pedersen et al. 1999).

Isolate n8d10 is haemolytic when cultured on horse blood agar and the haemolysin has properties similar to that of *V. anguillarum* ATCC 19264 in being a pore-forming haemolysin. Further evidence that n8d10 is indeed *V. anguillarum* was sought by PCR amplification of a section of the haemolysin gene. The 480 bp PCR product formed using the primers of Hirono et al. (1996) was of the same size and a similar sequence to that found by Hirono et al. (1996). The one difference in the 160 amino acid translation of the PCR product was leucine in place of isoleucine, the same difference being found with the type strain ATCC 19264. In summary, isolate n8d10 is a true *V. anguillarum* although its properties differ from those of strains normally associated with vibriosis in cod.

To test whether these isolates were lethal to larvae, cod larvae were transported to Glasgow from Seafish Ardtoe or Machrihanish and maintained for up to 7 days in individual wells of tissue culture plates. Control larvae, fed on enriched untreated rotifers, showed good survival (Figure 1). When cod larvae were fed on rotifers previously incubated with *V. anguillarum* NOR270-04 mortalities began on day 2 and rose to 60% by day 7, indicating that this organism is highly pathogenic for cod larvae. Interestingly, another *V. anguillarum* isolate (91079) that was highly pathogenic for turbot larvae (Munro et al., 1995) appeared much less pathogenic for cod larvae (Figure 1).

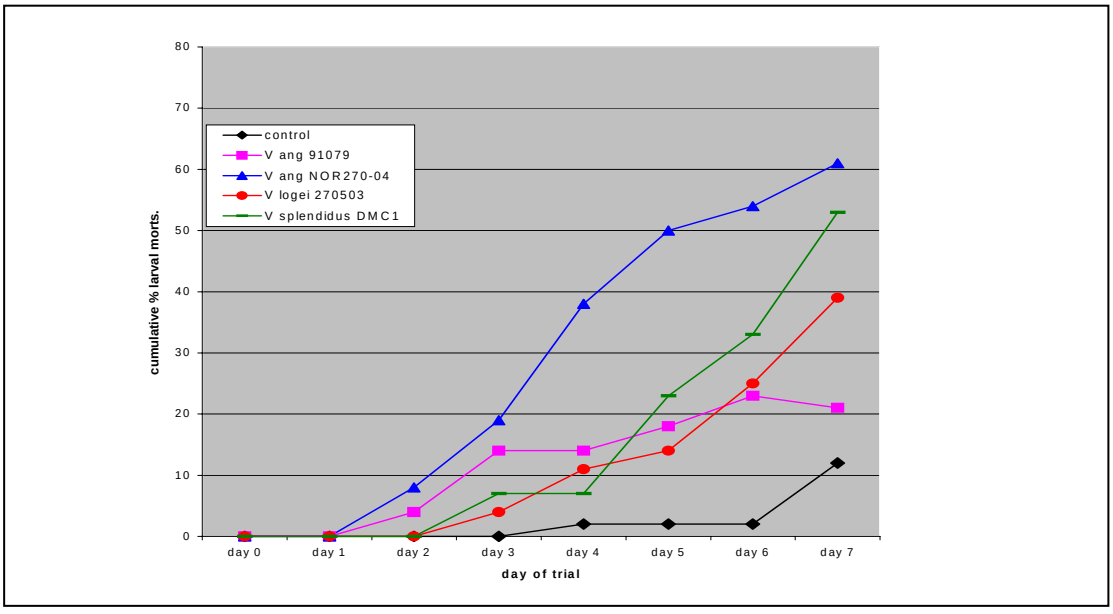

Figure 1. Mortalities induced in larval cod exposed to various bacteria. Control, no added bacteria; V ang 91079, *V. anguillarum* isolated from juvenile turbot; V ang NOR270-04, *V. anguillarum* isolated from moribund larval cod, Norway, 2004; V. logei 270503, *V. logei* isolate c1 from dying cod, Ardtoe 270503; V splend DMC-1, *V. splendidus* DMC-1.

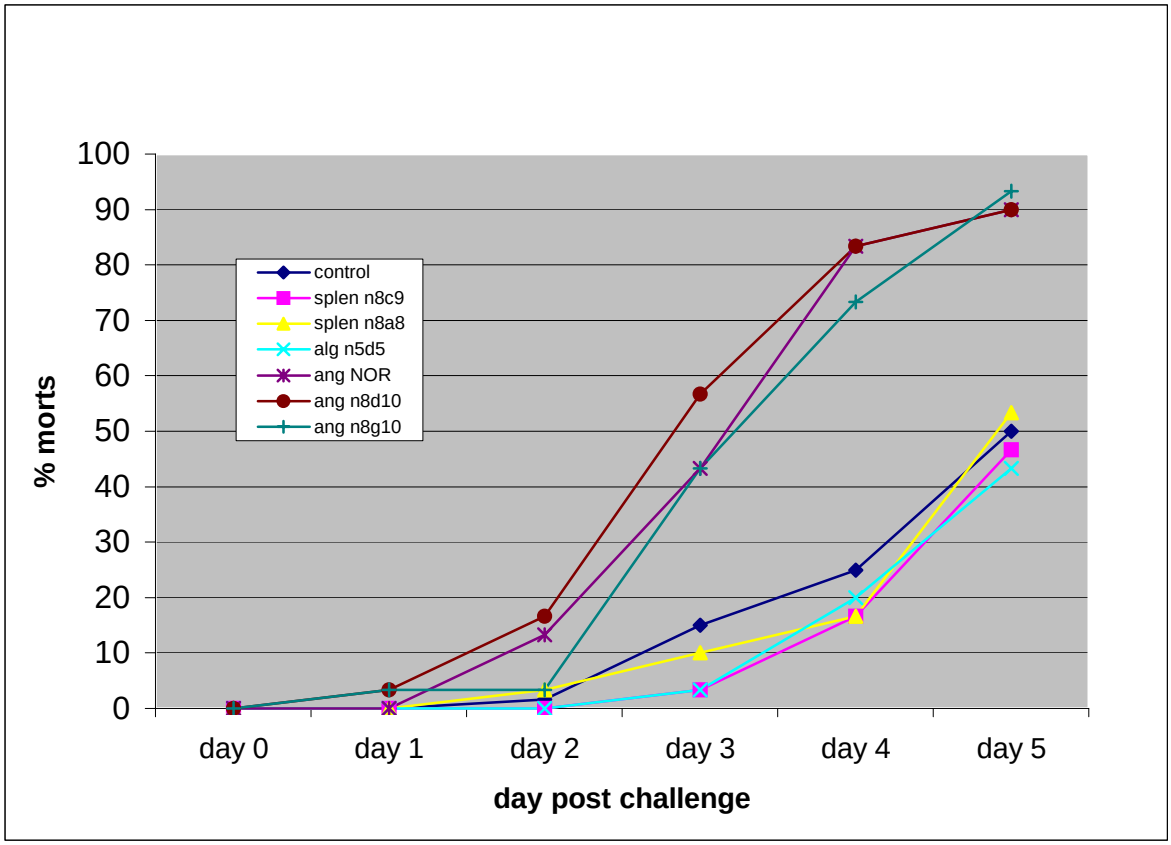

Figure 2. Mortalities induced in larval cod exposed to various bacteria isolated from cod larvae at Ardtoe. All isolates except *V. anguillarum* NOR270-04 were isolated from cod larvae at Ardtoe during a period of high mortalities in one rearing tank. Control, no added bacteria; splen n8c9 and splen n8a8, *V. splendidus*; alg n5d5, *V. alginolyticus*; ang NOR, *V. anguillarum* 270-04 (see Legend to Figure 1); ang n8d10 and ang n8g10, *V. anguillarum.*

In a second trial the virulence of *V. anguillarum* NOR270-04 was confirmed (Figure 2), and representative isolates of *V. anguillarum* from Ardtoe (n8d10 and n8g10) appeared equally virulent towards cod larvae.
V. anguillarum n8d10 was used as a control organism in 3 further trials and it was virulent towards cod larvae on every occasion. Ten further isolates of *V. anguillarum* from the Ardtoe rearing system were tested (Table 1). Of these, 7 very highly virulent, 2 were of low virulence and 1 was avirulent. The virulent organisms included two (n3c6 and n4b11) obtained from apparently healthy larvae on 070403, a time when no undue mortalities were found.

Route of infection by *V. anguillarum*
Macpherson (2004) showed that *Vibrio splendidus* must be delivered to the turbot larvae on live food so as to cause damage to the intestinal tract and subsequent mortalities. To determine whether the same applied for *V. anguillarum*, a trial was conducted in which cod larvae were challenged with *V. anguillarum* 270-04 or n8d10 added directly to the surrounding water or after incubation with rotifers for 1 hour (the standard challenge method). Both strains of *V. anguillarum* caused high mortalities when added directly to the water, as found for other isolates of this organism (O. Bergh, personal communication).

Table 1. Virulence against cod larvae of *V. anguillarum* isolates obtained from the Ardtoe cod hatchery

Isolate and isolation date	Trial number	virulence against cod larvae
NOR 270-04 (Norway 2004)	1, 2 and 5	high
91079 (turbot, Horne et al. 1979)	1	low
n8d10 (Ardtoe 270503, tank 8)	2, 3, 4 and 5	high
n8g10 (Ardtoe 270503, tank 8)	2	high
n8b11 (Ardtoe 270503, tank 8)	4	high
d1 (Ardtoe 270503, tank 8)	4	high
e1 (Ardtoe 270503, tank 8)	4	high
e2 (Ardtoe 270503, tank 8)	4	high
n8b7 (Ardtoe 270503, tank 8)	4	avirulent
n3c6 (Ardtoe 070403, tank 3)	4	high
n4b11 (Ardtoe 070403, tank 4)	4	high
n3a3 (Ardtoe 170303, tank 3)	4	low
n3e1 (Ardtoe 170303, tank 3)	4	low

Virulence is compared according to the statistical significance of the difference between survival in control and test groups of larvae.
High virulence= P value 0.1 – 1%
Low virulence = P value < 5%
Avirulent = P value > 5%

Vibrio logei

Whilst the bacterial flora of cod larvae was being monitored intensively during 2003 at Ardtoe we were fortunate to sample during a period when large scale mortalities occurred in one rearing tank. This resulted in identification of the above *V. anguillarum* isolates coded n8 (i.e. n8d10, etc.) but other isolates from the same samples were tentatively identified by denaturing gradient gel electrophoresis (DGGE) and PCR as *V. logei* or possibly the closely related *V. wodanis*. These bacteria were investigated in more detail in the current study, and a representative isolate (*V. logei* c1 270503) was found to be lethal for cod larvae (Figure 1). Further identification of isolate c1 270503 was done by comparison with type strains using over 50 biochemical identification tests and reference data from the thesis of Lunder (1992). Isolate c1 270503 was confirmed as being *V. logei* and was differentiated from *V. wodanis* on the basis of 7 key biochemical tests (indole production, production of acid from cellobiose, growth in 7% NaCl, lysine decarboxylase activity, degradation of starch, utilisation of mannitol and possession of gelatinase activity). This is the first time *V. logei* has been identified as a pathogen of marine fish larvae.

Vibrio splendidus

Vibrio splendidus has been identified as a pathogen of turbot larvae in French (Gatesoupe et al., 1999) and Spanish hatcheries (Thomson et al. 2005). It was shown by Macpherson (2004) in the EU PROBE project that *V. splendidus* strain DMC-1, originally isolated by Thomson *et al.* (2005), was lethal to turbot larvae only when administered on live food and not via water. Furthermore, Macpherson (2004) showed that *V. splendidus* DMC-1 produces a toxin of the aerolysin family (aerolysin is an enterotoxin causing gastroenteritis in humans) and that if the toxin gene is inactivated the bacteria are no longer harmful to turbot larvae. Production of

toxin appears to be controlled by an adjacent ToxR-like gene (ToxR regulates expression of virulence factors in *V. cholerae* according to environmental conditions).

Virulence of *V. splendidus* towards cod larvae

To determine whether *V. splendidus* might be important in UK hatcheries and may provide some explanation for the gradual losses of larvae seen in some rearing batches, cod larvae were exposed to rotifers colonised with *V. splendidus* DMC-1. As shown in Figure 1, high mortalities were induced in cod larvae showing that this bacterial isolate could kill cod larvae under the normal rearing temperatures for such larvae. Thomson et al. (2005) and Nicolas et al. (1999) both found that some strains of *V. splendidus* were virulent towards turbot larvae whereas others were not virulent. A similar situation was found here in that two *V. splendidus* isolates from the same larvae as the virulent *V. anguillarum* isolates did not cause mortalities in cod larvae (Figure 2). Halibut larvae were not available during the project and the virulence of DMC-1 against halibut larvae will be tested when they are available.

Presence of the aerolysin and ToxR-like regulatory gene in British isolates of *V. splendidus*

To determine whether isolates of *V. splendidus* from British hatcheries contained the aerolysin and ToxR-like genes required for virulence in *V. splendidus* DMC-1 (Macpherson 2004) a PCR assay was developed to screen a collection of isolates of *V. splendidus* from UK hatcheries stored from previous projects on cod [Reid, BBSRC project] and halibut larvae [Verner-Jeffreys et al. 2003, BMFA and FIN22 projects]. From 45 *V. splendidus* isolates from the digestive tract of Ardtoe cod larvae that were tested, 30 contained the haemolysin (toxin) gene and 3 of these (of 4 tested) contained the ToxR-like gene. A limited number of isolates from halibut were also tested; the haemolysin and ToxR-like genes were detected in all 3 "group 2" isolates [Verner-Jeffreys *et al.* 2003] tested. Nucleotide sequencing showed that the PCR products produced formed a closely related family distinguishable from the *V. splendidus* DMC-1 haemolysin and the aerolysin formed by *Aeromonas* species (Figures 3 and 4).

The entire ToxR-like gene has been sequenced for 5 isolates. Most of the sequence is conserved but a region with deletions of up to 24 amino acids was detected (Figure 5). The significance of this is not yet clear, but the closest identity was between strains from Ardtoe taken 5 years apart, from halibut and cod larvae, respectively (SFF2-20 and n8c9g5). Interestingly, isolates from the same batch of halibut larvae produced different ToxR-like sequences (SFF2-13 and SFF2-20), showing the diversity in *V. splendidus* populations. Larvae were fed on *Artemia* in all cases.

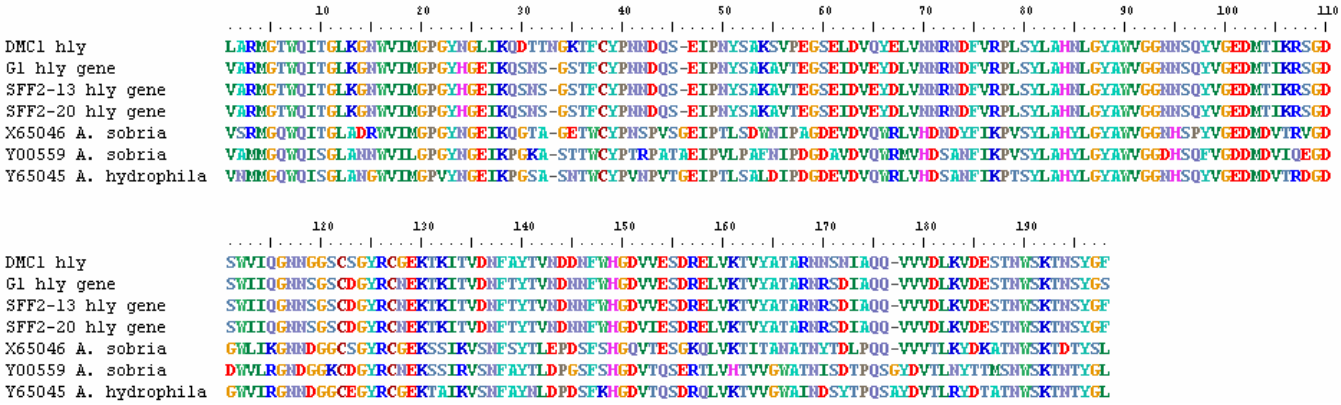

Figure 3. Comparison of the amino acid sequences of the region of the haemolysin gene amplified by PCR with those of known aerolysin genes.

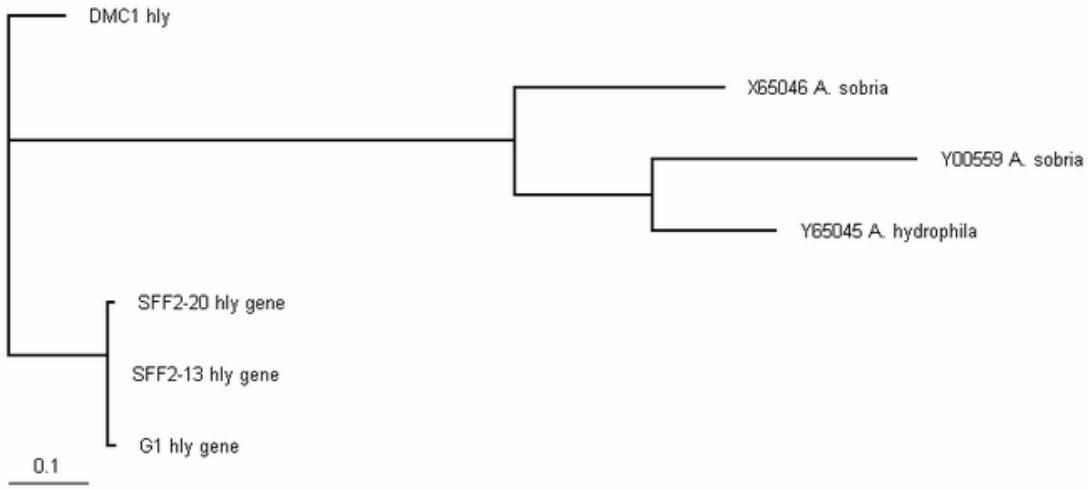

Figure 4. Dendrogram showing the relationship between the haemolysin (aerolysin) genes of *V. splendidus* DMC-1, SFF2-20, SFF2-13 and G1 (n8c9g5) with those of *Aeromonas sobria* and *A. hydrophila*.

Strain	Origin	Amino acid sequence at "deletion" region
SFF2-20	halibut UK -Ardtoe	SLSHTSSLGHSSTLGNRCSQGHDSSQGQSSI
n8c9g5	cod UK -Ardtoe	SLSHTSSLGHSGTLGNRCSLGHDSSQGQSST
SFF2-13	halibut UK -Ardtoe	SLSHTSSLG~~~~~~NRCSQGHDSSQGQSSI
MFF1-13	halibut UK -Mannin	SLSHTSSLGHSSTLG~~~~~~~~~~~~HESI
OFA1-19	halibut UK –Otter Ferry	PLDH~~~~~~~~~~~~~~~~~~~~~~~~SNI
DMC-1	turbot Spain	PL~~~~~~~~~~~~~~~~~~~~~~~~GQSNI

Figure 5. Comparison of the predicted amino acid sequence of the variable (deletion) region of the ToxR-like regulator of 6 *V. splendidus* isolates from 1 Spanish and 4 UK hatcheries.

DMC-1 was also shown to be pathogenic for cod larvae, the mortalities rising rapidly to 55% by day 7. The difference in mortality patterns between these organisms is explained because *V. anguillarum* is capable of invading and killing the larvae directly from the surrounding water. However, a few days must elapse before *V. splendidus* is accumulated in sufficient quantity in the intestinal tract and the enterotoxin produces enough damage to cause death. Plans to test the virulence of *V. splendidus* on halibut larvae were postponed until 2006 as insufficient numbers of larvae were available in 2005.

The conclusions from this section of the work are that *V. splendidus* DMC-1 can kill cod as well as turbot larvae and similar organisms are widely distributed in British hatcheries. It has not yet been shown that the isolates from British hatcheries are harmful to larvae but it is highly probable that *V. splendidus* could cause larval losses similar to those in turbot hatcheries.

Vibrio pelagius

This organism has been reported as a pathogen of turbot larvae in Spain (Villamil et al. 2003) but it has not yet been encountered in UK hatcheries.

Atypical furunculosis in haddock

An outbreak of atypical furunculosis was identified in juvenile haddock at Ardtoe in 2003 and because this was the first such report in haddock the causative strain of *Aeromonas salmonicida* was characterised by staff at Ardtoe, Marine Harvest and Glasgow University. Final characterisation has been completed during 2005/6 with the demonstration that the *A. salmonicida* isolate does cause mortalities on experimental infection of juvenile haddock with a median lethal dose of circa 10^6 bacteria. When the experiment was terminated, *A. salmonicida* was isolated from all 3 of the unchallenged cohabitants in the trial although there were no mortalities or obvious adverse effects in this group of fish.

Viruses

Nodavirus, aquareovirus and IPN virus are capable of causing serious losses in larval rearing. Nodavirus can cause viral encephalopathy and retinopathy (VER) in over 30 marine species worldwide (Munday and Nakai, 1997) including Atlantic halibut (Grotmol et al., 1997; Starkey et al., 2000) and Atlantic cod. Similarly, IPN virus has a wide host range including those species of interest in UK marine aquaculture.

Controlling bacteria in marine larval hatcheries by disinfection

Egg disinfection

Effectiveness of egg disinfection methods against viruses and bacteria

Egg disinfectants such as glutaraldehyde and Kick-start have previously been tested primarily against bacteria. However, IPN virus, aquareovirus and nodavirus are important pathogens of cod or halibut larvae, and Otter Ferry Sea Farms have expressed a specific interest in the effectiveness of disinfectants against such viruses.

We have confirmed that IPN virus is inactivated (>99%) within 1 minute exposure to glutaraldehyde (1/625), Kick Start (1/250) or Virkon (0.5%). The concentrations of glutaraldehyde and Kick Start are those used by Otter Ferry. Virkon was included only as a control as it is not used in egg disinfection.

PCR primers against IPN virus, aquareovirus and nodavirus have been prepared for future monitoring experiments as there was insufficient time for such work during this project.

The only bacterial pathogen known to cause serious losses of marine fish eggs is *Tenacibacter ovolyticus*, identified as an important pathogen of halibut eggs by Hansen (Hansen and Olafsen 1989; Hansen et al., 1992). Therefore, bronopol and Virkon were tested against several marine bacteria including *T. ovolyticus*. Bronopol at 50 or 100 µg ml^{-1} had no significant killing effect on *T. ovolyticus* within 1 hour. At 200 µg ml^{-1} the decimal reduction time ((D value; time to reduce bacterial concentration by 1 log, i.e. 90% killing) was approx 24 – 38 min, and > 99.99% killing was achieved within 2 hours.

Virkon at 0.05 or 0.1% (0.5 and 1 mg ml^{-1}) had no significant effect on *T. ovoloyticus* but 0.5% Virkon gave > 97% killing within 5 min, > 99% within 15 min and > 99.99% within 1 h. Virkon was slightly more effective against *V. anguillarum*, a 0.5% solution killing 99.9% of bacteria within 5 min and > 99.99% within 15 min. In contrast to bronopol, the MIC of Virkon was similar to the lethal dose, ~ 0.5 % against a range of bacteria including *T. ovolyticus* and *V. anguillarum*. [The concentration recommended for use of Virkon as a disinfectant is 1%]

Live feed disinfection

A major conclusion from the work showing that *V. splendidus* can kill turbot and cod larvae when ingested on food organisms is that the bacterial load on *Artemia* and rotifers should be minimised. We first studied this problem in 1993 in work funded by Golden Sea Produce and introduced phylatol as a disinfectant that was very effective in reducing bacterial contamination of *Artemia* post-enrichment. Phylatol disinfection markedly enhanced rearing success with larval turbot and the work remained confidential to GSP and their partners. As phylatol is no longer available this confidentiality is no longer relevant and, in collaboration with Otter Ferry, a significant effort has been put into finding a replacement for phylatol.

The use of Pyceze (50% solution of bronopol) for disinfection of cod eggs and live feed (rotifers and *Artemia*) was reported to BMFA in 2004 by Treasurer et al. (2004). As part of that study (funded by Novartis) we measured the minimum inhibitory concentration (MIC) of bronopol against a range of bacteria from cod and halibut eggs. The MICs ranged from 1-32 μg ml^{-1} for marine bacteria, with vibrios being more susceptible than most bacteria (Birkbeck et al., 2006). However, much greater concentrations of bronopol are required to kill bacteria within a reasonable time scale and in this study Beatrice Darde has shown the decimal reduction time for bronopol acting on *V. splendidus* at 20^{o}C is very similar to those published for *Escherichia coli* at 37^{o}C (Shepherd *et al.* 1988). For 200 μg ml^{-1} bronopol, 90% killing takes approximately 30 min; for 100 μg ml^{-1}, 50 min; for 75 μg ml^{-1}, 70 min; for 50 μg ml^{-1}, 120 min.

At Otter Ferry, David Patterson has tested 150 μg ml^{-1} Pyceze (75 μg ml^{-1} bronopol) as a replacement for INVE Hatch Controller as this is no longer available. In collaboration with Otter Ferry the effectiveness of this method was tested under controlled conditions. After decapsulated *Artemia* had been aerated overnight in seawater + 75 μg ml^{-1} bronopol in an open tank we found them to be completely free of viable culturable bacteria; over 100 seawater-rinsed *Artemia* nauplii added directly to marine broth gave no bacterial growth within 5 days showing that it is possible to eliminate bacteria at this stage of the live feed production process..

Pyceze and phylatol were then compared as disinfectants during enrichment of *Artemia.* Phylatol is normally used as a 1-hour rinse post-enrichment but here it was incorporated during enrichment at Otter Ferry. *Artemia* were hatched in the presence of bronopol as above, and 0.24 ml phylatol L^{-1} was added to an enrichment culture in the morning and afternoon; 0.15 ml Pyceze L^{-1} (75 μg ml^{-1} bronopol) was added to a parallel enrichment culture only in the morning. After 24 h the bacterial concentrations in the whole culture fluid and on rinsed *Artemia* were measured. As shown in Table 2, phylatol was relatively ineffective in controlling the concentration of bacteria yet none were detected in the bronopol-treated culture. In both cases the concentration of bacteria associated with washed *Artemia* was low.

Table 2. Concentration of culturable bacteria in tank water and associated with *Artemia* in Otter Ferry enrichment cultures disinfected with phylatol or Pyceze.

Disinfectant	concentration	concentration of bacteria in culture fluid (cfu ml^{-1})	concentration of bacteria in washed *Artemia* (cfu/nauplius)
Phylatol	0.24 ml L^{-1}	5 x 10^{6}	200
Bronopol	0.15 ml L^{-1} Pyceze	<10	4

The effect of different concentrations of bronopol on bacterial concentrations during enrichment was tested in the laboratory. *Artemia* hatched in the presence of 75 µg ml^{-1} bronopol were collected on a filter, washed with sterile seawater and dispensed into sterile Falcon tubes in 25 ml seawater and enriched with Algamac. Bronopol was added to 0, 25, 50, 75 and 100 µg ml^{-1}. To a parallel series of tubes *V. splendidus* was added to a concentration of c. 3 x 10^3 ml^{-1}. Cultures were illuminated and aerated for 24 hours. The culture fluid from each tube was serially diluted and 0.1 ml volumes plated onto marine agar. As shown in Table 3, very high levels of bacteria arose in undisinfected cultures yet 50 µg ml^{-1} bronopol or greater controlled bacterial concentrations to <300 cfu ml^{-1}. This experiment was deliberately conducted with open tubes to mimic conditions in hatcheries such that bacteria could be transmitted from tube to tube via aerosols. Therefore, the bacterial concentration in each tube will reflect the proximity of the tube to the highly contaminated control tubes, the degree of contamination that occurs and the rate of killing of the bacteria by bronopol.

Table 3. Effect of bronopol on bacterial concentrations during *Artemia* enrichment.

Bronopol	Bacterial concn. (cfu ml^{-1}) in *Artemia* cultures after 24 h enrichment	
(µg ml^{-1})	without added bacteria	with added *V. splendidus* (3 x 10^3 ml^{-1})
0	> 10^7	>10^8
25	8200	270
50	260	250
75	20	280
100	<10 (not detected)	180

A similar experiment was undertaken at Otter Ferry in which 0 – 75 µg ml^{-1} bronopol was tested in 100 litre enrichment cultures. As in the laboratory experiment, the concentration of bacteria in the untreated culture was extremely high (Table 4) but bronopol restricted bacteria to similar levels to those found in the laboratory experiment (<300 cfu ml^{-1}). Artemia viability was >90% in the bronopol-treated cultures, whereas, in the untreated culture it is possible that the lower viability was due to bacterial action.

Artemia nauplii from the untreated culture were washed for 10 min in running seawater, homogenised and serial dilutions plated onto marine agar showing very heavy contamination with a count of >10^6 bacteria per *Artemia* nauplius.

Table 4. Effect of bronopol on bacterial concn. during *Artemia* enrichment at Otter Ferry.

Bronopol	*Artemia* culture fluid after 24 h enrichment	
(µg ml^{-1})	Bacteria (cfu ml^{-1})	*Artemia* viability (%)
0	> 10^7	79.5
25	150	91.1
50	250	97.3
75	40	98.4

Aerosols generated in an Artemia enrichment system
If heavily contaminated *Artemia* cultures are aerated during enrichment there is likely to be contamination of other cultures in the room due to aerosol formation. To gauge the extent of this an agar plate was left open at the top of the control bin in the previous experiment. The number of bacterial colonies that grew on the plate after 20 min exposure was estimated to be 12500, equivalent to 10^5 cfu bacteria falling into a 1m x 1m tank per minute.

Fatty acid composition of *Artemia* enriched in the presence of bronopol
To determine whether bronopol affected the enrichment of *Artemia* the enrichment was carried out in the presence of bronopol or phylatol as described above. Comparative analysis of the fatty acid content of enriched *Artemia* at the University of Stirling showed no significant differences between the two samples of *Artemia* showing that bronopol had no adverse effect on enrichment (Appendix).

Possible use of probiotic bacteria
To determine whether hly- mutants of *V. splendidus* can act as model probiotic bacteria
To determine whether naturally occurring hly- isolates of *V. splendidus* can act as probiotic bacteria
These experiments were deferred due to the lack of availability of turbot larvae and the early departure of Dr Reid on maternity leave. It is planned to carry out the experiments in /April-July 2006.

Testing potential probiotic organisms for their ability to protect larvae from *V. splendidus* DMC-1
Routine screening of organisms prior to this project identified a small number of bacteria that inhibited growth of *V. anguillarum* and several further isolates have been identified during this study. The most promising 4 isolates (*Roseobacter* 27-4, Hjelm et al., 2004; *V. alginolyticus* n5d5 280403; unidentified n8c8 220403; unidentified n8b5 130303) were tested here against cod larvae to determine (a) whether they were harmful to larvae and (b) whether they could prevent mortalities in larvae challenged with *V. anguillarum*. None of the potential probiotics was harmful to larvae and lower mortalities were recorded in groups receiving 3 of these bacteria compared with untreated larvae controls. However, higher than normal mortalities in the control group meant that these differences were not statistically significant.

Recolonisation of *Artemia* after disinfection
The above experiments show that as the bacterial flora of *Artemia* can be removed efficiently it should be extremely easy to colonise *Artemia* with selected bacteria. A number of bacteria have been selected to test their ability to form a stable flora in the larval fish gut and to protect larvae from challenge by pathogens such as *V. splendidus* DMC-1

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

Vibrio splendidus DMC-1 can cause heavy losses in cod larvae as well as in turbot larvae, the species from which it was isolated. It is likely that this pathogen can cause mortalities in a wide range of marine species since the enterotoxin produced is of the aerolysin family that binds to surface receptors found on a very wide range of cells. Certainly, isolates very similar to *V. splendidus* DMC-1 are present in halibut and cod hatcheries in the UK and Isle of Man.
There are two scenarios for larval rearing losses in marine hatcheries. Sudden heavy larval mortalities (“crashes”) can occur from the action of well-recognised highly virulent

pathogens, such as *V. anguillarum*, that colonise, invade and kill larvae from low concentration in the water or digestive tract. Examples of such pathogens include the *V. anguillarum* isolates from Norway (NOR-270) and Ardtoe (n8d10) described here. However we believe that the more frequent gradual losses of larvae, particularly in the early feeding stages, could occur from damage to the digestive tract caused by enterotoxins produced by *V. splendidus* and other vibrios. Such bacteria do not multiply rapidly to high numbers in the larvae because they are not invasive and to cause damage they must be ingested in significant numbers on live feed. *Vibrio splendidus* can be found in low concentration in the gut flora of healthy turbot larvae and it is possible that lethal damage can occur if sufficient enterotoxin-producing bacteria, possibly of different species, accumulate in the digestive tract. The conclusion is that total bacterial levels on live feed should be reduced to as low a level as possible.

A further potential pathogen in the cod larval rearing is *V. logei*. This was not found routinely in the Ardtoe survey of Reid et al. and it is unclear whether it poses a significant threat to cod larval rearing or is an opportunistic pathogen. *V. logei* is a common marine bacterium but it is not known whether isolate c1 270503 is typical of the species or possesses virulence mechanisms not normally found in this organism.

Reduction of bacterial levels in live feed is identified as an important target for hatcheries. If *Artemia* enrichment cultures are not disinfected they can support growth of very high concentrations of bacteria and large numbers of bacteria can be released via aerosols that will contaminate neighbouring enrichment cultures. Bronopol is very effective in controlling bacteria during hatching and during enrichment. This has the effect of removing the source of contamination via aerosol release and it should be possible to provide enriched *Artemia* cultures almost free of bacteria. This could have a major impact on live feed quality. *Artemia* are extremely robust; swimming and feeding activities as well as enrichment (Appendix) were unaffected by the bronopol concentrations used.

Most marine fish larvae require rotifers as initial live feed prey and it is worth investing further effort into devising equally effective methods for rotifer disinfection. This was investigated by Munro et al. (1993, 1999) who found that rotifers were very sensitive to disinfectants. Killing of bacteria by bronopol requires concentrations of >100 $\mu g\ ml^{-1}$ (Shepherd et al. 1988) but bacterial growth is inhibited at concentrations of 1-32 $\mu g\ ml^{-1}$, i.e. the MIC. Cutts et al. (2004) used 15 $\mu g\ ml^{-1}$ bronopol to disinfect rotifers and it is worth developing their study further to determine whether low concentrations of bronopol can be used continuously in rotifer culture to minimise bacterial levels.

One aim of this study was to test model probiotic bacteria to determine whether they could protect from challenge of larvae with pathogenic bacteria. Although the potential probiotics tested were unable to cause significant reduction in mortalities in *V. anguillarum*-challenged groups it would be worthwhile to repeat this trial with lower challenge doses of *V. anguillarum* and to further investigate the apparent beneficial effect of the potential probionts in providing higher survival in unchallenged groups. Work in this area was limited due to the sporadic availability of larvae of the right species and age in a project of such short duration, the early departure of the PDRA on maternity leave and the need for a more stable aquarium challenge system. A suitable aquarium system is now operating and it is planned to complete the outstanding trials when larvae are available.

DRAFT LIST OF PUBLICATIONS ARISING FROM THE PROJECT

Birkbeck, T.H., Reid, H.I., Darde, B. and Grant, A.N. (2006) Activity of bronopol (Pyceze) against bacteria cultured from eggs of halibut, *Hippoglossus hippoglossus* and cod, *Gadus morhua*. *Aquaculture* in press

Macpherson, H.L., Bergh, Ø. Maura, D., Reid, H.I. and Birkbeck, T.H. T.H.Characterization of an aerolysin-like toxin from *Vibrio splendidus* and its role in causing mortalities in larval turbot, *Scophthalmus maximus* (L.) in preparation.

Reid, H.I. and Birkbeck, T.H. Identification by denaturing gradient gel electrophoresis of rpoBgene fragments of *Vibrio* isolates including the pathogens *Vibrio anguillarum* and *Vibrio logei* in larval cod. Submitted to *Appl. Env. Microbiol*

Treasurer, J., Laidler, A. and Birkbeck, T.H. Occurrence of atypical furunculosis in haddock *Melanogrammus aeglifinus.* In preparation

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Appendix. Fatty acid analysis profile of *Artemia*, enriched with or without continuous disinfection with 75 μg ml^{-1} bronopol

Fatty acid profile (% of total)	bronopol-treated	control
14:00	2.44	1.90
15:00	0.24	0.19
16:00	18.62	16.88
18:00	4.70	4.55
20:00	0.12	0.13
22:00	0.25	0.21
total saturated	26.37	23.87
16:1n-9	0.45	0.44
16:1n-7	1.43	1.50
18:1n-9	10.15	10.25
18:1n-7	4.84	5.06
20:1n-9	0.29	0.35
20:1n-7	0.00	0.04
22:1n-9	0.00	0.00
Total monounsaturated	17.14	17.64
18:2n-6	4.73	4.85
18:3n-6	0.38	0.42
20:2n-6	0.12	0.23
20:3n-6	0.30	0.29
20:4n-6	3.06	3.54
22:5n-6	6.75	7.91
Total n-6 PUFA	15.34	17.25
18:3n-3	13.90	14.63
18:4n-3	1.60	1.25
20:3n-3	0.47	0.52
20:4n-3	0.84	0.80
20:5n-3	5.83	5.71
22:5n-3	0.31	0.38
22:6n-3	17.52	17.53
Total n-3 PUFA	40.48	40.62
16:2	0.67	0.62
Total PUFA	56.49	58.49
Total	100.00	100.00