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The Bacteriology of Atlantic Halibut, *Hippoglossus hippoglossus* (L.) Larval Rearing.

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Presented for the degree of Doctor of Philosophy in the Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Faculty of Science, The University of Glasgow

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LIST OF ABREVIATIONS

ASW	Artificial Sea water
BP	Base Pair
CFU	Colony Forming Units
dH_2O	Deionised water
ddH_20	Double distilled deionised water
DEEB	Division of Evolutionary and Environmental Biology
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded DNA
EDTA	Ethylenediamine-Tetra-acetic Acid
EPA	Eicosopentaenoic acid
g	Gram
h	Hour
HUFA	Highly Unsaturated Fatty Acids
L	Litre
MA	Marine Agar
MB	Marine Broth
LMP agarose	Low Melting Point Agarose
mM	Millimolar
NCIMB	National Collection of Industrial and Marine Bacteria
ng	Nanograms
nm	Nanometres
nM	Nanomolar
OD	Optical Density
ONPG	O-Nitrophenyl-β-D-Galactopyranoside
PCR	Polymerase Chain Reaction
pmol	Picomoles
RDP	Ribosomal Database Project
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rDNA	Ribosomal DNA

rRNA	Ribosomal RNA
sASW	Sterile Artificial Sea water
sd	Standard Deviation
sdH_20	Sterile Distilled Water
sec	Second
SEM	Standard Error of the Mean
SFIA	Seafish Industry Authority
SSW	Sterile Sea Water
TBE	Tris-boric acid EDTA buffer
TCBS	Thiosulphate Citrate Bile Salts Sucrose
TE buffer	Tris-EDTA buffer
µg	Microgram
µl	Microlitre
µm	Micrometre
UMBS	University Marine Biological Station
UPGMA	Unweighted Pair Group Method with Averages
uv	Ultraviolet
VP	Voges-Proskauer
v/v	Volume per volume
w/v	Weight per volume

SUMMARY

A bacteriological survey of three different UK halibut hatcheries was undertaken. These were the Seafish Industry Authority (SFIA) Marine Farming Unit at Ardtoe in Argyllshire, Mannin Seafarms, Isle of Man and Otter Ferry Seafish . Otter Ferry operates different rearing practices to the other two hatcheries in that it uses marine copepods, as well as enriched *Artemia,* as a start feed for developing halibut larvae. SFIA Ardtoe and Mannin Seafarms rear their larvae intensively, only supplying first-feeding larvae with unenriched *Artemia* metanauplii and ongrown enriched *Artemia*.

The bacterial flora of hatchery-reared Atlantic halibut eggs, larvae, juveniles and adults were monitored. The water in the halibut rearing tanks, and some of the possible sources of microbial inputs into the rearing system, were simultaneously sampled and analysed. Characterisation was done using a combination of traditional biochemical tests, the BIOLOG GN bacterial identification system, PCR-RFLP of 16S rRNA genes and partial 16S rDNA gene analysis.

The gut microflora appeared to be seeded towards the beginning of the non-feeding yolk-sac stage; by the onset of first-feeding this exceeded 10^2 Marine Agar culturable CFU/ larva. First feeding saw a rapid increase in bacterial numbers within the gut to more than 10^4 CFU/larva, a level that was reached within a week of the start of feeding. Bacteria isolated from non-feeding yolk sac larvae were predominantly non-fermentative Gram-negative rods, in particular *Pseudoalteromonas* species, by contrast the presumptive gut microflora of first-feeding larvae was generally dominated by members of the *Vibrio* genus.

In the intensively-reared larvae surveyed, there was evidence of a bacterial succession after the larvae started to feed. Initially, first-feeding halibut reared on enriched *Artemia* were colonised by live food-associated bacteria, particularly *V. splendidus* and *V. alginolyticus*-type organisms. Later in their development *V. salmonicida* and *V. fischeri* type organisms predominated. The guts of the adult halibut analysed contained predominantly *Photobacterium phosphoreum* and the gut flora of the copepod-fed larvae sampled was predominantly of *V. salmonicida*-type organisms.

A trial was run to compare the performance of Atlantic halibut yolk-sac larvae reared in commercial scale incubators under different conditions. Four different treatments were tested;

1. recirculation, non-disinfected eggs;
2. recirculation, disinfected eggs
3. flow-to-waste, disinfected eggs;
4. flow-to-waste, disinfected eggs, addition of antibiotics.

The peracetic acid/ hydrogen peroxide based disinfectant, Kick Start, was used to surface disinfect the eggs (1:250 solution for 1 min) and, for the antibiotic addition treatment, a solution of amoxycillin, oxytetracycline and oxolinic acid, at a final concentration of 20ppm for each agent, was pumped into the incubators at 115 and 180 degree-days post-hatch.

The experiment was repeated four times; for each experiment, a batch of eggs was split into four 450 L commercial incubators, which were maintained under the appropriate treatment conditions until 220 day-degrees post-hatch (stocking density 22/L). At this point 400 larvae from each incubator were transferred to two separate 100 litre trial tanks. The balance from each incubator was pooled into a production tank. Start feed performance was assessed after a week for the trial tanks and within twelve days for the production tanks. Samples of yolk-sac larvae were also taken for aerobic gut bacteria at the point at which larvae were transferred to the first-feeding tanks.

Survival was best in the antibiotic-treated group (53%), whilst the poorest performing treatment was flow-to-waste/surface disinfected (28.5%). Both the recirculation treatments performed relatively well (42.5% for non-disinfected eggs and 48% for surface-disinfected eggs).

Addition of antibiotics appeared to prevent the colonisation of yolk-sac larvae by Marine Agar-culturable bacteria (<10 CFU/larva as compared to 9.6×10^2 CFU/larva for flow-to-waste, surface disinfected eggs). High levels of bacteria were recovered from larvae reared under both the recirculated water regimes, despite their relatively good performance (6.2×10^3 CFU/larva for non-disinfected eggs and 7.6×10^3 for disinfected eggs). This illustrated how colonisation of yolk-sac larvae by bacteria did not necessarily result in poor survival.

Differences in the types of bacteria that colonised the yolk-sac larvae under the different rearing regimes were revealed by characterisation of the isolates. There were apparent correlations between average survival and the presence, or absence, of certain phenotypes. In particular, the larval flora in the poorest performing treatment (flow-to-waste and surface-disinfected eggs) was dominated by isolates biochemically and morphologically distinct from the predominant *Pseudoalteromonas* species isolated from the better performing recirculation regimes. This lends weight to the hypothesis that certain types of bacteria may act as opportunistic pathogens in yolk-sac incubators whilst others are benign.

The generally good performance of larvae reared under recirculation conditions was encouraging for a number of reasons, particularly that it may avoid the need to add antibiotics prophylactically, with associated problems of an increased selective pressure for antibiotic resistant strains of bacteria in the hatchery.

No apparent differences in start feed success were found for the different treatments. In general, start feed performance was poor, particularly in the trial tanks (mean 8.6%).

A bacteria-free halibut larval rearing system was developed to enable the testing of selected bacterial isolates on larval survival under microbially-controlled conditions. Different egg disinfection protocols and rearing systems were tested, including multi-well tissue culture dishes and 5L round bottom flasks, before a reliable method of producing bacteria-free halibut yolk-sac larvae was developed. The best results were found with a cotton wool-stoppered 2L flask system; 25 eggs were stocked into each 1.5 L sterile seawater-containing flask and allowed to hatch *in situ*. This followed treatment of the eggs by disinfection in glutaraldehyde and Kick Start, and immersion in a combination of antibiotics (oxolinic acid, erythromycin, penicillin, kanamycin and streptomycin). Optimisation experiments revealed that water quality was a critical factor affecting yolk-sac larval survival. Larvae reared in artificial seawater or seawater collected from the Glasgow University aquarium performed significantly worse than those reared in matured seawater collected from the West Coast of Scotland.

Using the developed system, 23 isolates from the survey and the rearing trial were tested for their toxicity towards halibut yolk-sac larvae in three rearing experiments. These included *Pseudoalteromonas* species, *Halomonas marina*, *V.*

salmonicida-like, *Photobacterium phosphoreum V. splendidus* and less well characterised isolates recovered from poorly performing yolk-sac larvae in the rearing trial. In addition a turbot pathogen, *V. anguillarum* 91079, and *V. viscosus* 236 were also tested. Isolates were inoculated into the flasks, 3-4 days after the larvae had hatched, at a concentration of 5 x 10^2 CFU/ml. In all cases a minimum of three flasks were used for each treatment.

Control survivals to 220 day-degrees for the three experiments averaged 84, 51.5 and 49% respectively. With the exception of *V. anguillarum* 91079, there was no statistically significant difference in survivals between the controls and the different treatments. This suggests that most of the bacteria routinely isolated from halibut hatcheries are not harmful to yolk-sac larvae. Generally, most flasks contained in excess of 5 x 10^6 CFU/ml of the inoculated organism by the time the experiments were terminated. *V. anguillarum* 91079 was highly pathogenic towards halibut yolk-sac larvae, as was previously found for another strain of *V. anguillarum* (Bergh,1992).

Of 68 tested isolates from the trial and the survey, one *Pseudoalteromonas* organism, TG15-07, inhibited the growth of a wide range of other micro-organisms *in vitro*, as assessed by a cross-streaking assay. This organism may be a potential probiotic candidate.

Chapter 1 Introduction

In the search for alternative candidates to Atlantic salmon, *Salmo salar* L., the aquaculture industries in Northern Europe and Canada have identified Atlantic halibut, *Hippoglossus hippoglossus* L., as a promising species for mariculture. They show good growth rates at the low temperatures common off their coastlines and command a high price once market size is reached.

Unfortunately, considerable problems have been met in producing high quality metamorphosed juveniles in economic quantities. As well as quality problems, with respect to high indices of incomplete or abnormal metamorphosis, variable survival in these critical early stages remains a considerable problem. It is possible that there are microbial influences resulting in the often poor survivals so far reported.

1.1. Biology of the Atlantic halibut

1.1.1. Development

Firstly, a word of caution is appropriate, as the pattern of 'normal' development of Atlantic halibut outlined here has mainly been gleaned from observation and experimentation in halibut hatcheries. There is very limited evidence available from observation of naturally developing halibut larvae. Eggs have been recovered from gravid females and from the immediate surrounds of the presumed spawning grounds. Juvenile halibut, which have completed their metamorphosis, have also been recovered, but only one halibut larva has so far been recovered from the wild (Haug *et al.* 1989).

Aspects relating to the early life history of Atlantic halibut are well reviewed elsewhere (Haug, 1990), so only a summary is presented here. In the wild, Atlantic halibut spawn at very great depths in cold water off the coasts of far northern Atlantic countries (Norway, through Iceland to the Eastern coast of Canada). The spawning season is from February through to April and Atlantic halibut are batch spawners, releasing from 3 to 8 batches of eggs in total. The total number of eggs produced is size dependent, but is in the order of 1-2.2 $\times 10^4$ per kilogram (Norberg *et al.* 1991). Following an egg incubation period of some 110-120 degree-days (^{O}C x days), the Atlantic halibut is characterised by a very long yolk-sac stage of some 220 degree-days post-hatch, before the onset of first feeding (Pittman *et al.* 1990; Lein and Holmefjord, 1992). During this long yolk-sac stage the developing halibut is mainly reliant on endogenous reserves.

By the onset of first feeding the larva is some 11-12 mm long, possessive of the requisite apparatus to process and capture its natural early food supply - presumably marine copepods and other components of the zooplankton. It thus has a fully functional gut and a well-developed sensory motor system, pigmented eyes and a characterisable behaviour repertoire to various tactile and visual stimuli (Pittman *et al.* 1990; Huse, 1993; Mangor-Jensen and Naas, 1993).

The larva goes through a series of developmental stages, collectively termed metamorphosis, over the next 400- 600 degree days culminating in the juvenile, or pre-adult condition. At this point, the developing halibut is no longer translucent and of conventional round fish form but instead, as in other flatfish of the family Pleuronectidii, is dorso-ventrally flattened with a well-pigmented dorsal aspect and cream ventral aspect. The head has undergone an axial rotation of some 45 degrees about the long axis, resulting in both eyes being viewable dorsally. Its behaviour has changed as well; no longer is it a pelagic animal, evenly distributed through the water column, but, instead, is largely demersal, spending the majority of its time in close contact with the bottom substrate.

1.2. Rearing Systems

1.2.1. Maintenance of Broodstock

Sexually mature male and female halibut are maintained in large circular tanks with a constant throughput of clean, well-oxygenated water. Wild caught broodstock have generally been used until recently in UK hatcheries, although increasingly hatcheries are starting to rely on broodstock that have been reared from eggs. Norwegian (Holmefjord *et al.* 1993) and Scottish (Shields *et al.* 1999) hatcheries have successfully experimented with photoperiod manipulation of the natural rearing cycle. This gives the potential of year round production of eggs. As it is important that broodstock are spawned at temperatures of less than 7^0C, water in which the fish are maintained must be chilled in Scottish hatcheries for much of the year. At present, production of adequate quantities of viable eggs is a major bottleneck in the industry in Scotland, hence a substantial research effort has been directed towards solving this problem.

1.2.2. Stripping and Fertilisation

Stripping and artificial fertilisation of eggs appears to give more stable results than natural spawning, so is the preferred method in halibut hatcheries (Bromage *et al.* 1994;

Shields *et al.* 1997). It is, however, very demanding on the labour force. This is because the success of stripping procedures has to be synchronised with the ovulatory rhythms of the females (Shields *et al.* 1993; Bromage *et al.* 1994; Holmfjord,1996).

When a female is ready to spawn, eggs are stripped into a receptacle and milt added. Following fertilisation, the eggs are transferred to upwelling incubators where they are incubated in darkness until hatching, following which they are transferred to yolk-sac incubators. Various models of yolk-sac incubator have been investigated since the first acceptable results were achieved using very large, upwelling silos in Norwegian hatcheries (Harboe *et al.* 1994b). Recent UK hatchery experience shows such large volume incubators are not necessary; what appears to be critical is the rate and pattern of water flow through the incubators (Shields *et al.* 1999). The time of transfer from the yolk-sac incubators varies; in some hatcheries they are moved out at 150 day-degrees post-hatch (approximately 25 days), but in others the time of movement is delayed until the point at which they are ready to start feeding (220- 260 day-degrees) (Shields *et al.* 1999).

1.2.3. First-feeding

Following yolk-sac incubation, different rearing strategies are being used at present; for clarity these can be summarised as intensive versus extensive approaches, although in reality there is a continuum between the two. There are likely to be substantial differences in the types of microbial communities that develop under each system. These likely differences, and their possible health implications, are reviewed later.

1.2.3.1. Extensive and semi-intensive rearing using marine copepods as a start diet.

The Norwegians have pioneered this approach, reviewed by van der Meeren and Naas, (1997). Good growth rates, survival and patterns of metamorphosis are achieved when marine copepods are fed to the first feeding halibut larvae (Naess *et al.* 1995; Shields *et al.* 1999). This is presumably because, as will be discussed later, they are nutritionally close to the preferred diet of halibut larvae in the wild. Unfortunately, the supply of copepods is unreliable and strongly seasonal. Various strategies have been tested in an attempt to produce adequate quantities of copepods. The first of these is the mesocosm-based approach; here, inlets in fjords are blocked off, potential predators are removed and the larvae are allowed to graze on the zooplankton that develop in synchrony

with fertiliser-stimulated algal blooms (van der Meeren and Naas, 1997). Other methods involved rearing larvae in protected rearing containers (indoor or outdoor tanks and plastic bags) and supplying them with copepods, (principally *Eurytemora velox* and similar spp.) either collected from the wild or reared in large outdoor rearing tanks (Naess *et al.* 1995).

1.2.3.2. Intensive rearing- utilisation of cultivated prey organisms

To avoid the problems of supplying adequate numbers of prey organisms on a year-round basis, substantial research effort has been directed towards the production of nutritionally-optimal cultivated live feed, suitable for presentation to first-feeding Atlantic halibut larvae. The preferred candidate has, to date, been the brine shrimp *Artemia*.

Artemia are brachiopods that naturally inhabit hypersaline ponds and lakes around the world. A Particularly important source of this organism is the Great Salt Lakes in Utah, where substantial quantities are harvested every year for aquaculture needs. They possess some attributes that make them, in many respects, an ideal start feed diet for fish larvae (Léger *et al.* 1986). Firstly, they enter a state of dormancy (diapause) when the lakes they inhabit dry out in response to increased salinity and other cues. Whilst in this highly protected encysted state, they can be stored almost indefinitely until required; at this point the *Artemia* can be easily encouraged to 'hatch' by a combination of rehydration, and exposure to high oxygen levels and light (Léger *et al.* 1986). The free-swimming *Artemia* nauplii can then be collected and either fed directly to the developing larvae or cultured on to larger sizes. Thus, hatchery operators have a food source that can be stored for a long time and the production of which can be rapidly scaled up or down as required, rather than having to rely on a fickle food source such as marine copepods, the production of which is highly influenced by seasonal and other environmental factors.

Unfortunately, as a food source for developing halibut larvae, *Artemia* are nutritionally deficient compared to marine copepods. In particular, they lack the optimal ratios of important highly unsaturated fatty acids (HUFA) such as eicosapentaenoic acid [20:5 (*n*-3); EPA] and docosahexaenoic acid [22:6 (n-3); DHA] (Shields *et al.* 1999). The *Artemia* can be enriched with these important HUFA by various techniques, such as the use of microalgae, oil-based emulsions and micro-encapsulated preparations (Barclay and Zeller, 1996) before they are fed to the developing marine fish larvae, including halibut. However, halibut that are fed these enriched diets do not do as well, with respect to key

indices such as survival, pigmentation and retinal morphology, compared to larvae fed a diet containing copepods (Shields *et al.* 1999; (Naess *et al.* 1995). There has been speculation that this is because the DHA/EPA ratios in enriched preparations are generally less than 1, unlike the ratios substantially greater than 1 which are found in the early life stages of marine copepods (Sargent and Henderson, 1986). It is possible that this is not the entire story, as there is evidence to suggest that a mixed diet consisting mainly of HUFA-enriched *Artemia*, but supplemented with copepods at so-called 'critical stages' during larval development, may suffice (Naess *et al.* 1995). Other factors than DHA/EPA ratios, such as carotenoid and phospholipid content, are probably also important (Shields *et al.* 1999). Also, there is histological evidence that halibut larvae do not digest *Artemia* as readily as copepods (Luizi *et al.* 1999).

1.3. Development of larval-associated microflora

Although the internal organs of healthy teleosts are generally considered to be microbiologically sterile (Liston, 1956; Toranzo *et al.* 1993), external surfaces tend to be colonised by an adherent microflora (Cahill, 1990). However, there is some debate as to whether the assemblages of bacterial species commonly isolated from such surfaces as the skin mucosa, the gills and the intestinal mucosa, constitute specialised microfloras, or are merely transient associations of bacteria that have colonised these surfaces opportunistically.

1.3.1. Eggs

In relatively few studies have the adherent microfloras associated with marine teleost eggs incubated under intensive conditions been described (Hansen and Olafsen, 1989; Morrison and Macdonald, 1995). This is despite an early realisation that, firstly, bacteria readily colonise the eggs of marine fish (Dannevig, 1919) and, secondly, the suppression of bacterial growth in egg rearing units by the addition of antibiotics can improve both hatching rate and survival (Oppenheimer, 1955; Shelbourne, 1963).

As pointed out by Hansen & Olafsen (1999), the glycoproteinaceous nature of the eggshell is well suited for adhesion and colonisation by bacteria. They speculate also that the inorganic and low molecular weight organic compounds released by the developing

embryo will diffuse through the eggshell and establish a gradient outside the eggshell; this may act as a chemoattractant for bacteria able to utilize these secreted compounds. These factors, combined with the unnaturally close proximity of other eggs in egg incubators, may favour colonisation and overgrowth by opportunistic pathogens, resulting in reduced hatching rates or otherwise compromise the health of hatched larvae.

There is some evidence that this is indeed the case with Atlantic halibut. Hansen and Olafsen (1989) studied the attachment and colonisation of both Atlantic halibut and cod (*Gadus morhua* L.), from fertilisation to hatching, by scanning electron microscopy (SEM). They also characterised and grouped the adherent microflora. It was found that marked bacterial growth could be observed within two h of fertilisation and, by the time of hatching, the eggs were heavily overgrown. Eggs appeared to be colonised by a wide variety of bacterial species, which generally reflected the external tank water microbial community, including members of the genera *Pseudomonas*, *Aeromonas*, *Alteromonas* and *Flavobacterium*.

The egg-associated microfloras may vary from rearing unit to rearing unit. For instance, a dominant member of the microbial communities in the Atlantic halibut egg incubators surveyed by Bolinches and Egidius (1987), an *Actinomyces*-like bacterium, was not found at all in the study by Hansen and Olafsen (1989). No recognised fish pathogens were isolated in either study. However, Bergh *et al.* (1992) demonstrated that some of the *Flexibacter* strains isolated from halibut egg surfaces by Hansen and Olafsen (1989), induced significant mortalities when used to challenge both eggs and yolk-sac larvae. These organisms were subsequently identified as belonging to a new species, which they designated *Flexibacter ovolyticus* (Hansen *et al.* 1992). This shows that some, at least, of the adherent microflora may be opportunistic pathogens. Further, more indirect evidence that the bacterial flora associated with intensively incubated halibut eggs may be at least partly harmful is demonstrated by the improvement in hatching rate and yolk-sac larval survival when eggs are surface disinfected (Bergh and Jelmert, 1990; Harboe *et al.* 1994a).

The importance of intra-ovum infection of halibut and other marine fish eggs by bacteria is hard to assess as little work has been published on this subject (Hansen and Olafsen, 1999). This is despite the evidence that salmonid eggs are quite often infected with *Renibacterium salmoninarum* and other bacterial species, including *Flavobacterium*

psychrophilum (Evelyn *et al.* 1984; Barker *et al.* 1989; 1991; Brown *et al.* 1997). Hansen and Olafsen, (1999) report that halibut and cod eggs may also contain bacteria prior to fertilisation. They found *Enterobacteriacae* in cod eggs and *Corynebacterium-Nocardia-Mycobacterium* in halibut at an incidence of 16% and 20% respectively.

1.3.2. Gills

Only a few studies have been made on the microflora associated with the gills (Mudarris and Austin, 1988). As these authors point out, this is an important gap in the literature because the gills, as delicate structures in intimate contact with the external environment, are a potential colonisation site for pathogens.

Mudarris and Austin, (1988), in their study on turbot (*Scophthalmus maximus* L.) gills, reported bacterial densities of up to 7.0 x 10^5 g^{-1} over the lamella surface. As well as characterising the species present, they also examined the surface of the gills using SEM. They reported that the bacteria tended towards colonisation of protected niches over the surface of the lamellae, rather than becoming established on exposed surfaces. Unlike other authors (the results of which are summarised by Cahill, 1990), they also claim that there were differences between the communities of bacteria colonising the gills and those in the surrounding water. They also report that a low proportion of the gill-adherent bacteria appeared to be culturable on Zobell's 2216E Marine agar.

It is hard to see how bacterial colonisation of the gills alone could be easily studied in developing halibut larvae because the small size of the structures during this period would make the necessary manipulation difficult.

1.3.3. Skin

Few studies have characterised the bacterial flora of the skin of marine teleosts. Early studies, such as Gilmour *et al.* (1976), reported that the bacteria isolated from the skin were similar to those found in the intestine. It is possible to criticise this, and presumably other studies, on the basis of methodology. Firstly, Zobell's medium was used and this only selects a low proportion of marine bacteria. Secondly, samples of skin were incubated for four days at 15^0C in liquid Zobell medium prior to plating out; such a procedure may well result in active inhibition by overgrowth of some bacterial species present. If there are specialised commensal bacteria present on fish skin, as some authors have speculated, (Bernadsky and Rosenberg, 1992; Sar and Rosenberg, 1987) it is possible

that they are extremely fastidious and, thus, unlikely to be isolated by such crude techniques.

In a more recent study, Austin and McIntosh (1988) reported that after gently flushing with sterile freshwater very low bacterial levels were present on rainbow trout skin. They reached this conclusion after both observation using SEM and after making cultures from skin homogenate. In contrast, levels similar to those quoted in earlier studies, (such as Gilmour *et al.* 1976), were obtained if the skin was not rinsed. They thus conclude that the natural antibiotic compounds, such as lysozyme in the mucus of fish skin, discourage the establishment of a microbial flora on fish skin. Correlatory evidence for this view is provided by the much higher levels of bacteria that they isolated from rinsed fin surfaces, as compared to skin.

Little is published about the types of bacteria associated with the exterior of marine fish larvae, although there is some evidence about the types of bacteria that may be associated with halibut larvae. Skjermo and Vadstein, (1993) looked at the types of bacteria associated with rinsed Atlantic halibut larvae and concluded that the skin flora was closely related to the tank water floras.

1.4. Development of gut-associated microflora in teleosts

Adult marine and freshwater teleosts are generally characterised by an abundance of bacterial species occupying the favourable niches afforded by the intestinal mucosa. Here, a bacterial species, if it is able to resist dislodgement by sloughing off of the intestinal mucosa or removal by host defence mechanisms, is afforded a protected, well-nourished environment.

Various workers have attempted to characterise the 'normal' development of an intestinal microflora in developing teleosts, both freshwater and marine species (Campbell and Buswell, 1983; Muroga *et al.* 1987; Sugita *et al.* 1989; 1988b; Hansen *et al.* 1992; Munro *et al*, 1993;1994; Bergh *et al.* 1994; Blanch *et al.* 1997).

The Atlantic halibut larval gut appears to be colonised in a two-step pattern (Bergh *et al.* 1994), as in other marine fish larvae (Strøm and Ringø, 1993; Ringø *et al.* 1996; Ringø and Vadstein, 1998). The general pattern appears to be that, although the surface of the egg is readily colonised by epibiotic bacteria, the developing embryo itself is largely free of bacteria. Following hatching, the external surfaces of the larvae are rapidly

colonised. Colonisers tend to be representatives of common bacterial species found in the water column or components of the egg-associated epibiotic community. Data is scanty, but the gut of the larval halibut does not appear to be colonised until later in development. This appears to coincide with the time at which the yolk-sac larvae start imbibing water, presumably containing bacteria, for osmoregulatory purposes (Bergh *et al.* 1994; Tytler and Blaxter, 1988). Gut bacterial loadings at this stage are apparently low.

Changes in the gut microflora have been observed at the onset of first feeding in marine species that have been studied (Campbell and Buswell, 1983; Tanasomwang and Muroga, 1988; Munro *et al.* 1994), and Atlantic halibut are apparently no exception (Bergh *et al.* 1994). This shift appears to be both quantitative and qualitative, with both an increase in bacterial numbers as well as a shift from the predominance of non-fermentative species, such as *Cytophaga* and *Flexibacter* species, to fermentative types, such as *Aeromonas* and *Vibrio* species.

This shift is apparently rapid, for instance in turbot it may take place within four days of start feeding (Munro *et al.* 1993). Whether the species that colonise during this period are persistent and prevent attachment of later arriving bacterial species is more debatable (section 1.5.).

It appears that the gut microflora of developing larvae is influenced by their diet and rearing regime. Munro *et al.* (1994) found that in intensively-reared turbot larvae which were fed on rotifers, the gut microflora reached a maximum level of 10^5 CFU/fish by day 5 post-hatch. By contrast, in turbot fed on copepods, the maximal level of 10^5 CFU/fish was not reached until day 8. There were substantial differences in the types of bacteria that made up the gut microfloras of the copepod-reared and rotifer-reared turbot larvae. By contrast, there was little difference in the gut microfloras that developed in turbot larvae reared at different densities (extensive versus intensive rearing systems). This was despite the significantly better survivals reported for the extensively reared turbot larvae. If the types of bacteria present in larval rearing systems do influence the health of marine fish larvae, there does not appear to be a simple relationship between the types of bacteria that establish themselves in the gut microflora and survival.

Bergh (1995) discovered that a number of the bacterial species associated with the intestinal mucosa of first-feeding Atlantic halibut larvae, maintained on marine copepods,

were able to inhibit the growth of pathogenic *Vibrio* species *in vitro*. Possible implications of these findings are discussed later (sections 1.5 and 1.8.2.2.1).

Whether similar bacterial species establish themselves in intensively reared Atlantic halibut is not known at present. It may well be that, as in turbot larvae reared on copepods versus rotifers, there are substantial qualitative differences in the gut microfloras of larvae fed enriched *Artemia*, as opposed to marine copepods.

1.5. Is the microbiota present in the intestinal tract of adult marine fish autochthonous?

It is well established that the microorganisms that dominate in the gastrointestinal tract of adult mammals constitute members of a stable, autochthonous microbiota. As well as important digestive functions, this microflora also helps to protect the host from colonisation by possibly harmful microorganisms (Van der Waaij, 1992). Concern has been expressed that the artificial conditions under which marine fish, such as Atlantic halibut, are reared may hinder the normal successional process of microbial colonisation and development of such a stable microbiota, if it does exist in the natural state. For this reason it is worth establishing whether the microbial communities that make up the intestinal microflora of marine fish are indeed autochthonous.

1.5.1.1. Is the gut microflora merely a function of the food ingested?

Early work in this field suggested that bacteria present in the gut of fish are a function of the food ingested rather than any intrinsic property of the fish in question (Liston, 1956), since the intestinal tract of non-feeding fish is practically sterile (Margolis, 1953). It is possible, though, that these early reports were deficient because the isolation techniques used may have only cultured a limited proportion of the actual microbiota present.

Conway *et al.* (1986) claim that the intestinal microbiota of a marine teleost flatfish, the flounder (*Pseudopleuronectes* sp.) showed a rapid starvation survival response following withdrawal of nutrients. In this experiment flounder were sampled before and after only 24 h withdrawal of food. In their natural environment marine fish may undergo periods of months when they do not feed (Liston, 1956). Thus, although these results are of interest, in that they demonstrate that bacteria present in the intestinal microbiota may enter a dormant state upon nutrient depletion, the period of starvation of the host was probably

too short to form any useful conclusions, in regard to the abilities of these bacteria to persist in flounder intestinal mucosa under natural conditions.

Other evidence suggests that the gut microflora of marine fish is not merely a transient collection of bacteria that, following ingestion, have gained an opportunistic 'foothold'. Campbell and Buswell, (1983) found that the gut microflora of developing sole (*Solea solea* L.) was eventually dominated by *Vibrio* species which were not always present in the food that was being fed at this point.

Savage, (1977) defines autochthonous microorganisms as possessing the following characteristics:

i) the ability to grow anaerobically;

ii) always found in normal adults;

iii) colonise particular areas of the tract;

iv) colonise their habitats during succession in infant animals;

v) maintain stable population levels in climax communities in normal adults;

vi) may associate intimately with the mucosal epithelium in the area colonised.

It is debatable whether a definition designed to be applied to organisms inhabiting a very different environment - homeothermic, terrestrial animals with longer digestion and evacuation times - should be applied, unmodified, to fish. However, as a guide to the types of properties we might expect of an autochthonous microflora the scheme has its uses didactically. Available evidence is therefore reviewed using the scheme. Where appropriate, possible shortcomings of this 'working definition' are highlighted.

1.5.1.2. Is the intestinal microbiota of fish anaerobic?

Although facultative anaerobic bacteria have been isolated from marine species studied, the evidence for the presence of obligative anaerobes is less conclusive. For instance, Westerdahl *et al.* (1991) found that all of the strains they isolated anaerobically from the intestinal mucosa of turbot would also grow aerobically. This contrasts with findings from freshwater fish, where obligate anaerobic bacteria have been reported (Sakata, 1990). Whether there is a *de facto* requirement for anaerobiosis is possibly

unlikely. This is because, in contrast to the mammalian gut, the intestinal tract of even large adult carnivorous marine fish is probably not an anaerobic environment (Lésel *et al.* 1990). Hyposmotic marine teleosts have to take in water continuously, by drinking, to maintain homeostasis in the face of an external hyperosmotic environment. Thus, there is a continual flow of oxygenated water through the digestive tract, presumably bathing any bacteria present. In this context, the observation that the microbiota of freshwater species studied, confronted by the opposite physiological challenge of maintaining internal homeostasis in the face of a hypotonic environment, and consequently not actively drinking, appears to be dominated to a greater extent by both facultative and obligate anaerobes, is of interest (Sugita *et al.* 1988a).

1.5.1.3. ii-‘ are always found in normal adults’.

There is some evidence that the gut microbiota of adult marine fish is composed of similar bacterial phenotypes. Liston first characterised what he termed ‘gut vibrios’ as being the dominant genera in two phylogenetically very different ‘flatfish’- an elasmobranch (skate, *Raja* sp.) and a teleost (lemon sole, *Pleuronectes microcephalus* L.) (Liston, 1954). Identification, using then available techniques, indicated that this group was composed of nutritionally demanding, psychrophilic Gram-negative rods that ferment glucose and maltose, do not liquefy gelatin, reduce nitrates and trimethyl amine, and are unable to grow at 30°C. Sizeable proportions of these bacterial species were luminous. He also found that the intestinal microbiota was significantly different in structure to the microfloras associated with the skin and the gills of the fish he sampled. Although he tentatively identified all these species as ‘vibrios’, on the basis of antibiotic susceptibility, there were anomalies, in particular that a number of the strains produced gas from glucose; a characteristic of *Pseudomonas* spp.

Studies done later on other marine fish taken from a variety of geographical locations and habitats have also found that a substantial proportion of the intestinal microbiota was dominated by ‘gut vibrios’(Aiso *et al.* 1968; Onarheim and Raa, 1990). Others have also found, like Liston, that many of these strains were sensitive to the vibriostatic agent, O/129, but produced gas from glucose. Onarheim and Raa (1990) looked closer at strains isolated from a range of marine fish, showing such properties, and found that they had a similar percentage G + C content to more conventional

(biochemically) *Vibrio* strains, further confirming Liston's original grouping of such strains as members of the *Vibrionaceae*.

Subsequently, these organisms have been shown to be phylogenetically related to *Vibrio salmonicida* on the basis of 16S rDNA sequence analysis (Onarheim *et al.* 1994), confirming earlier work indicating that these isolates were serologically related to *V. salmonicida* (Onarheim and Raa, 1990).

From a health perspective, it is interesting that some of the *Vibrio* species isolated from the guts of both farmed and wild-caught marine fish appear to produce antimicrobial compounds, at least *in vitro* (Onarheim and Raa, 1990; Olsson *et al.* 1992; Bergh, 1995- section 1.8.2.2.1).

If 'normal', e.g. healthy wild-caught, adult marine teleosts, do possess an autochthonous gut microflora dominated by 'gut vibrios', of possibly protective function, it is worth investigating whether farmed fish have a similar gut microflora. Little is published to this effect and early reports are conflicting, again complicated by the difficulty of comparing studies where different methodologies have been employed. Strøm and Olafsen (1990) found that there were qualitative changes in the bacterial communities inhabiting the gut of wild-caught juvenile cod following domestication. In particular, they found that a group of *Vibrio*-type organisms, present in the freshly caught cod juveniles, were not present one month after capture; furthermore it did not re-establish itself over the time course of the survey (one year). The group of *Vibrio*-like organisms that was only found in the cod upon capture shared antigenic determinants with *Vibrio salmonicida*, in that 10-50% of the strains cross-reacted with monoclonal antibodies prepared against *Vibrio salmonicida*; there were biochemical differences, however. There were subsequent outbreaks of *V. salmonicida* amongst some of the studied groups of cod. Intriguingly the authors speculate that 'the gut mucosa associated vibrios are "relatives" of the pathogenic strains, occupying the same microenvironments'.

However, (Westerdahl *et al.* 1991) found that the guts of farmed turbot contained *Vibrio* species able to produce antimicrobial substances and multiply in intestinal mucus, *in vitro*. It is not possible to say whether these species were similar to the 'gut vibrios' that were apparently displaced in the captured and cultivated cod.

There is some evidence that hatchery-reared turbot may also be colonised by 'commensal' organisms. Blanch *et al.* (1991) sampled different batches of turbot larvae

from a hatchery in Northern Spain. Early in development the turbot larvae appeared to be colonised by a heterogeneous assortment of bacterial species, predominantly members of the *Vibrionaceae*. Towards the end of the monitoring period an organism, later designated *Vibrio scophthalmi*, was consistently found in the guts of batches of 90-day-old turbot fry. This organism appeared to be avirulent, was not isolated from the turbot feed and was rarely present in the early developmental stages. Whether it is present in other turbot hatcheries is not clear.

1.5.1.4.'colonise particular areas of the tract'

Carnivorous marine fish have relatively straight undifferentiated intestinal tracts and some teleost species, such as the mullet, do not even have a separate stomach as such. The Atlantic halibut possesses a stomach and a limited number of pyloric caecae (less than five). The opportunity for differentiation of bacterial communities along the length of a relatively undifferentiated gut (compared to a mammalian gut) is presumably lessened. Westerdahl *et al.* (1991) did not find any substantial differences in the types and numbers of bacterial species colonising different areas of farmed turbot guts.

1.5.1.5.'Colonise their habitat during succession in infant animals'

The colonisation of the gut of developing marine fish larvae has mainly been dealt with in section 1.4. It has to be borne in mind that those studies mentioned traced a succession of bacterial species in intensively reared marine fish larvae; the situation in naturally developing fish may be very different. It is not easy to see how 'normal' succession can be observed in the 'natural state', however.

1.5.1.6.'maintain stable population levels in climax communities in normal adults'

Few studies have followed the seasonal changes in the bacterial species that colonise the guts of adult marine fish, probably because it is a laborious procedure, which would necessitate the analysis of large numbers of samples over a minimum of two seasons.

Liston, (1956; 1957) monitored the quantitative and qualitative changes in bacterial counts on skin, gut and gill samples in freshly caught skate and lemon sole over 27 months. He found there was seasonal variations in the numbers and types of bacteria present in the guts of the fish sampled, with a peak in bacterial numbers coinciding with plankton blooms. As mentioned earlier, he also found there was a seasonal minimum of gut bacterial

numbers during the spawning season in flounder. Liston notes that these variations were gradual, not rapid oscillations, meaning that the gut microfloras of the fish he was studying were not just highly ephemeral collections of bacteria which were a reflection of those present in the outside environment. The gut microbiota were markedly different to those colonising the gills and skin, and seasonal variations were less marked than in those more environmentally exposed environments. It would not be surprising if there were seasonal changes in the abundance and structure of the gut microflora in temperate, poikilothermic species.

1.5.1.7. 'and may associate intimately with the area colonised'

There is a substantial literature on the specific, and non-specific, adhesion mechanisms possessed by the normal microbial inhabitants of the intestinal mucosa and epithelia in mammals (Savage, 1980). Whether the bacteria present in the guts of fish are associated in the same way is not clear, although some studies indicate that bacteria may be quite firmly attached to the gut mucosa of adult fish *in vivo* (Westerdahl *et al.* 1991; Hansen *et al.* 1992). Also, as discussed earlier, *in vitro* evidence indicates that certain 'gut vibrio species', are able to adhere strongly to salmonid gut mucosal surfaces (Knudsen *et al.* 1999).

1.5.2. Other possible functions of the gut microflora in fish

It has been speculated that the gut microflora may serve a role in nutrition, as it does in mammals. This could either be by direct utilisation of the bacteria themselves (Seki, 1969; Olafsen, 1984; Macdonald *et al.* 1986; Matena *et al.* 1995), or, alternatively, the fish may utilise exogenous enzymes produced by the gut bacteria as an aid to digestion. If this is the case, it does not appear to be important in the very early stages as good survivals are reported in bacteria-free larval rearing systems (Baker and Ferguson, 1942; Trust, 1974a; Battalora *et al.* 1985; Douillet and Holt, 1994).

1.6. Disease susceptibility of Atlantic halibut larvae - 'host factors'

The relationship between the microorganisms present within a rearing system and the individual halibut larvae is likely to be very complex, due to the large number of influencing factors. Firstly, there are what might be termed 'host factors', which will

influence the degree of susceptibility of a halibut larva to colonisation by potentially pathogenic microorganisms, opportunistic or otherwise.

1.6.1. The ontogeny of an immune response in larval fish

An important factor influencing the potential disease susceptibility of Atlantic halibut larvae will be its capacity to recognise and respond to potential pathogens. There is strong evidence that halibut larvae, like other teleosts, are of reduced immunocompetence at the time of hatch and only attain full immunocompetence later in life. This will have a bearing on their potential disease susceptibility.

Much of the available evidence on the development of an immune response in young teleosts, particularly in salmonids and other predominantly freshwater species, such as the carp and tilapia species is well reviewed by Ellis, (1988) and Manning (1994). Little work, however, has been done on marine species, such as the Atlantic halibut. As they are different from the other species studied, both phylogentically as well as in their normal reproductive mode of production of large numbers of small pelagic eggs, it is possible that there will be differences in the ontogeny of their immune responses as well (Chantanachookhin *et al.* 1991).

1.6.1.1. Development of specific immunity

The development of a specific humoral immune response, involving the production of specific antibody, by surface immunoglobulin bearing - mammalian B-cell equivalent - cells to antigen is apparently delayed until at least the start of first feeding (Manning, 1994). Preliminary indications (Bergh *et al.* 1995)- Abstract only) are that this is the same for Atlantic halibut.

The thymus is probably the first organ to become lymphoid during ontogeny (Ellis, 1977; Zapata and Cooper, 1990; Manning, 1994). The first development of a thymus in the three marine species, which included a relative of the Atlantic halibut, the Japanese flounder, *Paralichthys olovaceus*, studied by Chatanachookhin *et al.*(1991), also appeared to be delayed until some period post-hatching. Early indications (Bergh *et al.* 1995)- abstract only) are that a primitive thymus is only present at around the stage of first feeding in Atlantic halibut.

It is likely that the other organs implicated in the mounting of an immune defence also develop relatively late in Atlantic halibut. For instance the splenic rudiment in another marine species, the seabass, *Dicentrarchus labrax*, was only apparent some eighteen days post-hatch; the first lymphocytes in the organ are only to be seen some forty days post-

hatching (Quesada *et al.* 1994). Atlantic halibut have a longer yolk-sac stage than seabass and the other marine species so far studied, so it is possible that the appearance of an immunologically active head kidney may be even further delayed in this species.
It is possible that young teleosts may display a period of immunotolerance early in their development (Ellis, 1988). This is a phenomenon characteristic of mammals, where early, neonatal or early post natal, exposure to an antigen results in a reduced, or even abolished, antibody response on secondary exposure, compared to controls which have been exposed to the antigen for the first time at the later stage.

If immunisation of young halibut is to be attempted, it is important to know, firstly, whether Atlantic halibut larvae display a period of immunotolerance and, secondly, at what stage they first become capable of mounting an anamnestic response. Immunisation at too early a stage may be not only wasteful, in terms of manpower and other resources, but counterproductive as well.

1.6.1.2. Non-specific defence mechanisms

It is likely that Atlantic halibut, like other young fish, are reliant to a large degree on non-specific defence mechanisms before they become immunocompetent. It is known that phagocytes, particularly macrophages and granulocytes, are present at an early stage of development in salmonids, cyprinids and tilapia (Ellis 1977; Agius, 1981; Grace *et al.* 1981). The early presence of melanomacrophage centres was also discovered in tilapia and rainbow trout; interestingly, as in the development of specific immunity, this is around the normal onset of first feeding in both species studied (Agius, 1981). It is possible, then, that phagocytes are an important first line of defence in larval halibut, as they are in the other species so far studied.

Other humoral, non-specific, components are probably very important. In particular there is some evidence that the developing embryos and larvae of some are provided with a maternal complement of defensive components. Thus the developing yolk-sac larva has some measure of protectivity before it is able manufacture its own. Yousif *et al.* (1991; 1994) and Takemura and Takano (1995) both report the presence of lysozyme in the eggs of salmonids and tilapia respectively. C-reactive protein has been assayed in the eggs of several species (Alexander and Ingram, 1992).

Lysozyme is also considered an important component of many fish defence mechanisms (Ingram 1992). This is evidenced by its high concentrations in immunologically important sites, such as plasma, the kidney, the spleen and the skin

mucosa (Lindsay, 1986; Alexander and Ingram, 1992). Adult Atlantic halibut have particularly high levels of lysozyme, as measured in both the skin mucus and serum (Balasundaram, 1993). Providing a reserve of lysozyme to the young developing fish, before it is capable of synthesising its own in appreciable quantities, could represent a strategy by which the mother confers a measure of protection on her progeny against pathogenic bacteria.

The presence of IgM-like protein which is presumably maternally derived, has been discovered in the eggs of some fish species (Van Loon *et al.* 1981; Bly *et al.* 1986); Takemura and Takano, 1995; Olsen 1997). Recent work indicates that this maternal input of IgM is utilised by the developing larva until it can start to produce its own, endogenously generated supplies (Olsen, 1997;Takemura and Takano, 1997). It is possible that there may be a 'window of opportunity' for infective agents during that period when both exogenously (maternally) and endogenously-derived components are at a low level (Olsen and Press, 1997). This is quite possibly a problem in Atlantic halibut larvae, with a very long yolk-sac stage before the onset of first feeding and, by implication, a longer reliance on maternally derived factors than salmonids. It is interesting that some authors have identified key periods of high mortality in halibut larvae to be during the later stages of yolk-sac larval development (Harboe *et al.* 1994b).

To this author's knowledge, other non-specific components, such as serum antiproteases and iron-binding transport molecules, like lactoferrin and transferrin, which are important to the older animal, have not been assayed for in young developing teleosts. Again, there is no available evidence that teleost larvae and juveniles posses interferon and other mechanisms by which they can resist viral infection. This does not however preclude them from having them. Available evidence (Alexander and Ingram, 1992) indicates that they are synthesised by T-cell analogues as in mammals. The observation that Atlantic halibut are resistant to IPN virus when they have completed their metamorphosis, but not before (I. Bricknell, personal communication), could be an indication that these interferon-producing cells are not present until this late developmental stage (80-100 days post hatch).

1.6.2. Developmental status

It is likely that the resistance of halibut larvae to invasion by microorganisms will change over the period of its development. As well as the development of an immune response, dealt with in the previous section, there are other factors that may have a bearing on their susceptibility to bacterial infection.

For instance, as their preferred orientation within the water column changes with the onset of metamorphosis, the larvae may move to the base of the tank and, therefore, be exposed to higher bacterial levels. They will also probably become more resistant to physical abrasion as their outer tegument thickens and hardens, making them less susceptible to invasion by opportunistic bacterial species able to establish themselves at the site of insult. There are also important alometric considerations. As the larvae increase in overall size, the relative proportions of structures that are intimately exposed to the outside environment, such as the gills and intestinal epithelium, will decrease.

1.6.3. Nutritional status

There is evidence that the health and disease resistance of fish is influenced by their diet (Landolt, 1989). Recently, considerable attention has been devoted to the influence of dietary factors in disease resistance in fish, particularly salmonids. Attention has focussed on the potentailly positive effects of addition of dietary antioxidants, Vitamin C and Vitamin E. There are recent reviews on this subject (Landolt, 1989; Blazer, 1992; Lall and Olivier, 1993; Waagbø, 1994; Oliver 1997).

There are few reports on the effects of dietary factors on the disease resistance of larval fish in the mainstream literature, however. This is despite the practice of routinely supplementing *Artemia* enrichments with vitamin supplements, in particular Vitamin C.

1.6.4. Stress

The conditions under which Atlantic halibut are reared, as in other intensive culture systems, are very different from those likely to be experienced by halibut larvae developing in their natural environment. These conditions may be stressful and could influence their likely disease resistance. Immunosupression following exposure to a stressor is a characteristic of the so-called stress response or General Adaptive Syndrome (Seyle, 1950). The physiological basis to this phenomenon, which appears to be common amongst the vertebrates, is imperfectly understood in adult fish, let alone larval teleosts. Release of cortisol, following stress activation of the HPI axis (hypothalamus -pituitary-interrenal), is thought to induce a lymphocytopaenia (Pickering, 1984). Cortisol has been shown to have other immunosupressive effects in mammals (Bateman *et al.* 1989). *In vitro* and *in vivo* immunosupressive effects, following cortisol exposure, have also been demonstrated in various teleost models. These include, a suppression in phagocytic cell

activity (Narnaware *et al.* 1994), a reduction in levels of plasma IgM in masu salmon (Nagae *et al.* 1994), a decline in spontaneous serum haemolytic activity in Atlantic salmon (Carlson *et al.* 1993), and the inability of lymphocytes taken from cortisol-treated catfish to respond to mitogenic stimuli (Ellsaesser and Clem, 1987).

Examples of potential stressors in halibut hatcheries could include both acute stressors, such as grading and movement of animals between tanks, and chronic stressors, such as high population densities.

1.7. Disease susceptibility - possible pathogens of Atlantic halibut larvae.

1.7.1. Bacteria

1.7.1.1. Vibrio anguillarum

Vibriosis, caused by the pathogen *V. anguillarum*, is a serious disease of farmed marine fish worldwide. Atlantic halibut larvae, juveniles and adults all appear to be susceptible to infection by *V. anguillarum* 01 and 02 serotypes. (Bergh *et al.* 1992; Bricknell *et al.* 2000).

Bergh *et al.* (1992) showed that yolk-sac larvae of Atlantic halibut are highly susceptible to a *V. anguillarum* 02 serotype originally isolated from cod (NCIMB 6). Bricknell *et al.* (2000) showed that post-metamorphic and sub-adult Atlantic halibut were susceptible to bath challenge by a *V. anguillarum* 01 serotype originally isolated from Atlantic salmon. They also showed that vaccination of sub-adult halibut can be effectively protect them from challenge by *V. anguillarum.*

Although vaccination protocols are still at an early stage of development (T. J. Bowden, personal communication), it would be wise for hatchery operators to consider vaccinating their stock, and in particular their broodstock, against this potentially serious disease. As vaccination of yolk-sac larvae and first feeding stages may not be feasible for biological reasons, the main line of defence will have to remain a high level of surveillance to prevent this damaging organism gaining entry. There are no reports of *V. anguillarum* strains other than 01 and 02 serotype isolates being pathogenic to halibut. However, as has

been pointed out by other authors, most organisms grouped into the species *V. anguillarum* are avirulent environmental isolates (Larsen *et al.* 1994; Ringo and Birkbeck, 1999).

1.7.1.2. Aeromonas salmonicida

Aeromonas salmonicida subspecies *salmonicida*, typical and atypical strains, are the causative agents of furunculosis in salmonids and, increasingly, other fish species (Bernoth, 1997).

Atlantic halibut larvae appear to be only mildly susceptible to *Aeromonas salmonicida* subsp. *salmonicida* (Bergh *et al.* 1997). Approximately 40% of the challenged larvae survived to 29 days after hatching, as opposed to around 60% survival in the uninfected controls, and *A. salmonicida* was not easily reisolated from infected larvae and pathological changes, different to those found in the control larvae, could not be discerned.

Adult and juvenile Atlantic halibut do not appear particularly susceptible to either typical or atypical strains of *A. salmonicida* subsp. *salmonicida*. (Hjeltnes *et al.* 1995) challenged 180 - 200g juvenile halibut, by both cohabitation and injection routes, with the same isolate used in the above study. In each study only one halibut died and *A. salmonicida* was reisolated from both fish. More extensive experiments in which Atlantic halibut were challenged with a wide range of *A. salmonicida* subs. *salmonicida* typical and atypical isolates reinforced these findings (Bricknell *et al.* 1999). It was shown that 10^6 to 10^7 typical and atypical *A. salmonicida* cells was the minimum lethal dose in injection experiments.

On the basis of these results, *A. salmonicida* subsp. *salmonicida* does not, at present, appear to pose a significant threat to either Atlantic halibut hatcheries or ongrowing operations. It is always possible that A. *salmonicida* strains could adapt and become more adept at evading Atlantic halibut defences in the future as there is an increased level of contact between potential host and pathogen as a result of an increase in halibut farming.

1.7.2. Other recognised bacterial pathogens

In the absence of reports in the literature, it is presently unclear whether there is risk to halibut larvae and fry by other recognised bacterial pathogens of cold water fish, such as *R. salmoninarum*, '*Vibrio viscosus*', *Piscirickettsia salmonis*, *Photobacterium*

damselae, *Cytophaga maritimus* and *Vibrio salmonicida*. Other potential pathogens could also include *V. splendidus* and *V. alginolyticus* strains shown to be harmful to other reared marine fish larvae (Anguiano-Beltran *et al.* 1998; Gatesoupe, 1997; Gatesoupe *et al.* 1999; Pedersen *et al.* 1999)

1.7.3. Opportunistic pathogens

It is probable that the main risk to Atlantic halibut larvae in hatchery systems will not come from recognised bacterial pathogens, such as *Vibrio anguillarum*. Only rarely have demonstrably pathogenic bacteria been isolated from larval rearing systems, or correlations shown between the presence of particular bacterial phenotypes and larval survival (Kusuda *et al.* 1986; Munro *et al.* 1993; Munro *et al.* 1995; Tanasomwang and Muroga, 1988; Tanasomwang and Muroga, 1989).

However, Bergh *et al.* (1992) and Skiftesvik and Bergh, (1993) have induced infections experimentally in Atlantic halibut larvae, by challenge with a *Flexibacter* species originally isolated from halibut eggs. An enteric disease of larval Japanese flounder has been shown to be caused by a *Vibrio* species, named *Vibrio* INFL (Masumuro *et al.* 1989). This group reported, in a later paper, that this species induced a pathology quite different from *V. anguillarum*, with a local (non-systemic) infection of the intestine. The disease could not be reproduced in older animals with *Vibrio* INFL (Muroga *et al.* 1990). It seems possible then that there are bacteria opportunistically pathogenic to the larval stages, but not older animals. Such species may have been isolated from rearing systems, but not characterised as 'pathogens' by researchers.

1.7.4. Viruses

1.7.5. Infectious Pancreatic Necrosis (IPN) virus

Infectious Pancreatic Necrosis virus has been shown to be highly virulent to Atlantic halibut larvae and fry by both Norwegian and Scottish workers (Biering *et al.* 1994; Wood *et al.* 1996). An outbreak in a Scottish hatchery in 1996 caused losses of up to 100% in metamorphosed halibut fry. It was presumed to have been caused by cross-transfer of the virus from IPN carrying Atlantic salmon, which were present on the same site (Wood *et al.* 1996).

1.7.6. Viral Haemorrhagic Septicaemia (VHS) Virus

Viral Haemorrhagic Septicaemia has been considered primarily a disease affecting freshwater-reared salmonids. However it has recently become clear that VHS is present in the marine environment off the coasts of Europe and poses a significant threat to turbot, *Scophthalmus maximus,* a phylogentically close relative of the halibut (Stone *et al.* 1997; Dixon, 1999; Snow and Smail, 1999;). Outbreaks of VHS have severely affected turbot rearing operations in Scotland and Ireland in recent years .

There are currently no reports in the literature on the susceptibility of Atlantic halibut larvae or adults to VHS. However, sub-adult halibut may be susceptible to turbot pathogenic VHS isolates administered via the interperitoneal route, but not when bath-challenged (M. Snow, personal communication).

1.7.7. Nodavirus

Viral Nervous Necrosis has emerged as a serious threat to halibut farming. To date, it has only been reported in Norway, (Grotmol *et al.* 1995) but this situation could easily change, just as it has for Infectious Salmon Anaemia (Hastings *et al.* 1999).

The nodaviruses have been isolated as disease agents from a number of marine aquaculture species worldwide (Munday and Nakai, 1997). These include Japanese parrotfish, *Oplegnathus fasciatus* (Yoshikoshi and Inoue, 1990), larval barramundi, *Lates calcarifer*, in Australia and Tahiti (Glazebrook *et al.* 1990; Renault *et al.* 1991), sea bass, *Dicentrarchus labrax*, in France (Breuil *et al.* 1991), turbot, *Scophthalmus maximus*, in Norway (Bloch *et al.* 1991), and grouper species, *Epinephelus fuscogutatus* and *E. akaara*, in Taiwan (Chi *et al.* 1997).

Grotmol *et al.* (1995) first reported a nodavirus-like agent infecting Atlantic halibut larvae in Norway, causing very high mortalities. Subsequently, this group presented histopathological data supporting the original conclusions that the infective agent was a member of the Nodaviridae (Grotmol *et al.* 1997).

The Atlantic halibut nodavirus isolates have recently been shown to be phylogenetically very close to those of two Pacific coldwater species, Barfin flounder, *Verasper moseri*, and Pacific cod, *Gadus macrocephalus* (Aspehaug *et al.* 1999). Comparison of an 827 bp section of the coat genes of an Atlantic halibut nodavirus isolate (HHNNV) and a barfin flounder isolate (BFNNV) showed the two sequences to be

virtually identical. One possible conclusion is that nodavirus isolates have been transported to Europe, from the Pacific, very recently. Aspehaug *et al.* (1999) present two alternative hypotheses, firstly that the transfer could have been via ornamental fish transported between the Pacific and Europe or, secondly, that such viruses could have been introduced several times from an unknown natural reservoir, such as *Artemia* cysts.

Recently published work suggests a degree of host-specificity among nodavirus strains. In pathogenicity tests, isolates from Striped Jack were highly virulent to larval Striped Jack and Japanese flounder but not to Atlantic halibut. Conversely, a halibut isolate was virulent to larval halibut but not to other species of challenged marine fish larvae (Totland *et al.* 1999).

1.8. Possible Microbial Control Strategies

In a hatchery there are various means by which the culturist may seek to control and manipulate the bacterial populations that develop within his rearing system, and Vadstein *et al.* (1993) considered three different approaches. Firstly, there is the non-selective reduction of bacteria, secondly, selective enhancement of bacteria and, thirdly, enhancement of larval resistance against bacteria. The three approaches are not mutually exclusive and, as the authors point out, none of the approaches guarantees freedom from disease; rather, all may in some way help to alter probabilities in the culturist's favour.

1.8.1. Non-selective reduction of bacteria

Non-selective reduction of bacteria includes the use of disinfectants, antibiotics and other means of sterilisation of incoming waters (such as ozone and UV treatment).

1.8.1.1. Eggs

Surface disinfection of eggs is routinely employed in salmonid aquaculture and this may be a viable approach in Atlantic halibut culture. Bergh and Jelmert (1990 reported that application of buffodine for ten min significantly improved the survival of hatched larvae. Other agents may be more effective, particularly glutaric acid (Harboe *et al.* 1994a). The peracetic acid based surface disinfection 'Kick start' is an effective egg treatment, although survival of larvae hatched from eggs so treated was not followed in the original study (Kristjansson, 1995). Kick Start possesses some advantages over glutaraldehyde in that it is less harmful to hatchery operators as the active ingredients, hydrogen peroxide, peracetic

acid and acetic acid, are readily degraded to harmless compounds. Some UK hatcheries have now routinely used Kick Start to surface disinfect all of their eggs for three seasons.

1.8.1.2. Live food

A possible portal of entry for pathogens into marine fish rearing systems could be via the food chain (Campbell and Buswell, 1983; Muroga *et al.* 1987; Nicolas *et al.*, 1989).

The use of naturally collected marine copepods, compared to using hatchery reared live food organisms, such as *Artemia*, carries a potentially higher risk because pathogens, naturally present in the marine environment, may be associated with the copepods (Léger *et al.*, 1986).

There may also be a potential disease problem with the use of *Artemia*, though high bacterial loadings of the enriched *Artemia* used in one of the Scottish hatcheries, SFIA, Ardtoe were uncovered in an initial study- 10^3 - 10^4 CFU/ *Artemia* (Verner - Jeffreys, 1996). This was similar to levels reported in the literature from other *Artemia* rearing systems (Blanch *et al.*, 1991). Many of the bacteria associated with *Artemia* appear to be *Vibrio* species, which have been implicated as potential opportunistic pathogens of marine fish larvae (Nicolas *et al.* 1989).

Commercial hatcheries have experimented with various methods of removing this loading. It has been reported that rinsing of the *Artemia* in fresh water prior to feeding to the larvae may suppress halophilic *Vibrio* spp. (N.P. Brown personal communication). There are also commercial products available which seek to suppress the *Vibrio* blooms which are often observed during the enrichment process in *Artemia* culture, such as 'Hatch Controller' (INVE). There are no reports in the literature at present as to the effectiveness of this and other products.

Complete removal of the bacterial loading associated with *Artemia* may be problematical. In an earlier study by the author, *Artemia* were immersed for five min in the peracetic acid containing the disinfectant 'Kick Start'. Although a 2-3 fold log order reduction was observed folowing this exposure, appreciable numbers of bacteria were still associated with the *Artemia* following treatment. This was presumably because bacteria present within the gut of the *Artemia* were protected from surface-disinfection.

It is possible that this high, only partly removable, bacterial loading may compromise the health of the halibut fry to which the *Artemia* are being fed. Pérez -

It is possible that this high, only partly removable, bacterial loading may compromise the health of the halibut fry to which the *Artemia* are being fed. Pérez - Benavente and Gatesoupe (1988) report that turbot fry fed *Artemia* that have been reared under axenic conditions (by application of antibiotics to the rearing system) show better survival and growth rates compared to fry that have been fed normally cultured *Artemia*. Other, more indirect, evidence also supports the notion that high bacterial loading of the live food may be a potential health problem for developing marine fish larvae (Gatesoupe, 1982; Gatesoupe *et al.*, 1990). Challenge experiments have been done which demonstrate that other species of marine flatfish larvae are susceptible to bacterial disease if pathogens are incorporated into their live food, both rotifers, *Brachionus plicatilus*, and *Artemia* (Masumura *et al.*, 1989; Munro *et al.*, 1995; Grisez *et al.*, 1996). However, it is debatable whether such challenges adequately model likely hatchery circumstances. In most cases, the live food was immersed in a very high concentration of a potential pathogen, prior to presentation to the respective larval species.

1.8.1.3. Rearing tanks

Cleaning of tanks with surface disinfectants, prior to adding new stock, is part of normal hatchery hygiene practice and treating tanks with formaldehyde is a common procedure in UK hatcheries.

Although antibiotics are probably used routinely in many hatchery-rearing systems, there are surprisingly few reports in the literature as to their optimal usage and effectiveness. By contrast, well defined treatment protocols are available for treating adult and post-metamorphic halibut with a range of different antibiotics (Samuelsen and Lunestad, 1996; Samuelsen, 1997; Samuelsen *et al.* 1997; Samuelsen and Ervik, 1997; 1999).

Increased survivals have been reported following application to sea bream, *Sparus aurata*, larval-rearing tanks (Hernández *et al.* 1994). According to Vadstein *et al.* (1993) antibiotics have also been applied to Atlantic halibut yolk-sac rearing tanks with some success. However, it is generally agreed that the continued, indiscriminate, application of antibiotics to larval rearing tanks is probably unwise (Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999) because bacteria can readily acquire resistance to the antibiotics administered (Amàbile-Cuevas *et al.* 1995; Kruse and Sorum, 1994).

1.8.2. Selective enhancement of bacteria - the 'probiotic approach'.

Recognising that there are problems with indiscriminate application of antibiotics and disinfectants in a hatchery situation, other potential control strategies have been explored.

One of these strategies may be termed 'controlled substitution' under the scheme of Vadstein *et al.* (1993). The rationale is that potentially harmful bacterial species in the hatchery rearing system are substituted with beneficial species, or 'probiotics'.

The potential uses of probiotics in aquaculture have recently gained a lot of attention. Until recently there was an absence of suitable definitions and universally agreed standards as to how they should be evaluated and administered. Fortunately, reviews on this subject have recently been published (Ringø and Birkbeck, 1999; Ringø and Gatesoupe, 1998; Gatesoupe, 1999).

1.8.2.1. Definitions

Huis in't Veld and Havenaar (1994): define probiotics as " a mono- or mixed culture of live micro-organisms which, applied to man or animal affects beneficially the host by improving the properties of the indigenous microflora".

Whether this applies to aquaculture is debatable as it would probably exclude live microbial supplements added to the culture systems which seek to improve or influence water quality parameters. This is a problem recognised by Gatesoupe (1999). He accordingly reserves the term probiotic for strains that are transient or resident in the intestinal tract. He favours the definition bioremediation for microbes that break down pollutants or waste by the microbes. He defines a further subset of probiotics to be biocontrol agents, which are antagonistic to pathogens.

1.8.2.2. Potential mechanisms of action

Suitable probiotic candidates should be able to survive, either within or outside of the host, be harmless to the organism being cultured and ideally be able to inhibit the growth of potential pathogens. There are various ways suitable candidates could achieve this. These include:

- candidates that release antimicrobial substances;

- candidates that compete for potential receptor sites in the gut with pathogenic bacteria; this could be by either non-specific or specific (lectin-receptor) adhesion mechanisms.

- candidates that stimulate the host immune system.

1.8.2.2.1.Release of antimicrobial substances

It has long been recognised that certain species of marine bacteria are able to produce substances that can inhibit the growth of other microorganisms (Zobell). For instance, Dopazo *et al.* (1988) screened the ability of antibiotic-producing marine bacteria, isolated from intertidal seaweeds, to inhibit the growth of a range of bacterial pathogens. They found that, of the 23 pathogenic strains assayed, only two strains were resistant to all of the antibiotic-producing strains. However, Hansen and Olafsen (1989) attempted to manipulate and control the bacterial colonisation of the surface of cod eggs by incubating gnotobiotic eggs, dissected from the ovary and fertilised under sterile conditions, in cultures of these defined antibiotic strains. Unfortunately, this approach was unsuccessful, with the eggs rapidly becoming recolonised by the bacterial species present within the incubator. This illustrates how important it is to test potential probionts *in vivo* as well as *in vitro*.

Bacteria which are naturally in intimate association with marine organisms may also release inhibitory compounds. The shrimp, *Paleamon macrodactylus*, is apparently extensively colonised by an *Alteromonas* sp. If these bacteria are removed from *P. macrodactylus* embryos by antibiotics they are susceptible to infection when challenged with the pathogenic fungus *Lagenidium callinectes*. If the embryos are reinoculated with the bacteria, or with a substance released by these bacteria, 2,3- indolinedione, they are resistant to challenge (Gil-Turnes *et al.* 1989). This offers evidence that there is a mutualistic relationship between the shrimp and the colonising bacteria and that the protection inferred on the embryos is by release of microbial inhibitory compounds. This may be a general phenomenon amongst decapod crustacea, as is evidenced by the observation that the American lobster, *Homarus americanus*, also appears to be protected by an epibiotic bacterium that releases an inhibitory substance against *L. callinectes*, this time 4- hydroxyphenyl alcohol (tyrosol) (Gil-Turnes and Fenical, 1992). From an evolutionary perspective, it is interesting that apparently different bacterial species

appeared to have formed commensual relationships with different decapod hosts; possibly this is an example of convergent evolution.

There is some evidence that some so-called 'gut vibrios'(section 1.5.1.3) may produce antimicrobial substances. (Onarheim and Raa, 1990; Olsson *et al.* 1992; Onarheim *et al.* 1994; Sugita *et al.* 1997). Westerdahl *et al.* (1991) found that V*ibrio* species isolated from the guts of reared turbot (*Scophthalmus maximus*) produced antimicrobial substances, *in vitro*, against a range of *V. anguillarum* strains, *A. hydrophilia* and *A. salmonicida.*

Bergh (1995) found that the gut mucosa of Atlantic halibut reared on marine copepods contained *Vibrio* species that produce antimicrobial substances. Other potential probiotic candidates release substances that inhibit the growth of pathogenic bacteria, both *in vitro* and *in vivo*. Smith and Davey (1993) found strains of *Pseudomonas fluorescens* in the intestine of brown trout (*Salmo trutta*) that inhibited the growth of *A. salmonicida in vitro* and protected trout against furunculosis. It appeared that the *P. fluorescens* strain limited the growth of *A. salmonicida* by competition for free iron.

Competition for iron has been proposed as the mechanism by which another potential probiont, this time a *Vibrio* species isolated from healthy turbot larvae, may protect turbot from infection by a pathogenic *V. splendidus* species (Gatesoupe, 1997b).

Although probiotics are already used on a wide scale by shrimp culturists, very little published information is available. Gomez Gil (1995) reports that *V. alginolyticus* strains, isolated from healthy rearing systems, have been routinely employed as a probionts in Ecuadorian shrimp hatcheries since 1992.

Austin *et al.*(1995) looked closer at one of the strains to determine their possible mode of action. It appeared that some form of bacteriocin was released by the *V. alginolyticus* strain tested that inhibited the growth of *V. ordalii, V. anguillarum* and *A. salmonicida in vitro.*

There are reports that other genera of bacteria may be suitable probiotic candidates, in particular lactic acid bacteria (Ringø and Gatesoupe, 1998). Strøm and Ringø (1993) isolated a *Lactobacillus* species, *L. planarum*, from the intestinal mucosa of cod. They reported (cite a paper in Norwegian) that this species is able to produce a microbial inhibitor which depresses the growth of the fish pathogens *V. anguillarum* and *A.*

salmonicida. When *L. plantarum* was added direct to the rearing water of five-day-old cod larvae, the bacterial flora of the larvae became dominated by *L. plantarum* and other species, which proliferated in the untreated controls, were suppressed from growing. The total levels of bacteria in the *L. plantarum*- treated larvae remained at some 10^2 cfu/larva until nine days after hatching; in contrast the untreated larvae had bacterial counts of over 10^4 cfu/larva at the end of the same period.

1.8.2.2.2. Colonisation of intestinal mucosa

As well as production of antimicrobial substances, probiotics may colonise the intestinal mucosa and thereby act as a barrier to infection by potential pathogens. To date, few aquaculture-related studies have concentrated on this aspect of the use of probiotics, although there is some evidence that presumptive gut vibrios show the ability to both adhere to and multiply in the intestinal mucosa of salmon , *in vitro* (Knudsen 1999).

1.8.2.2.3. Stimulation of the immune system

Although probiotics are reported to have positive immunomodulatory effects in mammals (Van der Waaij, 1992) there is no evidence, at present, that this is the case in fish. It may be worth investigating whether so called 'gut vibrios,' and other members of the normal gut flora, have a role in immunological priming of the host against pathogenic bacteria.

Studies have demonstrated that endocytosis of bacteria may take place in the foregut of cod larvae, and there has been speculation that this is linked to processing and presentation of antigenic determinants to appropriate cell species (Hansen and Olafsen, 1990)

1.8.2.3. Bioremediation

There are other potential benefits in using live bacterial supplements in Atlantic halibut rearing systems than the control of the gut microflora. Control of bacterial proliferation in live food rearing systems may be of benefit, both in terms of reducing the bacterial loading of the live food and in improving the performance of the live food rearing system itself.

Live bacterial supplements have been used in a number of studies by Gatesoupe and co-workers on Japanese flounder and turbot rearing systems (Gatesoupe *et al.* 1989; Gatesoupe 1990; 1991; 1994). They report that some of the products they tried, such as Acosil, an extract from cereal grains, which had been seeded with lactic acid bacteria, may be useful in controlling the growth of bacteria in rotifer rearing systems; although the results were variable (Gatesoupe *et al.* 1989; Gatesoupe, 1990). Bacillus spores, "paciflor 9" (which contained Bacillus strain IP 5832) added to rotifer enrichments, substantially reduced the proportion of dominant *Vibrio* species in the rotifer rearing system.

1.8.2.4. Role in nutrition

Another use of probiotics may be as a nutritional aid rather than as protection against pathogens. Ringø *et al.* (1992) showed that a *V. pelagius* species, originally isolated from copepod-fed turbot larvae, produces a high proportion of eicosapentaenoic acid (EPA). As discussed earlier (1.2.3.2), marine fish larvae have a high requirement for EPA and DHA, which is not present in sufficient quantities in unenriched *Artemia* or rotifer cultures. Supplementation of rotifer or *Artemia* cultures with HUFA-containing bacteria could represent another way in which these important nutrients can be fed to developing marine fish larvae.

This approach has been used to enrich rotifers, in particular, with bacteria containing EPA prior to feeding the EPA-containing rotifers to developing marine fish larvae (Nichols *et al.* 1996; Watanabe *et al.* 1992; Yazawa *et al.* 1992).

As pointed out by Ringø and Birkbeck (1999), the effects of feeding such EPA-enriched rotifers, with respect to health and survival were not examined in these studies. When Ringø and co-workers attempted to administer the EPA-containing *V. pelagius* organism to developing turbot, it was found in one experiment to be present in high quanities in the guts of the developing larvae 14 days after it was originally administered to the tank water (Ringø *et al.* 1996). Unfortunately, later work did not show any improvement in EPA profiles of turbot larvae treated with EPA-containing *V. pelagius* (Ringø and Vadstein, 1998). .

1.8.3. Controlled substitution - microbially matured water

There are other ways of altering the balance of the microbial environment in halibut larval rearing systems than direct addition of live microbial supplements, or 'probiotics'.

Vadstein and co-workers (Vadstein *et al.* 1993; Skjermo *et al.* 1997; Salvesen *et al.* 1999) have developed a method of treating incoming waters that they have termed 'microbial maturation'.

The theoretical basis to this method is the application of some basic ecological principles. It is considered that the types of organisms that initially colonise rearing tanks are likely to be pioneer, 'r-selected' species. Such organisms are able to grow rapidly under conditions of high nutrient availability and limited competition from other organisms. These are then replaced by more specialised, 'K-selected' organisms which are better able to grow under the opposite conditions of competition from other species and limited nutrient availability. It is possible that 'r' selected organisms may be more likely to be opportunistic pathogens. Interestingly, early reports show that deliberate maturation of the water prior to addition to turbot larvae tanks appeared to result in an improvement of both survival and growth rates (Salvesen *et al.* 1999).

As Ringø and Birkbeck, (1999) point out, " the approach of Skjermo *et al.* (1997) represents an important conceptual advance in control of microbial flora which is applicable to many larval rearing systems".

1.9. The role of 'green water'?

It has long been realised that the presence of micro-algae in the water that marine fish larvae are reared in improves their survival and overall performance (Houde, 1975; Houde, 1978; Naas *et al.* 1992).

It is unclear what is the cause of this phenomenon, although various hypotheses have been proposed. Some authors have suggested that micro-algae serve a role in early larval nutrition (van der Meeren, 1991; Naas *et al.* 1992; Reitan *et al.* 1997). It has also been proposed that the algae may help to stabilise important water chemistry parameters (Houde, 1978). Other workers have suggested that algae may act to influence, directly or indirectly, the composition of the tankwater microfloras the larvae are exposed to (Nicolas *et al.* 1989; Skjermo and Vadstein, 1993).

1.10. Prophylaxis

1.10.1. Vaccination

As discussed previously (1.6.1.1), vaccination is unlikely to be a practical option in the treatment of developing halibut larvae until some way into their development because they are unlikely to be immunocompetent until they have at least started to feed. However, consideration should be given to immunising fry and juveniles against vibriosis, once suitable vaccination protocols are available.

1.10.2. Immunostimulants

The third option of Vadstein *et al.* (1993), improving the resistance of the marine larvae, is tied in closely with the possibility of young halibut being immunologically immature. Immunostimulants are, by definition, designed to boost the non-specific defences of the subject to which they have been administered (Anderson, 1992). If, as was discussed earlier (section 1.6.1.2), halibut larvae and fry are reliant on non-specific defence mechanisms to a much larger degree than when they are older, this could represent a means by which a measure of increased protection can be inferred on the young fish, before its own defences are functioning properly.

Various products have been investigated as to their suitability in other fish species. Encouraging results with a yeast cell wall extract, β1-3 glucans, have been obtained with Atlantic salmon (Robertsen *et al.* 1990; Engstad *et al.* 1992; Engstad and Robertsen, 1993; 1994; Jorgensen *et al.* 1993; Rorstad *et al.* 1993; Brattgjerd *et al.* 1994; Dalmo and Seljelid, 1995; Jorgensen and Robertsen, 1995; Solem *et al.* 1995), rainbow trout *Oncorhynchus mykiss* (Sakai *et al.* 1995) and channel catfish (Jeney and Anderson, 1993), via interperitoneal or oral administrative routes. Their mechanism of action is not well understood, although preliminary investigation indicate they act by increasing macrophage activity, such as their ability to adhere to glass (Sakai *et al.* 1995), produce lysozyme (Engstad *et al.* 1992) and the reactive bactericidal oxygen species O_2^- and H_2O_2 (Brattgjerd *et al.* 1994). There is also an indication that complement-mediated haemolytic activity is upgraded (Engstad *et al.* 1992); also, administration of glucans prior to immunisation resulted in higher antibody titres following antigen exposure (Chen and Ainsworth, 1992).

Other products have also been tried, sometimes with encouraging results. EF2O4, injected in conjunction with *R. salmoninarium* vaccine, resulted in higher survival in

rainbow trout so treated, following challenge with a pathogenic strain of *R. salmoninarum*, compared to fish only injected with the denatured bacterin (Sakai *et al.* 1995).

Considerable interest has been expressed in the use of immunostimulants in marine larviculture (Vadstein, 1997). Skjermo *et al.* (1995) administered an alginate immunostimulant with high mannuronic acid content to juvenile turbot, using *Artemia* as a vector. Subsequent challenge with *V. anguillarum* resulted in a significant protective effect being attributable to the immunostimulant dietary additive. As to the potential utilisation of immunostimulants in halibut larval culture, Vadstein *et al.* (1993) report increased survival, in a small scale experiment, of Atlantic halibut yolksac larvae following addition of the immunostimulant, FMI, to the tank water. Although there is some evidence that halibut yolk sac larvae are able to actively take up β1-3 glucans (Strand and Dalmo, 1997), whether this is of benefit to the larvae themselves is more questionable. When first-feeding halibut larvae that had been fed on *Artemia* enriched with β1-3 glucans for a period of 4 weeks were subsequently challenged with *V. anguillarum* they performed no better than larvae that had not been fed the immunostimulant (Verner - Jeffreys, 1996). If anything, the immunostimulant-fed fish died slightly faster than the untreated fish. It is unwise to draw conclusions from one isolated experiment, however, particularly when no effort was made to vary the level of β1-3 glucans administered to the larvae prior to challenge.

1.11. Objects of Research

This thesis initially sought to investigate the influence of bacteria on the health and development of Atlantic halibut larvae. This was with a view to eventual improvement of these parameters by the development and implementation of hatchery microbial control strategies. Accordingly, there were three main aims:

1. To characterise the types of bacteria associated with halibut larvae and eggs of different developmental stages in different hatcheries in the UK.

2 To determine how rearing halibut larvae under different conditions affected their survival and the development of a gut microflora.

3. To assess the effects of selected isolates on larval survival.

Chapter 2. Changes in the Gut-Associated Microfloras During the Development of Atlantic Halibut (*Hippoglossus hippoglossus* L.) Larvae in British Hatcheries

2.1. Introduction

As a first step towards controlling the bacterial floras that develop in halibut hatcheries, with the aim of improving larval halibut rearing performance, it is important to know the types, numbers and sources of bacteria commonly associated with halibut eggs and larvae of different developmental stages. This information can then be used to target microbial contamination pathways, identify potential pathogens, and may also aid in the identification of potential probiotic candidates.

Accordingly, a bacteriological survey of three UK halibut hatcheries was undertaken.

2.2. Materials and Methods

2.2.1. Supply of halibut larvae and eggs

Atlantic halibut larvae and eggs from three geographically well-separated UK hatcheries were sampled for the presence of gut-associated heterotrophic bacteria. In two of the hatcheries, SFIA Ardtoe, Argyll, Scotland and Mannin Sea Farms, Isle of Man, the larvae were reared intensively, being fed on a combination of *Artemia* nauplii and enriched metanauplii following yolk-sac absorption and transfer to first-feeding tanks at 220 day-degrees post-hatch. In the third hatchery, Otter Ferry seafarms, the larvae were reared semi-intensively and fed on a mixed diet of enriched *Artemia* and copepods.

2.2.2. Gut-associated bacterial flora of Atlantic halibut larvae

In all cases the larvae were sampled after the procedure of Tanasomwang and Muroga, (1988). Between one and five larvae were collected from the rearing tanks and placed, by means of a sterile plastic pipette, into sterile plastic tubes, of internal diameter 3.8cm with 10μm nylon mesh heat-welded to the bottom. They were then rinsed three times in sterile 25 $^{0}/_{00}$ artificial seawater (ASW; Sigma Seasalts or Instant Ocean^{TM}) prior to immersion for 60 sec in 0.1% benzalkonium chloride (Sigma UK). Larvae were rinsed three more times in ASW before being homogenised in 2ml ASW. Larval homogenate was serially diluted and plated onto MA in duplicate. Three separate subsamples of larvae were homogenised and plated out for each sampling point.

Plates were incubated at 10 ^{O}C for upwards of 30 days, following which viable counts were made, and between fifteen and twenty colonies randomly selected from each sampling point, as well as any distinct morphotypes that were not isolated during the initial random selection. These isolates were subcultured 2-3 times on MA until pure and retained for identification.

2.2.3. Bacteriological flora of halibut eggs

Atlantic halibut eggs from two hatcheries were sampled for adherent epibiotic and internal bacteria after the method of Hansen and Olafsen, (1989). Eggs were collected and transferred to sterile plastic filters as described above, rinsed three times in 25 $^{0}/_{00}$ ASW, and homogenised in 2ml ASW. The egg homogenate was serially diluted and plated onto MA. Plates were incubated at 10 ^{O}C for at least a month before enumeration and selection of isolates for later identification.

2.2.4. Bacteriological flora of live food

Various samples of live food were taken from each of the hatcheries. These included: unenriched freshly hatched nauplii; Algamac 200 and Super Selco-enriched two-day ongrown nauplii; *Artemia* cysts; copepods from the live food production facility at Otter Ferry. In general, samples of live food were removed from the rearing tanks in sterile 30 ml universal bottles, collected on sterile 100 μm filters and rinsed three times in SSW to remove non-adherent external bacteria prior to homogenisation.

The homogenate was then tenfold diluted in ASW and the dilutions plated onto MA. The samples of *Artemia* cysts were removed aseptically from previously unopened vacuum sealed packets of cysts (EG grade cysts INVE Aquaculture). *Artemia* cysts were rinsed, serially diluted and plated onto MA and Thiosulphate Citrate Bile Salt Sucrose agar (TCBS Oxoid) as for the other samples. Water samples from the *Artemia* nauplii and enriched-*Artemia* rearing tanks at Ardtoe were also taken, serially tenfold diluted and plated onto MA and TCBS. For all live food samples, plates were incubated at 20 ^{O}C for a minimum of seven days prior to enumeration and random selection of isolates for later characterisation.

2.2.5. Bacteriological flora of rearing water

On occasion, water samples from the larval rearing tanks were also taken, serially ten fold diluted in 25 $^{0}/_{00}$ ASW, and plated onto MA. The plates were incubated in parallel with the surface-sterilised larval samples taken at the same time. Colonies were randomly selected following incubation and characterised.

2.2.6. Storage of bacteria

Bacteria were subcultured two or three times on MA to obtain pure cultures then stab inoculated into 2ml MA in a screw-capped vial. After 72 h incubation at 10 ^{O}C 1ml of sterile paraffin was added and the cultures stored at room temperature, or lower, in the dark. Selected isolates were also stored using the Protect system (Technical Service Consultants Limited) at –70 ^{O}C according to the manufacturer's instructions, except that 100µl of 20 % NaCl was added to the culture medium.

2.2.7. Identification of bacteria

Isolates from each sample were initially screened on the basis of their colony morphology, a few simple biochemical and physiological properties and their susceptibilities to a number of different antibiotics by the use of the Mastring S scheme (Table 2), resulting in each isolate initially being characterised on the basis of 42 discrete characters. Following initial cluster analysis, representatives of the different presumptive phenons were further characterised by a combination of conventional biochemical testing, BIOLOG GN, PCR-RFLP and 16S rRNA gene sequencing. Selected cluster representatives were identified to the species level by

comparison of biochemical and physiological test results to those published in Bergey and on the basis of their 16S rRNA gene homology to sequences deposited in sequence databases.

Table 1 shows the coding system used for the different bacteria isolated during the survey. A number of the bacteria originally isolated from yolk-sac larvae during the rearing trial (Chapter 3) are also compared, they are coded as in Table 1c.

Table 1a Coding scheme used for bacterial identification and description of the different samples of live food, rearing water, halibut larvae and adult faecal material taken from SFIA Ardtoe.

Code	Date isolated	Batch	Sample description
E1	20/04/98		Eggs sampled immediately after fertilisation
O1	26/09/97		Eggs taken from incubator towards end of development. Apparently moribund 'red' eggs.
O2	14/04/98		Healthy halibut eggs
SYS3	06/10/97	4/IX	Healthy 6 days post-hatch (32 day-degrees) surface-sterilised yolk sac larvae.
YSW3	06/10/97	4/IX	Water from same incubator as SYS3
SYS4	31/10/97	4/IX	Healthy 30 days post-hatch (180 day-degree) surface-sterilised yolk-sac larvae from 950L incubator.
YSW4	31/10/97	4/IX	Water from same incubator as SYS4
SYS5	01/11/97	3/IX	Poor batch of 34 days post-hatch (212 day-degree) surface-sterilised yolk-sac larvae from 950L incubator.
YSW5	31/10/97	4/IX	Water from same incubator as SYS5
SYS6	02/11/97	3/IX	Healthy 35 days post-hatch (218 day-degree) surface-sterilised yolk-sac larvae from 950 L incubator
YSW6	02/11/97	3/IX	Water from same incubator as SYS6
SFF1	09/11/97	4/IX	Surface-sterilised 7 days post first-feeding halibut.

Table 1a (continued)

Code	Date isolated	Batch	Sample description
FFW1	09/11/97	4/IX	Water from same incubator as SFF1.
SFF2	14/11/97	2/IX	Apparently healthy first-feeding larvae from tank containing 'white gut' moribund larvae
FFW2	09/11/97	4/IX	Water from same incubator as SFF2.
SFF3	14/11/97	2/IX	Moribund 'white gut' first feeding larvae.
SFF4	15/11/97	4/IX	Surface sterilised first-feeding halibut.
SFF5	10/12/97	2/IX	Surface sterilised first-feeding halibut.
SFF6	10/12/97	4/IX	Surface sterilised first-feeding halibut.
W1	05/02/98	4/IX	Surface sterilised halibut fry just transferred onto an inert diet
W2	05/02/98	2/IX	Surface sterilised halibut fry weaned onto inert diet
AH1	4/12/97		Rinsed fresh-hatch *Artemia* nauplii
AE1	4/12/97		Rinsed 2 day ongrown enriched *Artemia* nauplii (Algamac 2000).
S1	1/4/98		Faeces from the hind gut of a male hatchery-reared 2.7 kg adult halibut.
S2	1/4/98		Faeces from the hind gut of a male hatchery-reared 3.1kg adult halibut.
S3	1/4/98		Fluid from the hind gut of a male hatchery-reared 3.4kg adult halibut.This individual had, apparently, not been recently feeding.

Table 1b Coding scheme used for bacterial identification and description of the different samples of eggs, live food and halibut larvae taken from Mannin Seafarms and Otter Ferry. MYS1, MYS2, MFF1, MFF2, MA1 and MA2 were sampled from Mannin. OFE1, OFYS1, OFF1, OFF2, OFC1 and OFA1 came from Otter Ferry.

Code	Date isolated	Batch	Sample description
MYS1	27/6/98		Surface-sterilised, healthy 30 days post-hatch (183 day-degrees)
MYS2	27/6/98		Same batch of yolk-sac larvae as MYS1 except sampled from a different incubator.
MFF1	27/6/98		Surface-sterilised halibut larvae. 7 days post first-feeding.
MFF2	27/6/98		Surface-sterilised halibut larvae. 7 days post first-feeding.
MA1	29/6/98		Rinsed 2 day ongrown Algamac 2000 enriched *Artemia*.
MA2	29/6/98		Rinsed fresh-hatch *Artemia* nauplii.
OFE1	11/03/99		Healthy halibut eggs. 13 days post fertilisation (65 day-degrees).
OFYS1	22/07/99		Surface-sterilised yolk-sac larvae 29 days post-hatch (170 day degrees). Sampled from 1500L first-feeding tank after transfer.
OFF1	22/07/99		First feeding halibut larvae maintained entirely on copepods. 41 days post-first-feeding (630 day-degrees post-hatch).
OFF2	22/07/99		24 days post-first feeding halibut larvae fed mixture of copepods and enrched artemia. Last 4 rations enrched *Artemia* (460 day-degrees post-hatch)..
OFC1	22/07/99		Rinsed copepods
OFA1	22/07/99		Rinsed 2 day ongrown enriched *Artemia*. Super Selco (INVE) followed by Kelco.

Table 1c Coding scheme used for bacterial identification and description of the different samples of yolk-sac larvae sampled during the rearing trial (Chapter 3). All larvae were sampled at the end of yolk-sac absorption at 220 day-degrees, surface-sterilised in 0.1% benzalkonium chloride to remove external adherent bacteria before being plated onto MA.

Code	Date isolated	Rearing conditions
TG1	May 1998	Flow-to waste; surface disinfected eggs; addition of antibiotics
TG2	May 1998	Flow-to waste; surface disinfected eggs
TG3	May 1998	Recirculated water; surface disinfected eggs
TG4	May 1998	Recirculated water; non-surface disinfected eggs
TG5	May 1998	Flow-to waste; surface disinfected eggs; addition of antibiotics
TG6	May 1998	Recirculated water; non-surface disinfected eggs
TG7	May 1998	Flow-to waste; surface disinfected eggs
TG8	May 1998	Recirculated water; surface disinfected eggs
TG9	June 1998	Flow-to waste; surface disinfected eggs
TG10	June 1998	Flow-to waste; surface disinfected eggs
TG11	June 1998	Recirculated water; surface disinfected eggs
TG12	June 1998	Recirculated water; non-surface disinfected eggs
TG13	June 1998	Flow-to waste; surface disinfected eggs
TG14	June 1998	Flow-to waste; surface disinfected eggs; addition of antibiotics
TG15	June 1998	Recirculated water; non-surface disinfected eggs
TG16	June 1998	Recirculated water; surface disinfected eggs

2.2.8. Initial screening of isolates.

All isolates were initially subjected to 28 tests, which are summarised in Table 2. Each isolate was examined and a note made of its colony morphology on MA and any pigmentation. After performing a Gram stain, motility was then determined by examination of an inverted drop of culture using the x100 oil objective of a light microscope (Vickers Instruments) after 24-48 h incubation in Marine Broth (MB). Growth on TCBS (Oxoid) was determined after upwards of seven days incubation. Cytochrome oxidase activity was determined using Kovac's reagent and freshly grown organisms from MA plates (see Appendix 1).

2.2.8.1. Antibiotic susceptibility testing

Each isolate was screened against a wide range of antibiotics using the Mast Ring S system (Mast Diagnostics Ltd). As there are substantial inter-strain, inter-family and inter-genus variations in the susceptibilities of bacteria to antibiotics, this is a way in which large numbers of isolates from a single source can rapidly be sorted into presumptive phenons. Phenon representatives were then taken through a fuller characterisation process.

Organisms to be tested were grown in 1ml MB at 10 ^{O}C until the OD_{600} reached 0.1; 100 μl aliquots of the MB cultures were then spread onto three individual MA plates and left to dry for 2-3 h. Mastring S discs (11, 14 and 46, see Table 2) were then placed onto the inoculated plates and incubated at 10 ^{O}C until a visible lawn of growth was discernible. Resistance to the vibriostat agent 0/129 was also determined by placing a 10 and 150 μg disc (Oxoid) onto the centre of the M14 and M46 plates respectively at the same time as the Mastring discs were placed upon the plates. Susceptibility of each isolate to the test antibiotics was then determined. For cluster analysis, the data was initially represented in binary form as sensitive or resistant. Sensitive isolates showed a zone of clearing greater than or equal to 2mm around the test disc. For later, more in-depth analysis of differences between strains of closely related species, a greater degree of gradation was introduced by scoring sensitivity on a scale between 0 –10; 10 represented a complete zone of clearing greater than 2mm

Table 2 Identification tests for initial clustering of sample isolates

Gram stain

Pigment production

oxidase

Morphology (rod or coccus)

Motility

Growth on TCBS

Resistance to: O/129 10μg

O/129 150μg

<u>Mastring S</u>

Resistance to:

Chloramphenicol 25 μg

Erythromycin 5μg

Fusidic acid 10μg

Methicillin 10μg

Novobiocin 5μg

Penicillin G 1 unit

Ampicillin 10μg

Ampicillin 25 μg

Cephalothin 5μg

Colistin sulphate 25 μg

Gentamycin 10μg

Streptomycin 10μg

Sulphatriad 200 μg

Tetracycline 25 μg

around the test disc, 5 was a zone of clearing of less than 2mm and 2.5 for discernible interruption of growth of the organism around the test antibiotic but not complete clearing.

2.2.9. Cluster Analysis

Isolates were grouped using a UPGMA clustering programme supplied by J. L. Nicolas (IFREMER, Centre de Brest) which was run on a PC.

2.2.10. BIOLOG GN

Cluster representatives of the different samples were identified phenotypically using the commercially available BIOLOG GN scheme (UK agent, Don Wheatley Scientific). Essentially, the manufacturer's protocol was followed with some modifications to the inoculation medium and incubation conditions to better support the growth of marine organisms. BIOLOG GN was mainly designed for the identification of Gram negative bacteria of medical and mammalian veterinary importance and the recommended culture conditions reflect this.

2.2.10.1. Method

Isolates were subcultured onto MA and grown at 20 ^{O}C for 24-48 h. A 20ml tube, prefilled with modified sterile inoculation medium (2.5% NaCl, 0.05% KCl, 0.8% $MgCl_2$ and 0.15% carageenan), was then inoculated with colonies from the freshly-grown plate to form a uniform suspension with a transmission level of between 53 to 59% on the BIOLOG turbidimeter. 150 μl of the cell suspension was then added to each well of the BIOLOG GN plate and incubated (typically for 48 h) in a sealed plastic box containing a source of moisture at 20 ^{O}C. The plate was then read in the Biolog microtitre plate-reader at 590nm, and compared to other strain profiles using the MICROLOG 3 software supplied with the plate-reader.

2.2.11. Conventional Biochemical testing

After antibiogram cluster representatives had been identified using BIOLOG GN and placed into phenotypic groupings, additional biochemical tests were carried out on isolates from the different phenotypic groupings as a further aid to identification. Tests done included: oxidation and fermentation; nitrate reduction, ONPG (O-nitrophenyl-β-D-galactopyranoside; L-lysine, L-arginine and L-ornithine

decarboxylase activity; Voges – Proskauer (VP); indole production; catalase activity; acid from salicin, glucose and arbutin; and aescuilin hydrolysis (see Appendix 1 for details). Other discriminatory observations from the initial characterisation were also included, such as colony morphology, pigment production, growth on TCBS, cytochrome oxidase activity, agarase activity, luminescence and swarming on complex media.

2.2.12. PCR-RFLP

The 16S rRNA genes of representative bacteria were amplified using the Polymerase Chain Reaction (PCR), following which the amplicons were digested using different restriction enzymes. The fragments were then resolved by gel electrophoresis and the resultant pattern viewed.

2.2.12.1. DNA Template preparation and PCR conditions.

Template DNA was prepared in one of two ways. The first method entailed suspending a colony harvested from a fresh MA culture of the organism of interest in 100 μl of TE buffer (10mM Tris, 10mM EDTA pH 8.0) in a microcentrifuge tube and boiling for 10 min; 5μl of the resultant supernatant was used in subsequent PCR reactions. If this proved unsuccessful, attempts were made to prepare template DNA using a commercial kit (Instagene Matrix, BIORAD). The manufacturer's protocol was followed with minor modifications. A single isolated colony from a MA pure culture of the organism of interest was suspended in 1 ml sdH_20 in a microfuge tube and centrifuged at 12000 x g. The supernatant was then removed and 200μl of InstaGene matrix added to the pellet prior to incubation at 56 ^{O}C for 15-30 min. The mixture was then vortexed at high speed for 10 sec and then heated at 100°C for 8 min. The suspension was vortexed again for 10 sec, centrifuged at 12000 x g for 3 min and 5 μl of the supernatant used in subsequent 100μl PCR reactions (instead of the recommended 20 μl per 50μl reaction). It was also possible to store the prepared sample at –20 ^{O}C for later use.

The respective DNA template preparations were subjected to PCR amplification using the universal eubacterial primers 27F and 1522R (Figure 1). For the PCR, essentially the method of Suzuki *et al.* (1997) was followed, except that

HotStarTaq DNA Polymerase (Quiagen) was used instead of conventional Taq DNA polymerase.

Figure 1 Synthetic oligonucleotides used for PCR amplification in RFLP and 16S rRNA gene sequence analysis.

27f	5'-AGAGTTTGACMTGGCTCAG-3'
1522r	5'-AAGGAGGTGATCCANCCRCA-3'
685r	5'-TCTACGCATTTCACYGCTAC-3'

2.2.12.2. PCR method

Incubations were carried out in 0.5 ml microfuge tubes. The reaction mixture contained 5μl of template DNA (approximately 250 ng), 0.1 μM of each primer, 10x PCR buffer (100mM Tris-HCl, pH 9.0; 15 mM $MgCl_2$; 500mM KCl; 0.1% [v/v]; 1% [v/v] Triton X-100) (Quiagen); 200 μM of each dNTP (dATP, dTTP, dCTP, dGTP); and 0.5 μl (2.5 units) of the HotStarTaq DNA Polymerase in a final volume of 100μl. Samples were incubated at 95 ^{O}C for 15 min to activate the HotStarTaq DNA polymerase and denature the template DNA before being subjected to 35 cycles of the following incubation sequence:

1. Denaturation at 94°C for 1 minute
2. Primer extension at 55°C for 2 min
3. Product extension at 72 ^{O}C for 3 min.

Finally, product extension at 72°C was maintained for 10 min. Thermocycling was carried out using a programmable dri-block (Hybaid OMN.E).

2.2.12.3. Analysis of PCR products

PCR products were viewed under UV light following electrophoresis through a 0.8% agarose gel containing 0.5 x TBE and ethidium bromide (0.5 μg ml^{-1}); a 1 kb ladder (Promega) was included asa marker. Successful amplification was indicated by the presence of a single band of approximately 1500 bp.

Initially, a faint band of approximately 1500 bp was present continually in the samples without added template DNA (negative controls). The contamination source was traced to the Hot Star Taq and the manufacturers (Quiagen) confirmed that the likely cause of the contamination was carry over of trace amounts of *E. coli* genomic DNA. This was unlikely to affect results because much larger quantities of sample template DNA would be present. None of the amplified genes subsequently sequenced appeared to be from *E. coli*, supporting this view; later samples of HotStarTaq DNA Polymerase did not appear to suffer from this problem.

2.2.12.4. Restriction digests

Successfully amplified samples were subjected to digestion with a range of Promega restriction enzymes. In general, for each reaction 7µl of the PCR product (approximately 40ng DNA) was added to 0.5µl of the restriction enzyme being used (generally *HaeIII*), 2µl buffer C as supplied with the enzyme, 0.2 µl 10 µg ml^{-1} BSA, the final volume being made up to 20µl with dH_20. Samples were then incubated at 37 ^0C for 1-4 h before resolving the fragments by gel electrophoresis in 2.5% low melting point (Sigma LMP) agarose in TBE stained with ethidium bromide (0.5 µg ml^{-1}).

2.2.12.5. PCR-RFLP analysis

Banding patterns were examined visually and fragment sizes estimated by reference to the 100BP (Promega) ladders included with each gel. Fragment sizes were later quantified more accurately using the Quantity One gel analysis software (BIORAD); this allowed better between-gel comparisons of different restriction profiles without having to manually realign and run a fresh gel. As the relative intensities of the different bands are automatically calculated, this also gave an indication of possible double or triple fragments of the same size.

2.2.13. 16S rDNA sequence analysis

The 16S rRNA genes from representative isolates were also partially sequenced and compared with each other and with sequences deposited in the databases. This allowed the construction of inferred phylogenetic trees and also sequence matching as an aid to identification.

PCR products were prepared as for RFLP analysis, following which they were purified on Quiaquick-spin columns (Quiagen) and samples then subjected to bi-directional sequencing. Some PCR samples were sent directly to PNACL (Leicester) for sequencing with appropriate universal eubacterial 16S rDNA primers (Figure 1). In most cases the initial sequencing reactions were done in the laboratory.

2.2.13.1. Sequencing Reactions

4.5 μl of the purified PCR product to be sequenced (50-75 ng) was placed in a 0.5 ml microfuge tube, to which were added 4μl of ABI Prism BigDye Terminator Ready Reaction Mix (PE Applied Biosystems), 1.5 μl of the appropriate sequencing primer (1 pmol/μl) and ddH_20 to a total volume of 10μl. The reaction mixture was placed in a thermal cycler (Hybaid OMN. E) and then subjected to 25 cycles of melting at 96°C for 30 sec, annealing at 50°C for 15 sec and extension at 60°C for 4 min.

Completed sequencing reactions were then ethanol/sodium acetate precipitated; 1 μl of 2M sodium acetate (pH 4.5) and 25 μl absolute ethanol were added to the reaction tube which was then vortexed gently and placed on ice for 10 min. The sample was centrifuged at 15000 rpm for 30 min and as much of the supernatant as possible carefully removed. The pellet was then rinsed by adding 250 μl 70% ethanol, vortexed briefly before being spun again for 5 min at 15000 rpm, and as much as possible of the supernatant was then removed. The sample was then spun again briefly before removing the residual ethanol. Samples were then air dried before being sent to PNACL for processing.

2.2.14. Identification and construction of phylogenetic trees

Sequences were initially aligned and assembled automatically using the assembly tool Seqman on a Macintosh microcomputer. All the contigs were then visually checked by inspecting the automatically-aligned trace signals. This often helped to resolve otherwise ambiguous nucleotide positions. Assembled sequences were compared against other 16S rRNA gene sequences using the online tool Sequence Match (version 7.2 written by Neils Larson) on the Ribosome Database Project website (http://www.cme.msu.edu/rdp/html/analyses.html).

Phylogenetic trees were constructed by initially aligning the sequences to be compared using the tool CLUSTAL W online at the European Bioinformatics Institute website (http://ebi.ac.uk). The alignment files produced were edited and trimmed to form a mask of unambiguously aligned nucleotides, these alignments included invariant and gap regions. The edited alignments were then exported to the Institute Pasteur website (http://bioweb.pasteur.fr./) where they were automatically converted into a PHYLIP format infile suitable for submission for online phylogenetic analysis using the programs PUZZLE v. 4.0.2 (Strimmer and von Haeseler, 1996) and the PHYLIP package (Felsenstein, 1991.)

The resultant tree topologies were tested for robustness by bootstrapping the datasets. Output tree files were graphically represented using the program TreeView v.1.6.1 (Page, 1996).

2.3. Results

2.3.1. Scheme for the classification of marine agar culturable bacteria.

Samples were obtained for 3 UK hatcheries over a 21 month period (Table 1) and analysed initially for antibiotic sensitivity using Mastring discs, as well as simple biochemical and morphological tests. Representative isolates were then subjected to analysis with BIOLOG GN plates, PCR RFLP and 16S rRNA partial gene sequencing.

Detailed results for the isolates characterised in depth are contained in Appendix 2, but for convenience the 19 different species or genera of bacteria identified are summarised in Table 3. RFLP profiles of the groups are shown in Figure 2.

Table 3 Description of the 22 different genera or species of bacteria recovered from different samples of larvae, water, live feed and the guts of adult halibut taken from three UK halibut hatcheries.

PCR-RFLP pattern	Morphotype and discriminatory characteristics	Biochemical identification	Closest 16S rRNA identification (RDP score in parentheses)	Scheme Identification
Ia	Yellow pigmented; zone of clearing. Gram negative rods. Gliding motility. Reduce nitrate.	*Flexibacter ovolyticus*-like	*Flexibacter maritimus* str. R2 ATCC 43398 M64629 (0.608)	*Flexibacter ovolyticus*-like
Ib & II	Yellow pigmented, Gram negative rods. Gliding motility.	*Cytophaga* sp.		Other *Cytophaga/ Flexibacter*
III	Cream smooth regular edged colony. Non-fermentative	*Pseudomonas* sp.	*Pseudoalteromonas veronii* AF064460 (.936)	*Pseudomonas* sp. RFLP gp III
IV	Cream, smooth regular edged colony.			*Pseudomonas* sp. RFLP gp IV
Va	Pale cream colonies. Non-TCBS culturable, non-fermentative, Gram negative, cytochrome oxidase positive, motile rods	*Pseudoalteromonas* sp.		*Pseudoalteromonas* gp1
Vb	Smooth regular colony with black pigmented centre and cream edges. Agarase and cytochrome oxidase positive motile Gram negative rods.		*Pseudoalteromonas nigrifaciens* NCIMB 8614 (1.00)	*Pseudoalteromonas* gp2

Table 3 (Continued)

PCR-RFLP pattern	Morphotype and discriminatory characteristics	Biochemical identification	Closest 16S rRNA identification (RDP score)	Scheme Identification
Vb	Pale cream, lustrous. Non-fermentative cytochrome oxidase positive motile Gram negative rods.		*Pseudoalteromonas nigrifaciens* NCIMB 8614 (0.964)	*Pseudoalteromonas* gp3
Vc	Brown pigment production on MA. Lustrous growth; non-fermentative cytochrome oxidase positive motile Gram negative rods.		*Pseudoalteromonas* sp. AF022407 (0.904)	*Pseudoalteromonas* gp 4
VI	Cream colonies on MA. Motile Gram negative, non-TCBs culturable			Unidentified RFLP group VI
VII	Cream colonies on MA. Motile Gram negative , non-TCBs culturable		*Colwellia psychroerythraea* ACAM 605 U85843 (0.645)	Unidentified RFLP group VII
VIII	Cream colonies on TCBS		*Pseudoalteromonas haloplanktis* subsp. *tetraodonis* str. GFC IAM 14160 (T). x82139 (0.697)	Unidentified RFLP group VIII
IX.	Cream colonies on MA.			Unidentified RFLP pattern IX

Table 3 (Continued)

PCR-RFLP pattern	Morphotype and discriminatory characteristics	Biochemical identification	Closest 16S rRNA identification (RDP score)	Scheme Identification
X	Pale yellow colony. Cream on TCBS. Luminous.		*Photobacterium phosphoreum* ATCC 11040 x74687 (0.961)	*Photo. phosphoreum*
X	Pale yellow colonies. Sucrose positive on TCBS, non-luminous.		*Vibrio fischeri* NCIMB 1281 X74702 (0.883)	*V. fischeri*-like
Xb	Pale yellow colonies. Sucrose positive on TCBS, non-luminous		*Photobacterium profundum* (0.883)	*Photo. profundum*-like
XIa	Utilises sucrose on TCBS (yellow). Regular smooth cream colonies on MA. Reduce nitrate. Voges Proskauer negative. Many haemolytic on SRBC.		*V. splendidus* CIP 102893 X74724 (0.911)	*V. splendidus* gp1
XIb	Utilises sucrose on TCBS (yellow). Regular smooth cream colonies on MA. Reduce nitrate. Voges Proskauer negative. Many haemolytic on SRBC.		*V. splendidus* CIP 102893 X74724 (0.942)	*V. splendidus* gp 2
XIb	Cream on TCBS		*Vibrio* ANG.218 str. (0.948)	*V. splendidus* gp 3

Table 4 continued

PCR-RFLP pattern	Morphotype and discriminatory characteristics	Biochemical identification	Closest 16S rRNA identification (RDP score)	Scheme Identification
XII	Yellow on MA. Cream on TCBS.		*V. salmonicida* subgroup *V. logei* x74708 (0.8611)	*V. salmonicida*-like
?	Swarming on MA; yellow on TCBS generally failed PCR-RFLP. Voges Proskauer positive.		*V. alginolyticus* str. XII-53 (0.912)	*V. alginolyticus*
	Large regular cream colonies on MA. No growth on TCBS. Sensitive to 0/129. Reduces nitrate.		*V. tubiashii* CIP 102760 (0.911)	*V. tubiashii*
	Cream isolate		*Halomonas marina* (0.782)	*Halomonas*-like

Figure 2a Different RFLP patterns of isolates encountered during the survey. Patterns shown are the *Hae*III digestion products of the PCR amplified 16S rRNA genes of the different representative isolates resolved by electrophoresis on a 2.5% LMP agarose gel. All isolates are coded as in Table 1. A 100 BP ladder was used as a marker at each end; the brightest band is 500BP.

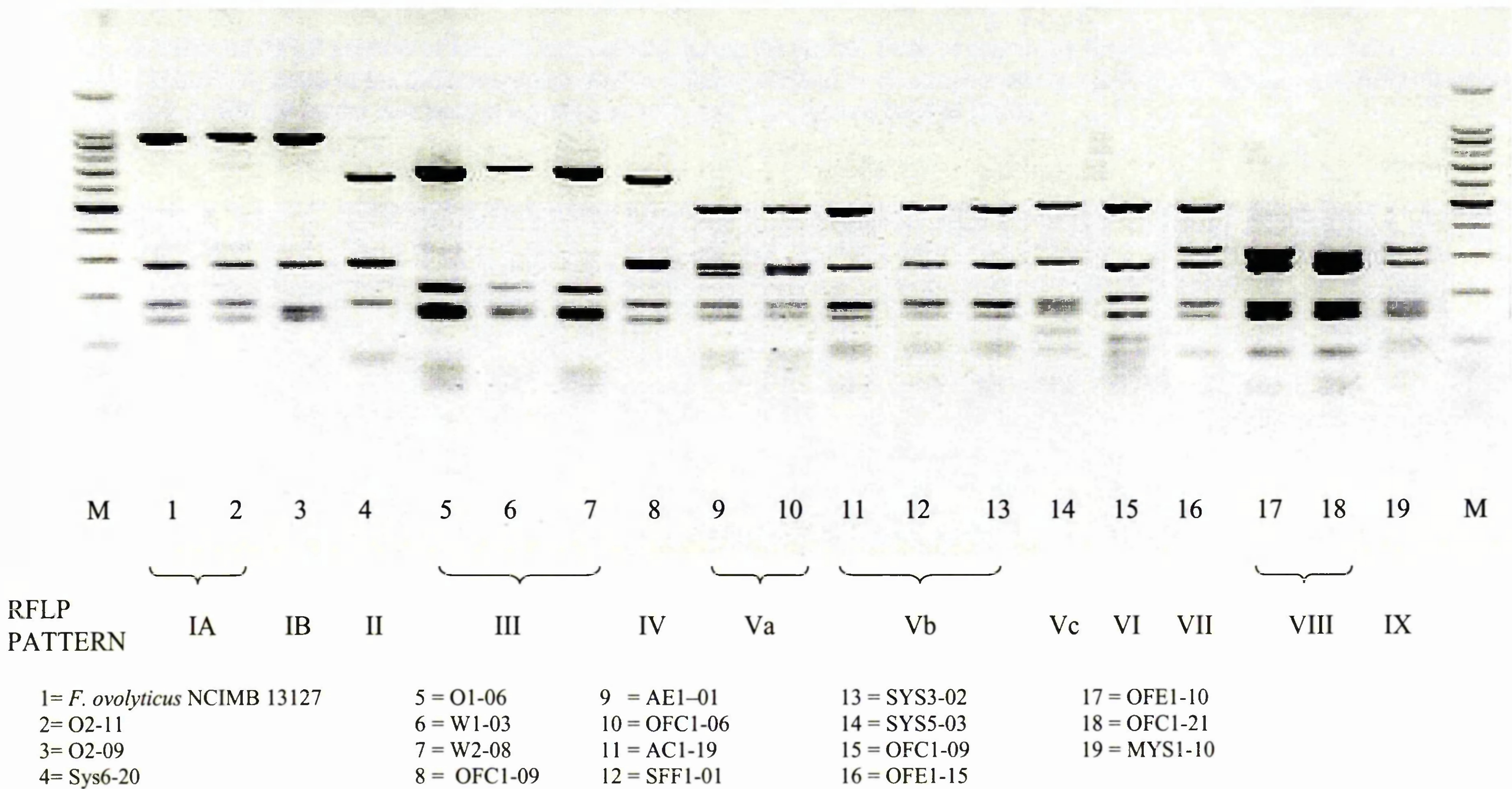
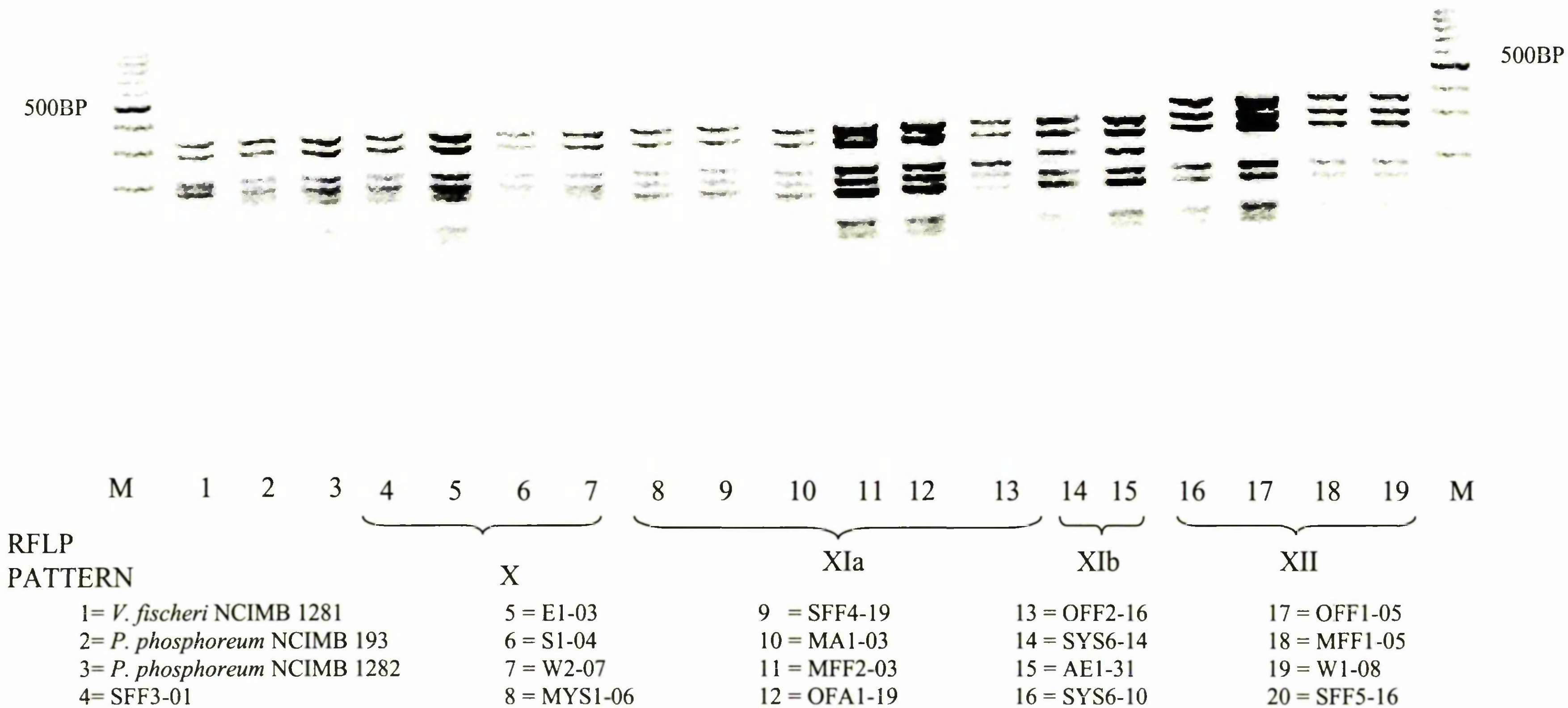


Figure 2b RFLP patterns of different *Vibrio* species isolated from three UK hatcheries. Patterns shown are the *Hae*III digestion products of the PCR amplified 16S rRNA genes of the different representative isolates resolved by electrophoresis on a 2.5% LMP agarose gel. All isolates are coded as in Table 1. A 100 BP ladder was used as a marker (M) at each end; the brightest band is 500BP. X are *Photobacterium phosphoreum* (SFF3-01, E1-03 and S1-04) and *Vibrio fischeri* –like (W2-07) isolates. XIa are *V. splendidus* Group 1 organisms; except SYS6-14 which has a slightly different RFLP pattern to the other organisms in this group. XIb are *V. splendidus* group 2 (AE1-31) and group 3 (SYS6-10) organisms. Group XII (OFF1-05, MFF1-05, W1-08, SFF5-16) are all *V. salmonicida*-like organisms.



2.3.2. Bacterial flora of eggs

Four samples of eggs were analysed (Table 4). The adherent bacterial flora of the eggs sampled immediately after fertilisation was mixed; although dominated by *Pseudoalteromonas* group 3 species, other genera were also present, particularly *Cytophaga/Flexibacter*-like isolates as well as luminous *Photo. phosphoreum* organisms.

The flora of two samples of eggs isolated from Ardtoe towards the end of their development was dominated by organisms that characteristically formed pale yellow colonies on Marine Agar. After approximately one week's growth at 10 ^{O}C a zone of clearing appeared in the centre of the colony; this appeared to coincide with a decrease in viability. Accordingly, all isolates originally identified were lost preventing further biochemical characterisation. Successful PCR-RFLP amplification was achieved in some cases from otherwise unviable colonies six month following initial isolation. The *Hae* III digest profile of one of these isolates (O2-11) is shown in in Figure 2. The *Hae* III digest of a *F. ovolyticus* type strain NCIMB 13127 is also included. It should be noted that there was no difference in the respective digest patterns of the Seafish Ardtoe halibut egg isolate and the *F. ovolyticus* type strain, which was originally isolated from Norwegian hatchery-reared halibut eggs. Little difference in the composition of the epiflora adhering to moribund and apparently healthy halibut eggs was discernible, except for a greater bacterial loading in the unhealthy egg sample.

By contrast, the single batch of Otter Ferry eggs sampled showed an epiflora dominated by organisms dissimilar to those isolated from the Ardtoe eggs. Based on their PCR-RFLP patterns and the limited biochemical data available. The two dominant organisms appeared to be a probable *Vibrio* sp. and a *Pseudomonas* sp.

Table 4 Composition of bacterial flora associated with Atlantic halibut eggs sampled from Seafish Ardtoe and Otter Ferry Seafarms. Isolates were characterised using a variety of techniques as described in section 2.3.

Sample description and code.	Hatchery	Age (day-degrees post-fertilisation).	Average CFU/egg (± SEM)	Identification	Number of isolates
Eggs sampled immediately post-fertlilisation. (E1)	Ardtoe	0		*Photobacterium phosphoreum*	3
				Pseudoalteromonas gp 3.	8
				Pseudomonas sp.	7
				Cytophaga/ Flexibacter spp.	2
Apparently moribund 'red' eggs taken from incubator towards the end of development. (O1)	Ardtoe	65	1.16×10^7	*Flexibacter ovolyticus*-like	18
				Cytophaga/Flexibacter spp.	2
Healthy halibut eggs (O2)	Ardtoe	60	8.3×10^4 ($\pm 2.1 \times 10^4$)	*Flexibacter ovolyticus*-like	13
Healthy halibut eggs (OFE1)	Otter Ferry	65	8.38×10^6 ($\pm 1.11 \times 10^6$)	RFLP gp V11	10
				Pseudomonas sp. RFLP gp V111	6
				Flexibacter/Cytophaga	1

2.3.3. Bacterial flora of yolk sac larvae

Table 5 shows the numbers and composition of the bacterial flora associated with samples of surface-sterilised yolk-sac larvae from the three hatcheries. A wide variation was found in both the numbers and types of bacteria recovered from the larvae, not just between larvae from different hatcheries but also between larvae from the same initial batch of eggs reared in different incubators. This phenomenon will be explored in greater depth in Chapter 4 but it should be noted that there are some apparent patterns to colonisation.

Pseudoalteromonas and *Vibrio* species are the dominant constituents of the gut flora of yolk-sac larvae shortly before transfer into first-feeding incubators. The high variation in average numbers recovered between samples and the high sample confidence intervals indicates that colonisation of yolk-sac larvae is likely to be a stochastic process. Also, there may well be variation in the numbers of bacteria present within individual yolk-sac larvae.

Table 5 Presumptive gut microflora of yolk-sac larvae from SFIA Ardtoe. Larvae were surface sterilised in 0.1% benzalkonium chloride to remove external adherent bacteria prior to homogenisation and plating onto Marine Agar. Isolates were characterised using a variety of techniques as described in section 2.3.

Sample description	Batch	Average CFU/larva (± SEM)	Identification	Number of isolates
Healthy 32 day-degree yolk-sac larvae from 950L incubator. SYS3	4/IX	< 5	*Flexibacter/ Cytophaga* spp	2
			Pseudoalteromonas gp 1	2
			Pseudoalteromonas gp 2	1
Healthy day-degree yolk-sac larvae from 950L incubator. SYS4	4/IX	106 ± 30.7	*Pseudoalteromonas* gp 1	6
			Vibrio splendidus gp 1	8
			Unidentified *Vibrio* spp.	4
			*Cytophaga/flexibacte*r spp	2
			Lost	10

Table 5 (Continued)

Sample description	Batch	Average CFU/larva (± SEM)	Identification	Number of isolates
Poorly performing 218 day-degree larvae, 450L incubator. SYS5	3/IX	2.01×10^4 $\pm 2.41 \times 10^4$	*Pseudoalteromonas* gp 4	13
			Pseudoalteromonas gp 3	4
			Pseudoalteromonas sp 2	4
			Flexibacter/ Cytophaga spp	2
			[a]Unidentified *Vibrio* group	5
			Lost	2
Healthy 218 day-degree yolk-sac larvae from 950 L incubator SYS6	3/IX	83 ± 17.8	*V. splendidus* gp. 1	6
			V. splendidus gp 3	3
			Flexibacter/ Cytophaga spp.	2
			Unidentified *Vibrio* spp.	4
			Unidentified other	2
			Lost	3

[a] Group of organisms that clustered to 85% level after antibiotic susceptibility testing and simple biochemical tests. They formed cream colonies on MA, were Gram-negative, motile, oxidase-positive rods that grew slowly on TCBS. One organism analysed using BIOLOG GN appeared to be a *Vibrio* species, but cultures were lost, which prevented subsequent analysis.

Table 6 Presumptive gut microflora of yolk-sac larvae from Mannin and Otter Ferry. Larvae were surface sterilised in 0.1% benzalkonium chloride to remove external adherent bacteria prior to homogenisation and plating onto Marine Agar. Isolates were identified using a combination of methods according to the scheme presented in section 2.3.

Sample description and code	Average CFU/larva (± SEM)	Identification	Number of isolates
Mannin 183 day-degree healthy yolk-sac larvae (MYS1)	368 (± 228)	*Pseudoalteromonas* spp	6
		Photobacterium profundum	3
		Unidentified *Vibrio* RFLP gp	1
[a]Mannin 183 day-degree healthy yolk-sac larvae (MYS2).	13.3 (± 11.4)	*Halomonas marina*-like	3
		[b]Unidentified Gram -ve motile rods	3
		Lost	4
Otter Ferry 170 day-degree healthy yolk-sac larvae from 1500L first-feeding tank	<5		

[a]The Mannin yolk-sac larvae samples were taken from two incubators which had been separately stocked with eggs from the same batch.

[b]Gram negative, motile rods that did not grow on TCBS and had a different antibiotic resistance profile to other organisms sampled.

2.3.4. Bacterial flora of first-feeding Atlantic halibut larvae

Tables 7 and 8 show the types and numbers of MA-culturable bacteria isolated from first-feeding and weaned halibut larvae reared at the three hatcheries. The numbers of bacteria associated with the gut of halibut larvae varied during their development (Figure 4) and the numbers recovered after larvae began feeding were higher than those associated with surface-sterilised yolk-sac larvae. In the particular batch monitored, levels rose after the larvae start to feed, and reached a peak of 10^5 marine agar culturable organisms/ larva within a week of starting to feed.
The types of bacteria recovered varied at different sampling points. *Vibrionaceae* were the dominant organisms recoverable from first-feeding halibut larvae but, as shown in Table 8, *Pseudoalteromonas* species may transiently colonise larvae immediately after they start to feed. Another example of transient colonisation is shown in Table 7. Here larvae were colonised by a luminous *Photo. phosphoreum* isolate. This species was not detected when the batch of larvae were sampled again 26 days later. *Vibrionaceae*, particularly *V. salmonicida*-like and *Photobacterium* species, other than those routinely associated with the live food, were recovered in high numbers from the guts of halibut larvae and juveniles in all three hatcheries. They were generally present in later developing halibut, but this pattern was not predictable. In the Mannin first feeding larvae (Table 9), for instance, *V. salmonicida*-like organisms were associated with a batch of sampled 7 days post first-feeding larvae. The older batch (59 days post first-feeding) did not appear to have this organism though, and was dominated instead by the *V. splendidus* organisms more commonly associated with younger larvae.

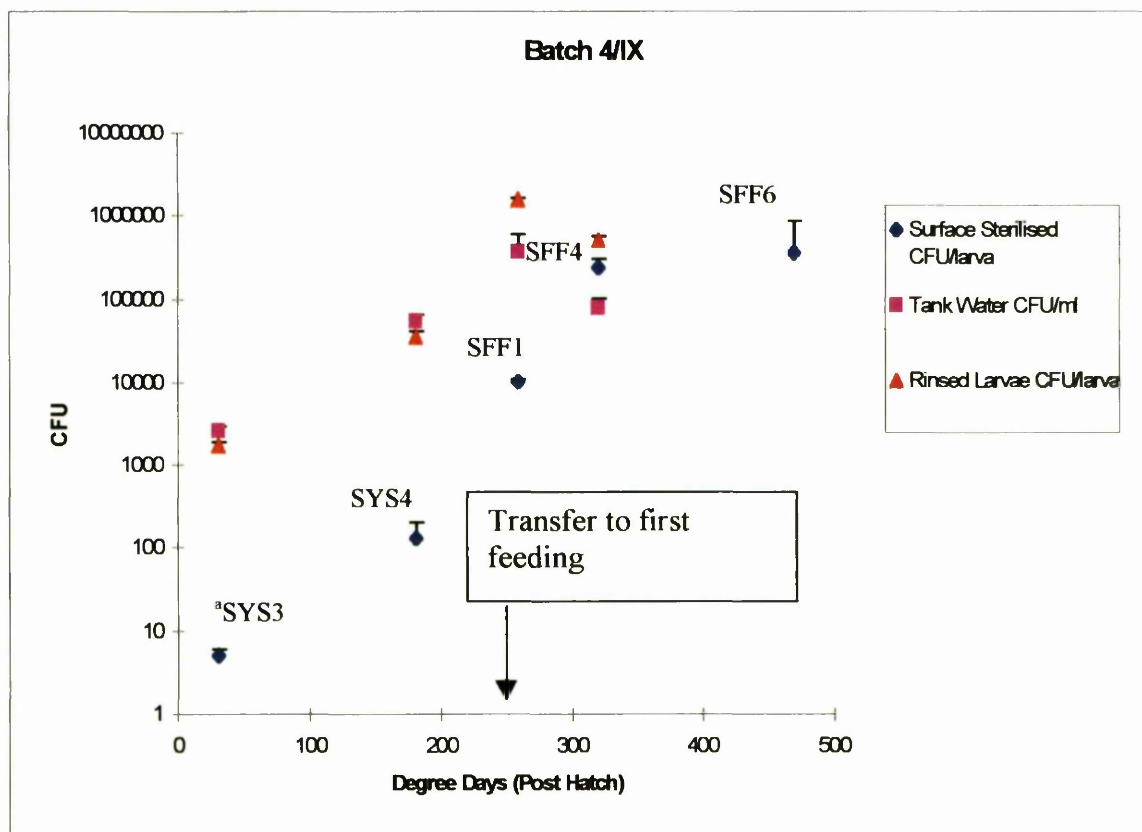
The two groups of Otter Ferry first feeding larvae analysed were also dominated by groups of organisms similar to those found in the other two hatcheries (Table 10). The younger group of larvae (OFF2), which had been fed on a mixture of enriched *Artemia* and copepods, contained a number of different organisms. These included both *Artemia*-associated *V. alginolyticus* and *V. splendidus* gp1 species, as well as *V. salmonicida*-like organisms. The group of larvae that had been solely fed on copepods (OFF1) was colonised almost exclusively by the *V. salmonicida*-like

organism, although this species was not present in the samples of copepods that were analysed.

The phylogenetic affiliations of the *V. splendididus* and *V. salmonicida*-like organisms isolated from the different hatcheries are explored in more depth later.

The fry that had been weaned from *Artemia* onto an inert diet appeared to be dominated by *V. salmonicida*, *Pseudomonas* and *V. fischeri*-like organisms. No live feed-associated organisms were recovered from either group of larvae. The *V. fischeri*-like organism was present in high quantities in both groups of weaned fry analysed but was not encountered in any of the first feeding larval samples.

Figure 4 Total levels of presumptive gut, tank water and larval surface bacteria isolated from a batch of Ardtoe-reared halibut larvae sampled at different developmental stages. Refer to Tables 5 and 7 for a description of the composition of the surface-sterilised (presumptive gut bacteria) samples.



[a] Isolate codes for surface-sterilised samples indicated.

Table 7 Presumptive gut micro-flora of a single batch (2/IX) of Ardtoe-reared halibut larvae sampled at three different times during development from the same first feeding tank and then from the weaning tank following transfer.

Sample description	Average CFU/larva (± SEM)	Identification	Number of isolates
Apparently healthy first-feeding larvae from tank containing 'white gut' moribund larvae	5.13×10^5	*Photo. phosphoreum*	8
	($\pm 4.46 \times 10^5$)	*V. splendidus* gp. 3	9
SFF2		Unidentified *Vibrio* spp	3
Moribund 'white gut' first feeding larvae.	7.77×10^4	*Photo. phosphoreum*	18
SFF3	($\pm 2.33 \times 10^4$)	Unidentified *Vibrio* spp	2
First-feeding larvae samples from the above tank 26 days later	7.43×10^5	[a]*V. alginolyticus* gp.	8
SFF5	($\pm 3.18 \times 10^5$)	*V. splendidus* gp 1	6
		V. salmonicida-like	6
Weaned halibut larvae feeding on an inert diet	8.33×10^4	*V. fischeri*-like	15
W2	($\pm 2.33 \times 10^4$)	*P. veronii* gp.	6
		Flexibacter/Cytophaga spp.	1

[a]Presumptive *V. alginolyticus*, as PCR-RFLP and 16S rRNA gene sequencing proved problematical due to difficulties encountered with PCR amplification

Table 8 Presumptive gut micro-flora of a followed batch (4/IX) of halibut larvae sampled at three different times during development from the same first feeding tank and then from a weaning tank following transfer.

Sample description and code	Average CFU/larva	Identification	Number of isolates
First feeding larvae 3 days post transfer (SFF1)	1.03×10^4	*Pseudoalteromonas* gp. 2	19
	(± 557)	*V. splendidus* gp. 2	1
First feeding larvae 11 days post-transfer (SFF4)	2.36×10^5	*Pseudoalteromonas* gp 2	1
	$(\pm 4.47 \times 10^4)$	*V. splendidus* gp. 1	18
		Unidentified *Vibrio* sp.	1
35 days post-transfer (SFF6)	3.57×10^5	[a]*V. alginolyticus* gp	17
	$(\pm 3.57 \times 10^5)$	*V. tubiashii*	3
Weaned (freshly) 89 days post-transfer (W1)	5.25×10^3	*V. salmonicida*–like	1
	± 250	*P. veronii* gp	2
		Photo. phosphoreum-like	6
		Unidentified Gram -ve rods	2

[a] see previous table foot note

Table 9 Identity of bacteria isolated from Mannin Seafarms first-feeding larvae.

Sample description	Diet	Average CFU/larva	Identification	Number of isolates
7 days post first-feeding larvae	*Artemia* nauplii (un-enriched)	8.45×10^4	*V. splendidus* gp 1	9
(78 day-degrees)		($\pm 2.6 \times 10^4$)	*V. salmonicida*-like	4
MFF1			[a]Unidentified *Vibrio*	1
			Unidentified *Vibrio* sp. (other)	1
59 days post first-feeding halibut larvae	Combination Algamac 2000 and Super Selco enriched Artemia	2.69×10^3	*V. splendidus* gp 1	10
MFF2		($\pm$ 759)	Unidentified *Vibrio* spp	3
			Lost	1
			Unidentified non-TCBS culturable rod.	1

[a] '*Vibrio splendidus* gp3'-like (sucrose negative on TCBS and same PCR-RFLP pattern) but unable to compare further without 16S rDNA sequence data.

Table 10 Numbers and identity of MA-culturable bacteria isolated from first-feeding halibut larvae at Otter Ferry Seafarms. Isolates were initially clustered on the basis of colony morphology allied to PCR-RFLP digest pattern. Representative isolates were then characterised further by a combination of biochemical testing (BIOLOG GN) and 16S rRNA gene sequencing.

Sample description and isolate code	Diet	Average CFU/larva (± SEM)	Identification	Number of isolates
24 days post-first feeding halibut larvae. OFF2	Fed on a mixture of copepods and enriched *Artemia.*	4.55×10^3 ($\pm 2.6 \times 10^3$)	*V. salmonicida*-like	5
			V. alginolyticus-like	4
			V. splendidus gp1	2
			[a]Unidentified RFLP pattern 2	1
			[b]Unidentified *Vibrio* sp.	7
630 day-degree halibut larvae OFF1	Entirely maintained on copepods	2.3×10^4 ($\pm 1.67 \times 10^4$)	*V. salmonicida* –like	16

[a]Haemolytic cream organism with RFLP pattern similar to *Pseudoalteromonas* spp., does not grow on TCBS and BIOLOG GN profile well separated from *Vibrio.*

[b]Non-swarming organism, not amenable to PCR analysis. Identified as a distinct *Vibrio* species by BIOLOG GN

2.3.5. Bacterial flora of the live food in the different hatcheries

Different samples of freshly hatched *Artemia* nauplii, enriched ongrown *Artemia* and copepods were analysed from three hatcheries (Table 11). Enriched ongrown *Artemia* samples from both Mannin Seafarms and Otter Ferry had 10^3 - 10^4 CFU/ rinsed *Artemia,* similar to that found in previous work for Ardtoe enriched-*Artemia* (Verner - Jeffreys, 1996). This appears to be the typical loading associated with enriched *Artemia* at the point at which they are fed to halibut larvae in UK hatcheries at present.

The composition of the bacterial flora associated with enriched rinsed *Artemia* was mixed. As well as *Pseudoalteromonas*-like organisms, a high proportion appeared to be *Vibrio* spp., particularly *V. splendidus* gp1, which was present in all three live food sytsems investigated.

Two samples of unenriched *Artemia*, from different hatcheries, were looked at, and in each case the numbers of culturable bacteria associated with the *Artemia* nauplii were lower than the enriched *Artemia* samples that were collected in parallel. It was also noteworthy that there was not the same domination by *V. splendidus* and *V. alginolyticus*.

Figure 5, illustrates how, in the Ardtoe production system, only a small fraction of the types of bacteria associated with *Artemia* cysts, unenriched nauplii and enriched ongrown *Artemia* appear to be the *V. alginolyticus* and *V. splendidus* organisms which appear to settle within the guts of first feeding halibut. Few, if any, of these organisms have been found in association with *Artemia* cysts and nauplii. Generally, they appear to be a normal part of the flora of enriched *Artemia*.

For copepods, the numbers of associated culturable bacteria was low compared to any of the *Artemia* samples analysed (Table 11) and the bacterial flora associated with the sampled copepods was mixed. The flora included *V. splendidus* organisms, as well as *Pseudomonas* species, and one organism that closely resembled an organism that was previously found in association with Otter Ferry eggs (see Table 4).

Table 11 Marine Agar-culturable bacteria isolated from live food from the hatcheries. Isolates were initially clustered on the basis of colony morphology allied to PCR-RFLP digest patterns and/or antibiotic resistance profile. Representative isolates were then characterised further by a combination of biochemical testing (BIOLOG GN) and 16S rDNA gene sequencing. Where indicated, samples were initially enumerated after growth on Marine Agar and/or TCBS.

Sample description	Average MA culturable CFU/live food particle, g or ml	Average TCBS culturable CFU/live food particle, g or ml	Identification	Number of Isolates
Artemia cysts	1.36×10^6 g^{-1} ($\pm 8.75 \times 10^5$)	None detected		
Ardtoe fresh hatch *Artemia* nauplii water.	6.06×10^6 / ml ($\pm 8.52 \times 10^5$)	2×10^4/ml ($\pm 7.07 \times 10^3$)		
Ardtoe enriched *Artemia* water.	$\times 10^8$ /ml ($\pm 4.5 \times 10^6$)	4.68×10^6/ml ($\pm 1.79 \times 10^6$)	MA isolates were non-TCBS culturable.	20 (MA)
			TCBS isolates were *V. splendidus* gp 1 & 2	5 (TCBS)
Rinsed Mannin enriched *Artemia*	1.18×10^4 ($\pm 5.39 \times 10^3$)	[a]ND	*V. splendidus* gp 1 & 2	4
			Non TCBS-culturable	11
Rinsed Mannin fresh hatch *Artemia* nauplii	1.08×10^2 (± 8.15)	ND	Non TCBS culturable	10
			Lost	5

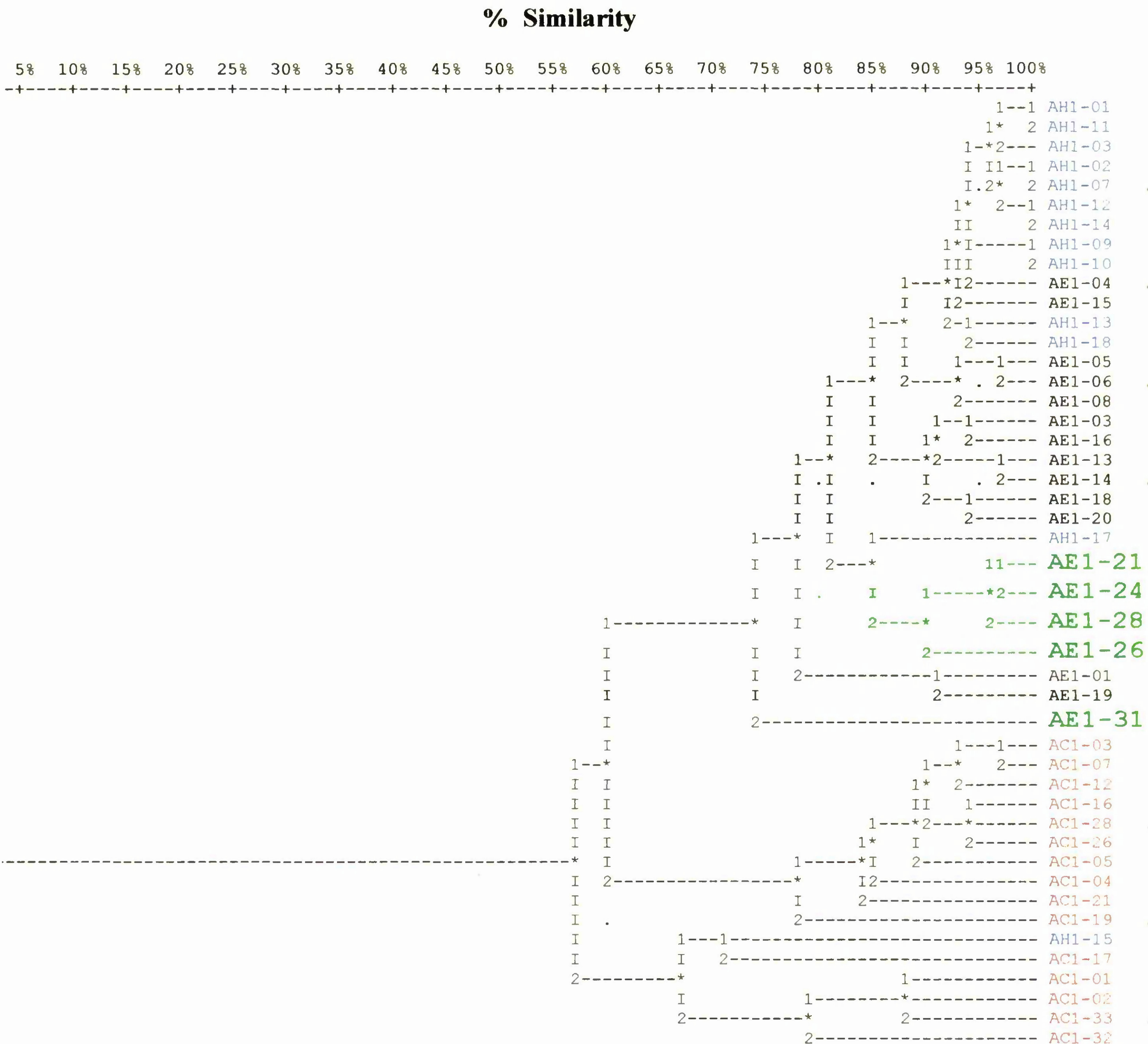
[a] ND = not done

Table 11 (continued)

Sample description	Average MA culturable CFU/live food particle, g or ml	Average TCBS culturable CFU/live food particle, g or ml	Identification	Number of Isolates
Otter Ferry enriched *Artemia*	1.41×10^4 ($\pm 1.86 \times 10^3$)	[a]ND	*V. alginolyticus*	10
			V. splendidus gp1	6
			Non TCBS-culturable	4
Otter Ferry copepods	5.67×10^2 ($\pm 3.12 \times 10^2$)	ND	[b]Unidentified	7
			Vibrio splendidus gp 2	3
			Yellow pigmented	3
			RFLP group VIII	1

[a] ND = not done
[b] Unidentified copepod-associated organisms. Not amenable to PCR-RFLP analysis. Gram -ve rods, sucrose negative growth on TCBS, clustered as distinct phenotype by BIOLOG GN (see Appendix 2f).

Figure 5 Dendrogram showing % similarity of isolates taken from Ardtoe *Artemia* cysts, *Artemia* hatching water and *Artemia* enrichments (based on sensitivities to antibiotics and simple biochemical and morphological tests). Isolates in bold resemble isolates later recovered from first-feeding halibut larvae. Isolates are coded as in Table 1. Some of these isolates have been further characterised by partial sequencing of their16S rRNA genes. AE1-26 is a group 1 *V. splendidus* organism. AE1-31 is a group 2 *V. splendidus* organism and AE1-24 is a *V. alginolyticus* organism.



Green = *Vibrio* species from enriched *Artemia* water
Black = *Pseudoalteromonas* species from enriched *Artemia* water
Blue = Isolates from freshly hatched *Artemia* Water
Red = Isolates from *Artemia* cysts

2.3.6. Gut microflora of adult Atlantic halibut

The gut heterotrophic microflora of adult farmed Atlantic halibut from SFIA Ardtoe was investigated by culture on MA. A high proportion of bacteria recovered (Table 12) appeared to be *Photo. phosphoreum* isolates, with the exception of the individual that had not apparently been feeding recently.

2.3.7. Bacterial flora of rearing water

On various occasions, water samples from the larval rearing tanks were also taken at the same time as Ardtoe surface-sterilised larval samples. In-depth characterisation was not performed but, as can be seen in Table 13, there were apparent differences in the relative proportions of different groups of bacteria present in the guts of larval halibut and the water surrounding them.

In the case of the yolk-sac larvae, *Vibrio* and *Pseudoalteromonas* spp. appear to be present in the water and surface-sterilised larval samples. There is some evidence that they tend to be over-represented in the guts of the larvae compared to their relative proportions in the water surrounding them; 69% of all the surface-sterilised yolk-sac larvae were identified as belonging to these groups as opposed to only 25.4% of the organisms isolated from yolk-sac incubator water samples.

A similar pattern is evident in the first feeding larvae. *Vibrio* and *Pseudoalteromonas* species are present in both the water and the guts of the larvae but other organisms, apparently present at high densities in the tank water, do not appear to be able to establish themselves in the guts of first-feeding halibut larvae. The high numbers of lost organisms in the water samples is noteworthy. Most of these were very slow growing strains that could not be retained using traditional culture techniques on MA. By contrast, a low proportion of the organisms isolated from first feeding larval samples were lost on subculture.

Table 12 Marine Agar culturable bacteria isolated from the hindgut of adult male halibut from SFIA Ardtoe. All fish were from a tank of 1995 yearclass fish that had been hatched and raised at the hatchery and fed on a moist diet. All fish sampled were adult males in spawning condition.

Sample description	Average CFU/g	% Luminous	Identification	Number of isolates
Faecal samples from four adult Atlantic halibut that had recently fed. Mean weight 2.9 kg	$3.13 \times 10^6 \pm 3.61 \times 10^6$	>70%	*Photo. phosphoreum*	12
			Other G-ve, oxidase positive motile rods	4
Fluid from hind gut of adult halibut devoid of solid faecal material. 3.4 kg.	1.6×10^5	2.14	G-ve motile rods	8
			[a]*Photo. phosphoreum*	2

[a] *Photo. phosphoreum* isolates were selected from a low dilution plate so are not representative of random sampling.

Table 13 Analysis of bacteria present in the guts of 5 different groups of Ardtoe larvae and the water surrounding them. In each case, samples of tank water were sampled and plated onto MA at the same time as the larvae. Isolates from each sample were randomly selected and clustered using Clustan Graphics Focal Point analysis on the basis of their antibiotic susceptibilities and simple biochemical testing; and placed into 7 different phenons, which are described in more detail in Table 14. Samples are coded as in Table 1. In summary SYS4, SYS5 and SYS6 are surface sterilised yolk-sac larvae samples and YSW4, YSW5 and YSW6 their respective tank water samples. SFF1 and SFF2 are surface-sterilised first-feeding halibut samples with FFW1 and FFW2 being the water samples in each case.

Phenon	Presumptive phenon identification	Number of isolates from the following samples within each phenon									
		SYS4	YSW4	SYS5	YSW5	SYS6	YSW6	SFF1	FFW1	SFF2	FFW2
I	*Vibrio* group, including *V. splendidus* isolates	5	1	4	2	5	5	1	0	11	6
II	*Pseudoalteromonas* spp., including *Pseudo. nigrafaciens* gp 2 isolates	0	1	4	4	0	2	15	6	0	1
III	Group of slow growing Gram -ve, oxidase positive motile rods otherwise not well characterised in the survey	2	4	0	7	0	1	0	0	0	3
IV	*Vibrio* group, including *Photo. phosphoreum* isolates and a group of slow growing organisms that did not grow sufficiently well for BIOLOG GN identification.	0	0	1	0	9	0	0	1	9	0
V	*Pseudoalteromonas* spp. Including black pigmented group 2 and brown pigment producing group 4.	8	0	17	1	0	0	1	0	0	2
VI	Orange pigmentented *Cytophaga/Flexibacter* spp	1	12	0	2	0	0	0	0	0	0
VII	Orange pigmentented *Cytophaga/Flexibacter* spp	0	1	2	3	2	5	0	0	0	0
lost		8	4	4	1	3	7	3	12	0	8

Table 14 Summary of percentage positive test results for the different phenons into which the water and larval isolates were divided in Table 13. Tests are as previously described, with sensitivity to an antibiotic scored as being a positive character marked on a scale of 0–1 with 1 counting as a zone of clearing of at least 2mm around the test disc. Membership of the 7 phenons was determined after clustering the data with the K means analysis program Focal Point Clustering in Clustan Graphics (500 random trials specified to find the globally optimal solution).

	Percentage positive tests for isolates in the following phenons.						
Test	I	II	III	IV	V	VI	VII
	n = 40	n =33	n=17	n=20	n=29	n=25	n=13
Gram stain	0	0	0	0	0	0	0
Motility	98	88	79	100	95	6	10
Pigment production	0	6	7	0	83	81	100
Morphology	100	100	100	100	100	100	100
Oxidase	100	97	86	100	93	6	23
Growth on TCBS	91	3	0	79	3	0	0
Luminescence	0	0	0	38	0	0	0
Chloramphenicol 25 μg	91	63	100	90	100	88	54
Erythromycin 5μg	2	76	43	4	93	13	77
Fusidic acid 10μg	2	0	57	13	0	94	92
Methicillin 10μg	0	2	64	0	1	19	0
Novobiocin 5μg	100	14	43	100	59	19	92
Penicillin G 1 unit	1	0	79	31	0	13	8
Streptomycin 10μg	7	0	36	4	3	6	4
Tetracycline 25 μg	91	88	79	100	88	84	100
Tetracycline 100 μg	98	82	100	100	97	100	92
Cephalothin 5μg	15	2	93	100	2	6	46
Colistin sulphate 25 μg	98	94	86	75	47	13	0
Gentamycin 10μg	70	70	61	58	85	9	4
Sulphatriad 200 μg	18	4	30	32	0	88	62
Ticarcillin 75 μg	47	10	64	80	44	100	15
Ampicillin 10μg	80	62	93	81	90	84	33
Ampicillin 25 μg	82	67	100	88	90	97	46
Nalidixic acid 30 μg	94	11	93	100	0	94	54
Ticarcillin 75 μg	78	21	100	88	95	88	31
Nalidixic acid 30 μg	48	86	64	85	100	88	81
Trimethoprim 2.5 μg	3	1	0	74	7	94	0
Sulphamethoxazole 50μg	0	0	4	39	0	94	0
O/129 10μg	0	1	11	88	0	58	23
O/129 150μg	30	4	39	100	1	97	42

2.3.8. Comparison of the 16S rRNA genes of survey isolates

Figure 6 shows the apparent phylogenetic affiliations of a number of different isolates from the survey and the rearing trial (Chapter 3)based on their 16S rRNA gene homologies, both to each other and to database deposited sequences of reference strains. Firstly, there is a large cluster consisting of isolates identifed as belonging to *V. splendidus* groups 1 and 2 (RFLP groups XIA and XIB). The *V. splendidus* reference strain ATCC 33125T (x74724) is contained within this cluster, identifying all these strains as being *V. splendidus*-like. These organisms were apparently present in the live food and guts of first-feeding halibut larvae in all three hatcheries surveyed.

Figure 7 shows the relationship between the different *V. splendidus* isolates as inferred by comparing a 551 bp section at the beginning of their 16S rRNA genes. The dendrogram shown is based on the results of the maximum likelihood algorithm based-PUZZLE analysis, but a similar relationship between organisms was uncovered when the same data was analysed by other tests (e.g. FITCH, NEIGHBOR and other tools in the PHYLIP phylogeny package- results not shown).

SFF5-03 and AE1-24, organisms isolated from Ardtoe first-feeding halibut and enriched *Artemia*, appear to be most closely related to each other but also cluster with *V. alginolyticus*-type organisms. The sequence data was not optimal for these two organisms, and their similarities may be underestimated, both to each other and the reference organisms, due to false mismatches at ambiguous or incorrectly identified base positions.

On the basis of the limited sequence data available, TG4-07 and SYS6-10 appear to be examples of a *Vibrio* species that has not so far been described. For convenience it has been called *V. splendidus* gp III, but as can be seen in Figure 7, it is, if anything, closer to *V. cholerae* (x74694). The closest RDP match was a so far unidentified *Vibrio* species originally isolated from squid.

OFF1-05, MFF1-05, SFF5-16 and W1-08 (Figures 6 and 8) are organisms isolated from Otter Ferry, Mannin and Ardtoe first-feeding larvae and Ardtoe recently-weaned halibut. All appear to be examples of *V. salmonicida*-like organisms with a similar RFLP pattern (XII).

The phylogenetic affiliations of some of the *Photobacterium* and *V. fischeri*-like organisms recovered are also indicated in Figure 8.

The *Pseudoalteromonas* isolates from both the survey and the rearing trial (Chapter 3) form a distinct cluster. Most of those represented here are members of *Pseudoalteromonas* groups 2 and 3 (RFLP group Vb), which are closely affiliated to *Pseudo. nigrifaciens*. TG15-07, which was isolated during the trial and inhibited the growth of other micro-organisms *in vitro* (see Chapter 5), and SYS5-03 form a separate cluster and RFLP group (Vc).

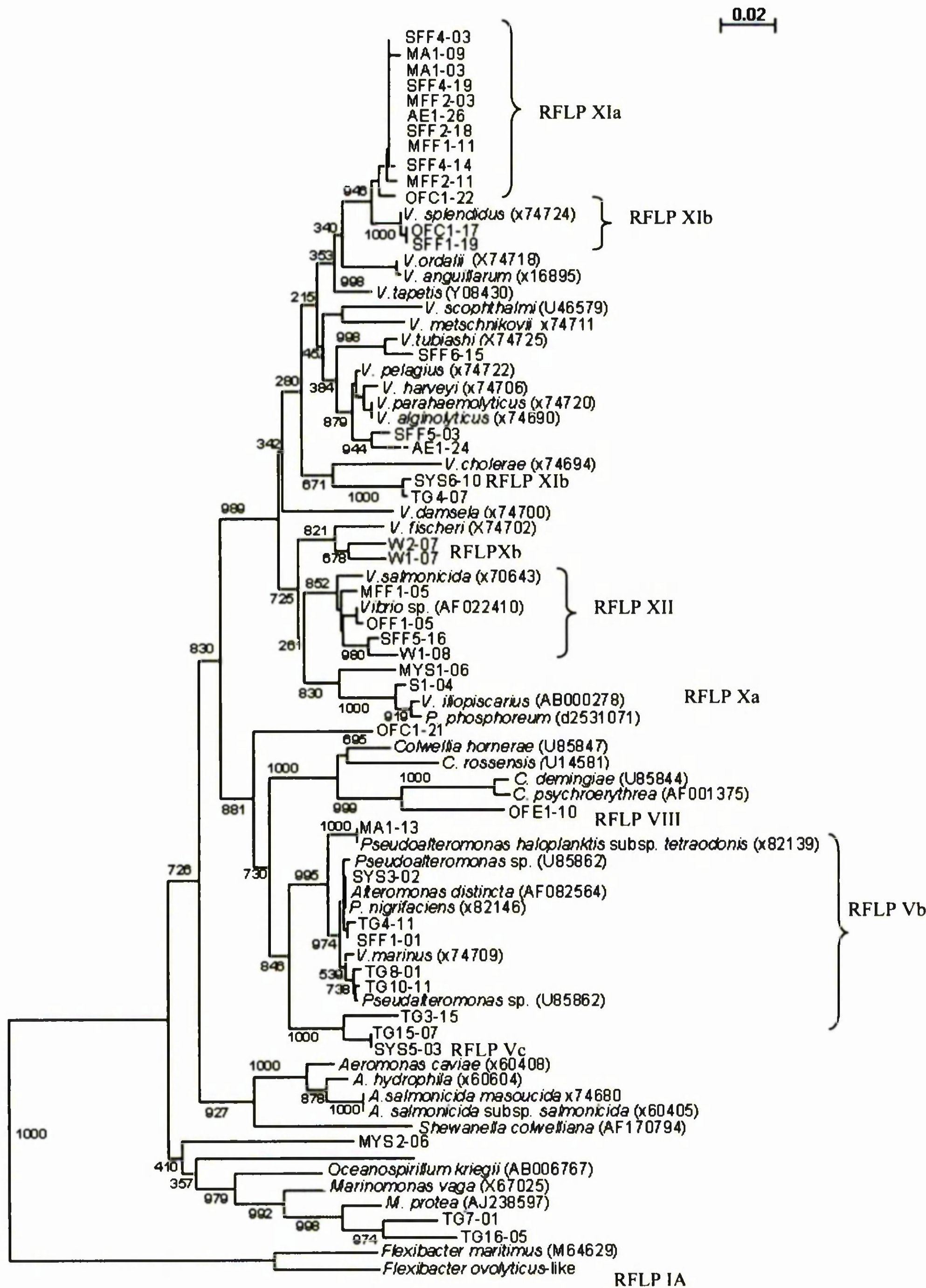


Figure 6 Phylogenetic tree based on the partial 16s rRNA gene sequences of bacteria isolated from the live feed and halibut eggs, larvae and from three UK hatcheries. The unrooted tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) applied to DNA sequence divergence corrected for multiple substitutions (Kimura, 1983). Multiple alignments were generated using the Clustal W program (Thompson et al. 1984b). Isolates are coded as in Table 1. Also included are a number of reference strain sequences, their GenBank accession numbers are given in parentheses. Where applicable, RFLP patterns, as illustrated in Figure 2, are indicated. The numbers at the nodes are bootstrap values (maximum value 1000).
The length of the horizontal branches is proportional to the numbers of nucleotide substitutions.

Figure 7 Molecular phylogenetic tree deduced from analysis of the partial 16S rRNA gene sequences of 23 different organisms (codes as in Table 1). The majority are representatives *of Vibrio splendidus* groups 1, 2 and 3 isolated from live feed and halibut larval samples from three UK hatcheries. As well as a number of *Vibrio* type strain sequences, two *V. splendidus* turbot pathogens are also included (DMC-1 and aj132986vs). aj132988vs is a scallop pathogen. Where appropriate, the sequence database accession numbers are shown. SYS6-10 and TG4-07 are samples from yolk-sac larvae; SFF1-19, SFF4-19, SFF4-14, SFF5-03, MFF2-03 were taken from Ardtoe and Mannin first-feeding larvae; MA1-09 and AE1-26 were isolated from Ardtoe and Mannin enriched *Artemia* samples. OFC1-17 and OFC1-22 were isolated from Otter Ferry copepods. Trees were constructed using the maximum likelihood-based program PUZZLE. Puzzling steps, equivalent to bootstrap values, are shown at the nodes. The length of the horizontal branches is proportional to the numbers of nucleotide substitutions.

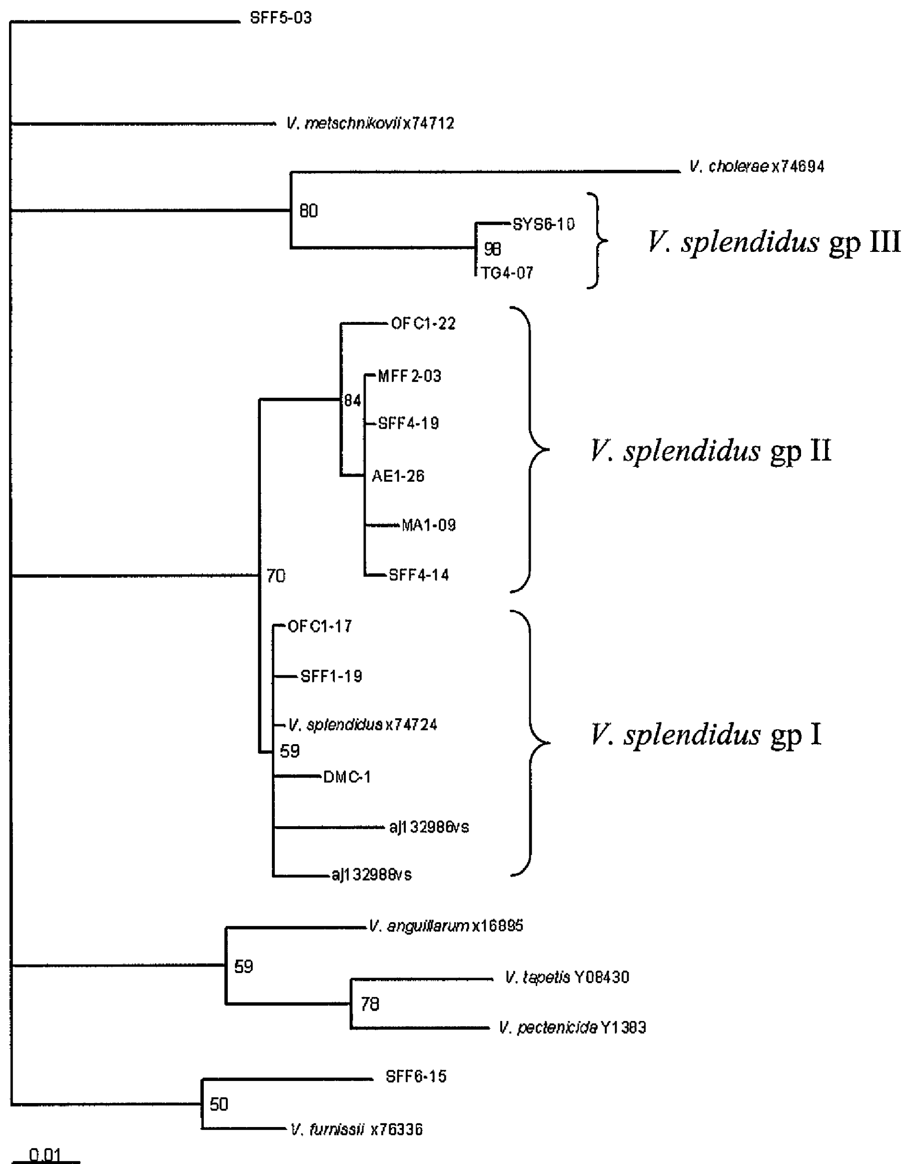
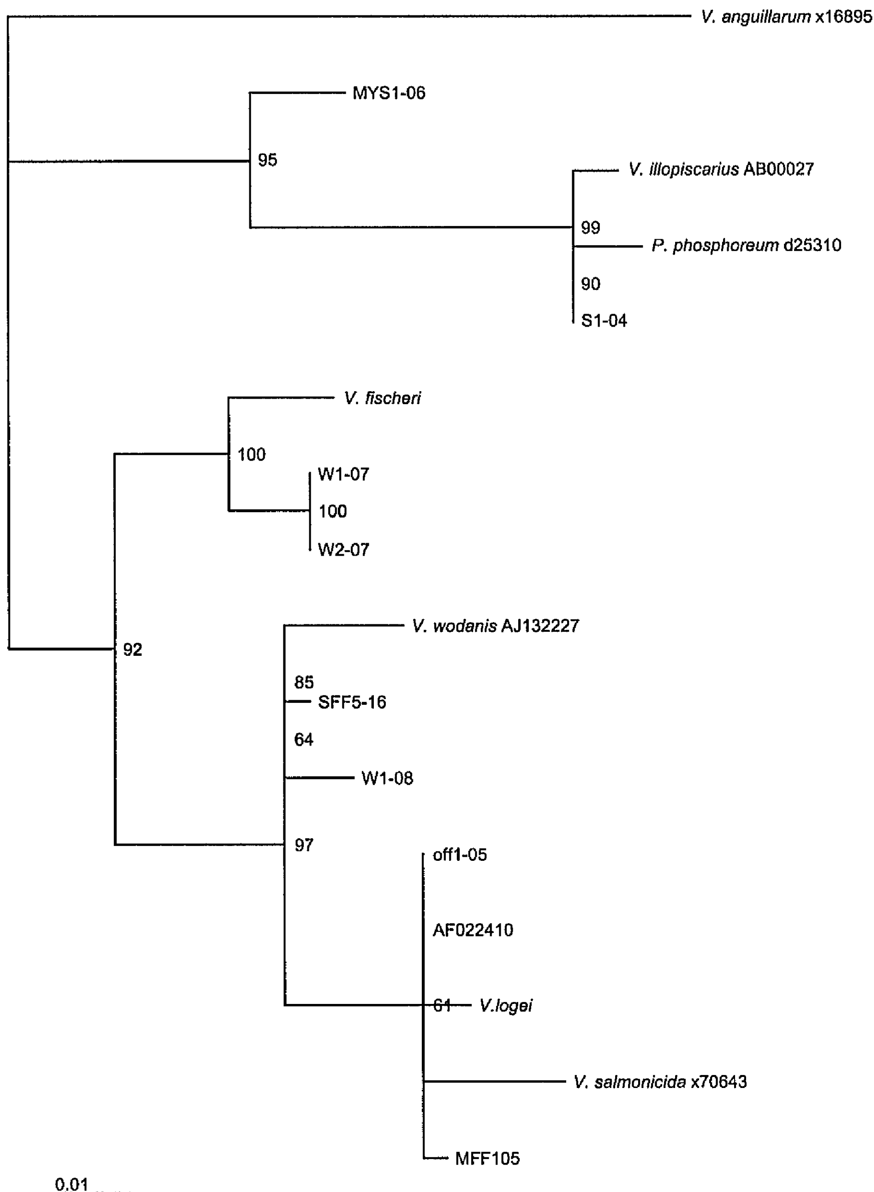


Figure 8 Molecular phylogenetic tree deduced from analysis of the partial 16S rRNA gene sequences of *Vibrio salmonicida*-like and *Photobacterium* organisms. Included are isolates recovered from first feeding halibut larvae from three different hatcheries (MFF1-05, OFF1-05, SFF5-16). Mannin Seafarms yolk-sac larvae (MYS1-06), Ardtoe larvae that had recently transferred to an inert diet (W1-07, W1-08, W2-07,) and a luminous isolate from the gut of an adult halibut are also included (S1-04). The unrooted tree was constructed by the neighbor-joining method (Saitou & Nei, 1987) applied to DNA sequence divergence corrected for multiple substitutions (Kimura, 1983). Multiple alignments were generated using the Clustal W program (Thompson *et al.* 1984b). Isolates are coded as in Table 1. Also included are a number of reference strain sequences, with their GenBank accession numbers. The numbers at the nodes are bootstrap values (maximum value 1000). The length of the horizontal branches is proportional to the numbers of nucleotide substitutions.



2.4. Discussion

In general, the types and numbers of bacteria isolated from larvae and eggs in UK hatcheries appear to be similar to those reported in Norwegian halibut hatcheries (Bolinches and Egidius, 1987; Bergh *et al.* 1994). There was a transition from predominantly non-fermentative organisms associated with the eggs and non-feeding yolk sac larvae to *Vibrio* species once the larvae started to feed. As has been found with other cultured marine fish, some of these *Vibrio* species appear to originate from the live feed (Campbell and Buswell, 1983; Muroga *et al.* 1987; Nicolas *et al.* 1989; Munro *et al.* 1994). Similar patterns of colonisation were found in all three hatcheries, particularly after the larvae had started to feed.

Eggs in the Ardtoe incubators were colonised by an organism that appeared to be very similar to the *F. ovolyticus* species isolated from Norwegian halibut eggs (Hansen and Olafsen, 1989; Hansen *et al.* 1992). This bacterial species was not present in the sample of Otter Ferry eggs sampled or in the freshly-fertilised Ardtoe egg sample. This suggests that, whilst the Ardtoe egg incubator system contained this organism, it does not necessarily occur in all Atlantic halibut egg culture systems. The *F. ovolyticus*-type organism was found in association with both moribund and healthy samples of eggs. A similar pattern was found by Norwegian workers, despite its demonstrated pathogenicity in a model system (Hansen and Olafsen, 1989; Hansen *et al.* 1992).

In contrast to an earlier study, *Vibrio* species were often found in association with yolk-sac larvae as well as *Pseudoalteromonas* species (Bergh *et al.* 1994). The study by Bergh *et al.* (1994) did not look at larval samples from a range of hatcheries and incubators, and as was found here, there is a wide variation in the types of bacteria which are likely to be isolated from yolk-sac larvae in different hatcheries.

There is evidence that one of the *Vibrio* groups isolated from the Ardtoe yolk sac larvae may be members of an otherwise previously undescribed species. Despite being tentatively assigned to *V. splendidus* group 3, isolates TG4-07 and SYS6-10, are not readily placed phylogenetically with the limited 16S rRNA gene sequence data available. Their RDP scores and the results of DNA PARSIMONY analysis place them between *V. anguillarum* x16895 and *V. splendidus*. While the Neighbor Joining

algorithm (Figure 6), FITCH and KITCH analysis of a bootstrapped DNA DIST matrix (results not shown) show them to be closer to *V. anguillarum* x16895 and *V. cholerae.*

They are also phenotypically distinct in that they do not utilise sucrose when cultured on TCBS agar, unlike the majority of the other *V. splendidus* organisms identified. These two organisms were isolated from different batches of Ardtoe yolk-sac larvae sampled a year apart from each other, one as part of the survey and the other, TG4-07, from the rearing trial (Chapter 3).

In the case of the yolk-sac larvae, *Vibrio* and *Pseudoalteromonas* spp. appear to be present in the water and surface-sterilised larval samples. However, there is some evidence that they tend to be over-represented in the guts of the larvae compared to their relative proportions in the water surrounding them (Table 13). This suggests that, although the ultimate source of bacteria entering the gut of non-feeding larvae may be the water surrounding them, certain groups of bacteria are better able to both enter and proliferate than other groups, such as *Cytophaga* and *Flexibacter* species. These organisms, by contrast, appear to be proportionally under-represented in the yolk-sac larval gut compared to their relative frequencies in the water of the yolk-sac incubators.

A similar pattern is evident in first feeding larvae. *Vibrio* and *Pseudoalteromonas* species are present in both the water and the guts of the larvae but other organisms, apparently present at high densities in the tank water, do not appear to be able to establish themselves in the guts of first-feeding halibut larvae. The high numbers of lost organisms in the water samples is noteworthy. Most of these were very slow growing strains that could not be retained using traditional culture techniques on MA. By contrast, a low proportion of the organisms isolated from first feeding larval samples generally were lost on subculture.

In general, yolk-sac larvae appear to be potentially colonised by a wide variety of different bacterial species. This colonisation process may be partly stochastic, as illustrated by larvae from different yolk-sac incubators, which otherwise have the same developmental history, being colonised by different bacterial species. The

potential health implications of this findings will be explored in depth later (Chapter 3).

After the larvae start to feed on *Artemia* they appear to be colonised by *Vibrio* species which originate from their start feed. *V. splendidus* organisms were recovered in high quantities from all three hatcheries, particularly *V. splendidus* group 1 organisms. To date, studies from larval flatfish rearing systems have not characterised in detail the types of organisms associated with larvae and fry. This more in depth characterisation, which included 16S rDNA sequence analysis, illustrates that certain metabolically active *Vibrio* species are likely to be found within halibut larvae early on during their development, particularly *Vibrio splendidus* and *Vibrio alginolyticus* type organism associated with the live feed; later in their development a different group of more fastidious *Vibrio* species are likely to be present.

It appears that at least three genetically distinct clusters of *Vibrio splendidus*-type organisms were present in the three hatcheries surveyed (Figure 7). Cluster one corresponds to organisms identified in the survey as *Vibrio splendidus* group 1 on the basis of their PCR-RFLP patterns and BIOLOG GN profiles. Organisms with this distinctive PCR-RFLP pattern were isolated in quantity from all three hatcheries, both from the live food production systems and the guts of first-feeding larvae themselves. It therefore seems reasonable to assume that the organisms isolated from the larvae originated from the enriched *Artemia* they were being fed. The presence of such genetically similar isolates in geographically separated hatcheries is noteworthy.

Group 2 includes organisms that are apparently closer genetically to the *V. splendidus* type strain x74724. Examples of these organisms were isolated from Ardtoe yolk-sac larvae, enriched *Artemia*, first-feeding Ardtoe halibut larvae and Otter Ferry copepods. Also included in this cluster are *V. splendidus* isolates that have been shown to be pathogenic towards turbot and scallops (Gatesoupe *et al.* 1999).

Figure 9 and Table 15 indicates some of the phenotypic variation in the *Vibrio* isolates. Most of the *V. splendidus* isolates form a natural cluster, group one. Members of this group were isolated from Otter Ferry and Ardtoe enriched *Artemia* and halibut larvae, as well as Ardtoe and Mannin yolk-sac larvae.

Figure 9 *Vibrio* and *Photobacterium* isolates clustered by the least sum of squares method according to sole carbon source utilisation patterns (BIOLOG GN) and sensititivity to a range of antibiotics. Phenon I are *V. splendidus* gps 1 & 2 isolates. Phenon II are *V. splendidus* gp1 isolates from Mannin Seafarms. Phenon III are *V. alginolyticus* isolates. Phenons IV and V are *Photobacterium* spp. and *V. salmonicida*-like organisms. Isolates are coded as in Table 1.

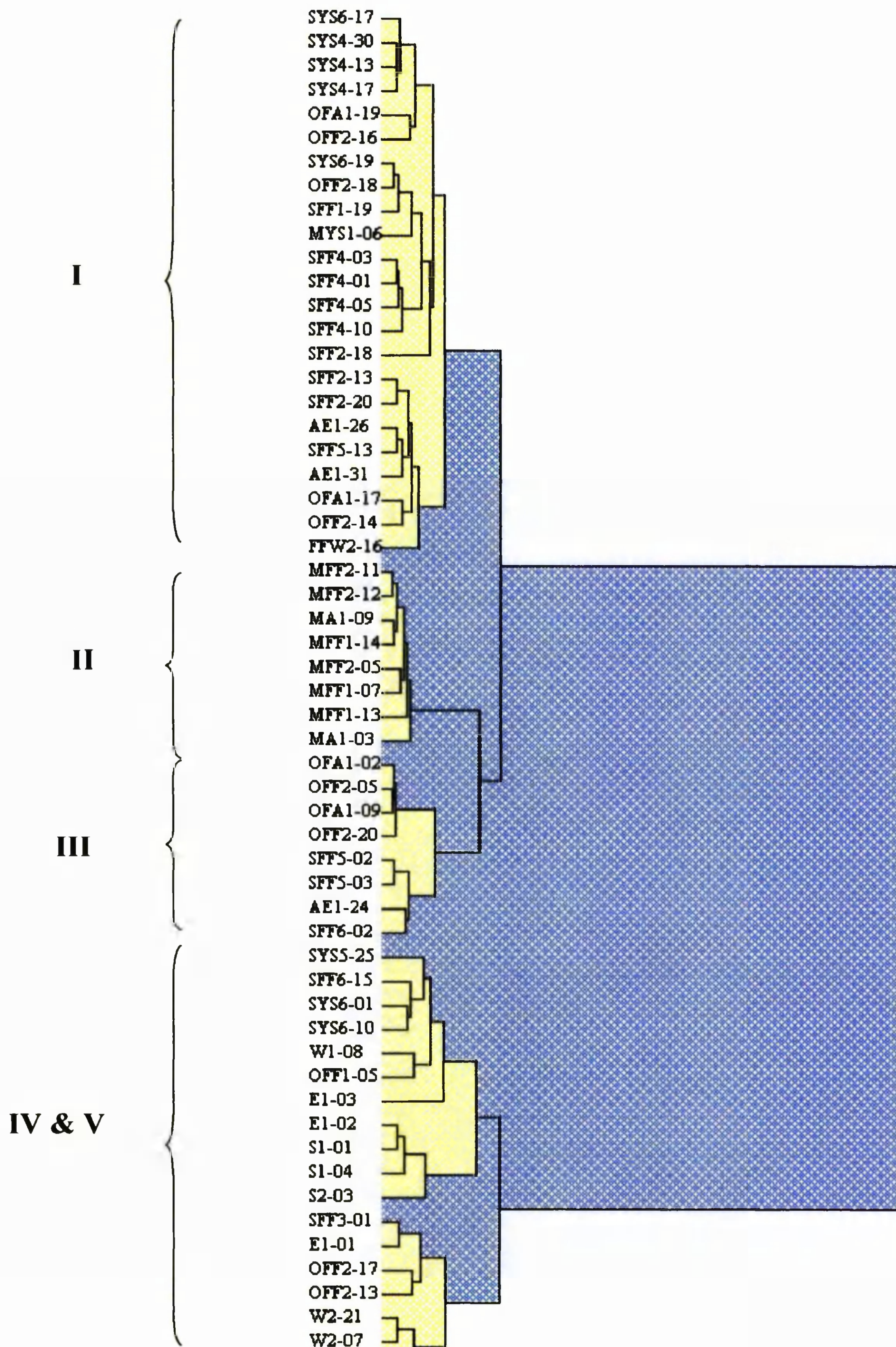


Table 15 Average metabolic activities (% positive of phenons) in Biolog Gn microplate assay and antibiotic sensitivities for the *Vibrio* phenons illustrated in Figure 9. Phenon I is *Vibrio splendidus* gps 1 &2 isolates. Phenon II is *V. splendidus* gp1 isolates from Mannin Seafarms. Phenon III are *Vibrio alginolyticus* isolates. Phenons IV and V are *Photobacterium* spp. and *V. salmonicida*-like organisms.

Test	Phenon				
	I	II	III	IV	V
	N=8	N=6	N=4	N=1	N=8
Single carbon source utilisation:					
Acetic acid	72.9	87.5	87.5	45	0
N- Acetyl-D-galactosamine	6.3	0	0	40	7.1
N- Acetyl-D-glucosamine	100	100	100	100	100
cis-Aconitic acid	97.9	100	93.8	15	0
Adonitol	2.1	6.3	0	10	0
D-Alaninamide	12.5	25	0	5	0
Alanine	93.8	100	100	35	0
L-alanine	100	100	100	90	7.1
L-Alanyl glycine	100	100	100	100	7.1
2- Amino ethanol	2.1	6.3	6.3	5	0
γ-amino butyric acid	4.2	12.5	18.8	0	0
D-L-arabinose	6.3	6.3	0	10	0
Arabitol	6.3	0	0	0	0
L-Asparagine	97.9	100	100	70	50
L-Aspartic acid	93.8	93.8	56.3	65	21.4
Bromo-succinic acid	72.9	62.5	87.5	30	0
2,3 Butanediol	2.1	0	0	10	0
D,L-Carnitine	2.1	6.3	12.5	0	0
Cellobiose	95.8	93.8	68.8	30	14.3
Citric acid	97.9	100	100	20	0
α-Cyclodextrin	4.2	0	100	20	0
Dextrin	100	100	100	90	71.4
I-Erythritol	2.1	0	0	0	0
Formic acid	39.6	31.3	87.5	0	0
D-Fructose	100	93.8	100	90	100
L-Fucose	0	18.8	12.5	15	0
D- Galactonic acid lactone	6.3	0	0	0	0
D-Galactose	100	100	75	55	28.6
D-Galacturonic acid	4.2	12.5	0	0	0
Gentiobiose	20.8	18.8	0	10	14.3
D-Gluconic acid	95.8	100	100	50	50
D-Glucosamic acid	8.3	18.8	0	0	0
α-D-Glucose	100	93.8	100	95	100
Glucose-1-phosphate	72.9	75	100	45	28.6
Glucose-6-phosphate	91.7	100	100	40	42.9
Glucuronamide	4.2	6.3	0	10	0
D-Glucuronic acid	2.1	12.5	0	0	0
L-Glutamic acid	100	100	100	75	71.4
Glycerol	95.8	100	100	60	42.9
D-L-α-Glycerol phosphate	81.3	100	87.5	55	0
Glycyl-L-aspartic acid	100	100	56.3	60	7.1
Glycyl-L-glutamic acid	97.9	100	93.8	70	21.4
Glycogen	97.9	100	100	45	21.4
L-histidine	70.8	75	100	10	0
α-Hydroxy butyric acid	2.1	12.5	6.3	5	0
β-Hydroxy butyric acid	0	0	0	20	0
γ-Hydroxy butyric acid	0	0	6.3	0	0
Hydroxy L-proline	6.3	0	100	10	7.1
p-Hydroxy phenylacetic acid	2.1	0	0	10	0
Inosine	95.8	100	100	95	100
meso-inositol	2.1	0	0	0	0
Itaconic acid	2.1	0	0	15	7.1

Table 15 (continued)

Test	Phenon				
	I	II	III	IV	V
	N=8	N=6	N=4	N=1	N=8
Single carbon source utilisation:					
α-Ketobutyric acid	0	0	0	0	0
α-Ketoglutaric acid	93.8	100	100	10	0
α-Ketovaleric acid	0	0	0	0	0
D,L-lactic acid	100	100	100	70	0
α-Lactose	12.5	31.3	0	0	7.1
α-D-Lactose-lactulose	4.2	6.3	0	0	0
L-Leucine	2.1	0	43.8	0	0
Malonic acid	4.2	25	0	5	0
Maltose	100	100	100	100	92.9
D-Mannitol	100	100	100	35	42.9
D-Mannose	95.8	93.8	25	90	42.9
D-Melibiose	18.8	50	6.3	0	0
Methyl pyruvate	85.4	93.8	100	60	0
β-methyl-d-glucoside	8.3	12.5	6.3	10	7.1
Mono-methyl succinate	37.5	18.8	75	15	0
L-Ornithine	29.2	75	25	10	0
L-Phenyl alanine	2.1	0	0	5	0
Phenyl ethylamine	2.1	0	0	0	0
L-proline	97.9	100	100	50	0
Propionic acid	56.3	81.3	100	20	7.1
Psicose	79.2	25	37.5	65	7.1
Putrescine	4.2	6.3	50	5	0
L-Pyro glutamic acid	4.2	18.8	0	15	0
Quinic acid	4.2	18.8	0	0	0
D-Raffinose	4.2	25	0	5	0
L-Rhamnose	4.2	12.5	0	0	0
D-Saccharic acid	2.1	6.3	0	5	0
Sebacic acid	0	0	0	0	0
D-Serine	8.3	6.3	0	10	0
L-Serine	100	100	100	100	14.3
D-Sorbitol	39.6	50	0	5	0
Succinamic acid	12.5	37.5	18.8	10	0
Sucrose	58.3	93.8	100	35	57.1
Succinic acid	97.9	87.5	100	75	21.4
L-Threonine	97.9	100	100	50	14.3
Thymidine	93.8	100	100	80	71.4
D-Trehalose	100	87.5	100	50	28.6
Turanose	45.8	12.5	18.8	10	0
Tween 40	95.8	87.5	100	80	14.3
Tween 80	85.4	62.5	81.3	50	0
Uridine	95.8	100	100	90	64.3
Urocanic acid	10.4	0	0	5	0
Xylitol	6.3	6.3	0	0	0
[a]Antibiotic sensitivity:					
Chloramphenicol 25 µg	87.5	93.8	93.8	100	85.7
Erythromycin 5 µg	4.2	0	6.3	0	0
Fusidic acid 10 µg	0	0	3.1	0	14.3
Methicillin 10 µg	0	0	0	0	0
Novobiocin 5 µg	91.7	100	93.8	50	57.1
Penicillin G 1 unit	2.1	0	0	0	0
Streptomycin 10 µg	14.6	56.3	34.4	5	0
Tetracycline 25 µg	79.2	6.3	62.5	92.5	78.6
Tetracycline 100 µg	87.5	0	71.9	92.5	85.7
Ampicillin 10 µg	64.6	0	18.8	20	3.6
Ampicillin 25 µg	75	0	18.8	27.5	35.7
Cephalothin 5 µg	21.9	18.8	12.5	50	75
Colistin sulphate 25 µg	100	100	100	100	75
Gentamycin 10 µg	66.7	87.5	68.8	75	64.3
Sulphatriad 200 µg	10.4	0	3.1	15	32.1
Cotrimoxazole 25 µg	55.2	0	28.8	55	46.4
Nitrofurantoin 50µg	100	100	100	92.5	85.7
Ticarcillin 75 µg	70.8	0	12.5	30	17.9
Nalidixic acid 30 µg	37.5	12.5	53.1	75	78.6
Trimethoprim 2.5 µg	10.4	0	3.1	7.5	14.3
Sulphamethoxazole 50µg	4.2	0	0	0	0

[a] Sensitive was scored as a positive character.

Note the very close similarity in terms of both phenotypic and 16 S rRNA gene homology between the isolates from two hatcheries in different parts of the West Coast of Scotland.

The representatives of the potential new species, genotype 3, form a cluster that is slightly different to the other organisms tested (Figure 9). This indicates that they are both genetically and phenotypically different. However, the differences are not large. Indeed, if the isolates are clustered by UPGMA they appear to be very close to the other Ardtoe *Vibrio splendidus* isolates.

The Mannin isolates form a separate cluster to the *V. splendidus* isolates found in the other two hatcheries. However, as is shown in Figure 5, they are genetically closely related and, indeed, when they are clustered solely on the basis of their BIOLOG GN profiles they appear to be very similar to the members of phenon 1 (data not shown). The main difference appears to be that they are more resistant to oxytetracycline, ampicillin and other antibiotics (see Table 15).

V. splendidus organisms are often found in association with turbot larvae and some have been implicated as potential pathogens (Gatesoupe *et al.* 1999). Also, many of these organisms were shown to be haemolytic towards sheep red blood cells. (Nicolas *et al.* 1989) found a high proportion of the bacteria isolated from turbot larvae fed on *Artemia* and rotifers were haemolytic and speculated that these may be harmful. However, as has been shown here, the species *V. splendidus* encompases a range of different organisms. It is likely that there is variation in the pathogenicity of these isolates towards halibut as there is towards turbot (Gatesoupe *et al.* 1999).

In general, separating the *V. splendidus* isolates on the basis of phenotypic variation does not appear to be a reliable method. Many of the discriminatory characters show within-phenon variation (see Table 15 and Appendixes 2d and 2e). Genetically different organisms are also clustered closely together, perhaps reflecting similar selective pressures operating at the hatcheries, particularly with regard to the acquisition or maintenance of antibiotic resistance.

The isolates designated as *V. alginolyticus* in this study were not readily PCR amplified using the simple genomic DNA extraction protocols used. Recent work in our laboratory indicates that reducing the quantity of DNA template used may increase the efficiency with which related organisms, originally isolated from turbot, are PCR amplified. It is possible that there may be substances produced by these *V. alginolyticus* strains *in vitro* which inhibit the PCR amplification process.

V. alginolyticus has been implicated as a potential pathogen of both humans and aquaculture species in a number of studies (Anguiano-Beltran *et al.* 1998; Balebona *et al.* 1998; Kusuda and Kawai, 1998; Barbieri *et al.* 1999; Chen *et al.* 1999; Kim *et al.* 1999; Lane and Birkbeck, 1999; Zanetti *et al.* 1999) . The species *V. alginolyticus* encompases a wide variety of organisms, only some of which are likely to be pathogenic. This is illustrated by the findings of Sechi *et al.* (2000) who found that *V. cholerae* virulence genes were carried by some, but not all, *V. alginolyticus* strains that they analysed. Munro *et al.* (1995) found that the survival of turbot reared in the presence a *V. alginolyticus* species was no different to control larvae reared in the absence of culturable bacteria. There is no indication that the isolates characterised as *V. alginolyticus* here were pathogenic to halibut larvae either. In some cases, they may even be beneficial parts of the gut and tankwater microfloras. Recent work has shown that *V. alginolyticus*-related strains were commonly associated with healthy haddock larvae reared in Canadian hatcheries (S. Griffiths personal communication). An organism identified as *V. alginolyticus* has also been implicated as a potential probiotic treatment (Austin *et al.* 1995).

Although the gut flora of start feed halibut larvae fed on *Artemia* appears to originate from organisms associated with their diet, the pattern is not straightforward. Only a fraction of metabolically active *Vibrio* species appear to be able to settle within the guts of the larvae. Other organisms, such as *P. veronii* and *Pseudoalteromonas* spp., which are present in large quantities in the *Artemia* rearing systems, were not generally found in the guts of first-feeding larvae. This indicates that there is selection taking place at some point. This could either be at the level of the *Artemia* after they are introduced into the feeding tanks or inside the guts of the larvae.

The *V. splendidus* and *V. alginolyticus* isolates may possess adhesins, such as chitin binding proteins (CBP), which would allow them to adhere to the *Artemia* at the expense of other components of the *Artemia* rearing tank microbiota. Various *Vibrio* species, including a *V. alginolyticus* species, have been shown to posses CBP (Yu *et al.* 1991; Montgomery and Kirchman, 1993; Montgomery and Kirchman, 1994; Pruzzo *et al.* 1996; Tarsi and Pruzzo, 1999).

Selection may also be taking place at the level of the gut itself. It could be that the conditions within the gut limit the growth of some of the *Artemia*-associated organisms. Alternatively, *V. splendidus* and *V. alginolyticus* may outcompete other organisms in the larval gut, either by overgrowth or direct inhibition.

Luminous *Photobacterium phosphoreum* isolates were recovered from the guts of first-feeding Ardtoe halibut larvae, freshly fertilised halibut eggs and the guts of adult halibut (Tables 4, 7 and 12 and Appendix 2e). Representative isolates all had identical PCR-RFLP patterns when their 16S rDNA genes were digested with 3 different enzymes (*Hae*III, *Rsa*I, *Sau*3AI). A representative of each is shown in Figure 2 after digestion with *Hae*III. There is no apparent difference between the PCR-RFLP patterns of two *Photo. phosphoreum* type strains, NCIMB 193 and NCIMB 1282, and the isolates recovered in this study.

Phenotypically, all isolates were also identified as *Photo. phosphoreum.* This identification was confirmed when one of the isolates, S1-04, had part of its 16S rRNA gene sequenced.

Other isolates, recovered from Ardtoe halibut fry and Mannin yolk-sac larvae, which had a similar *Hae*III PCR-RFLP pattern to the *P. phosphoreum* isolates, were subsequently shown to be different organisms. As well as being non-luminous, 16S rRNA gene sequence analysis showed them to be members of at least two further species. The 16S rRNA gene sequence of an isolate recovered from the yolk-sac larvae had a high sequence holomology to *P. profundum.* A number of the organisms isolated from Ardtoe larvae that had been transferred to an inert diet were homologous to *V. Fischeri* (Figure 7).

Organisms that formed cream colonies on TCBS (sucrose negative), were sensitive to the vibriostat O/129, and had a distinctive yellow colony morphotype on marine agar were recovered from halibut larvae and fry from all three hatcheries. All isolates tested had identical PCR-RFLP patterns when digested with three different enzymes (*Hae*111, *Rsa*I and *Sau*3AI). 16S rDNA gene sequence analysis of a representative from each of the hatcheries showed them to be genotypically similar to *V. salmonicida*.

The presence of *V. salmonicida*-like organisms in all three hatcheries was interesting. This organism was generally found in older samples of first-feeding or weaned larvae and was not isolated from any of the live food samples. This organism is slower growing and less metabolically active *in vitro* than the *V. splendidus* and *V. alginolyticus* species commonly found in less developed larvae. It could be that this is an example of a successional process. Initially the larvae are colonised by opportunistic live food-associated 'r-selected' organisms before giving way to organisms better adapted to survival and growth in the guts of developing larvae and fry ('K-selected'). It may also be that the *V. salmonicida*-like, and *Photobacterium* spp. found in older batches of halibut larvae fry and adults are examples of the so called 'gut *Vibrio*' group. It is interesting in this context that the guts of the adult halibut analysed contained high quantities of TCBS-culturable luminous *Photo. phosphoreum* isolates. Liston noted that a high proportion of the 'gut *Vibrio*' organisms he isolated from the guts of wild adult marine fish were luminous. The *Photo. phosphoreum* NCIMB strain 193 illustrated in Table 2 was originally isolated by Liston from the gut of a wild-caught lemon sole. Onarheim *et al.* (1994) identified one group, previously characterised as gut *Vibrio* organisms with antigenic similarity to *V. salmonicida*, as *V. salmonicida*-like after 16 S rRNA gene sequence analysis. Their deposited sequence has a high degree of homology to the *V. salmonicida*-like organisms encountered in this study.

There has been some speculation that the source of the *Vibrio* organisms infesting *Artemia* production systems could be the *Artemia* cysts themselves (Austin and Allen, 1981). None of the results of this survey bear this out. No TCBS-culturable organisms have so far been isolated from any cyst sample so far analysed (n = 8 and

other work not reported here). Also, the types of organisms seen in the *Artemia* nauplii production systems do not resemble the typical *V. splendidus* gp 1 organisms commonly isolated from enriched *Artemia*.

It could be that these *V. splendidus* and *V. alginolyticus* strains ultimately originate from the seawater used in the hatcheries. As is shown by the presence of *V. splendidus* organisms in association with the copepods reared in outdoor tanks at Otter Ferry, these species are common members of estuarine and inshore waters. Despite UV sterilisation of incoming water and other microbial control measures to reduce the risk of cross-contamination, it theoretically requires only one such organism to gain entry to a live food production system. Once it has established itself within a tank, it will be able to exploit the favourable niche of high nutrient loadings and limited competitive pressure from other microorganisms and easily transfer between the *Artemia* rearing tanks by the high aerosol levels found in live feed production rooms.

The examples of colonisation of Ardtoe first feeding halibut by a *Pseudoalteromnas* species and a luminous *Photo. phosphoreum* organism are interesting for a number of reasons (Tables 7 and 8). Firstly, in each case, these organisms were not recovered from the respective batches of larvae when they were later analysed, as such, they are examples of transient colonisation of first feeding larvae by an organism.

It is not clear where the luminous *P. phosphoreum* organism found in the first feeding Ardtoe larvae (Table 5) originated from, as it was not present in any of the live feed samples analysed or other tanks of first feeding larvae, that had been fed from the same source of enriched *Artemia*. Although a similar organism was found in association with freshly fertilised halibut eggs (Table 4) it was not found when freshly hatched yolk-sac larvae and egg incubator water samples from the affected batch were analysed earlier on during their development.

Also, it is also unclear whether this organism is pathogenic to first-feeding halibut larvae, as will be discussed later in chapter 4.

Previous work (Verner - Jeffreys, 1996) has shown that rinsed enriched *Artemia* collected from the Ardtoe rearing system is dominated by high loadings of *V. splendidus* type organisms (although these were not characterised in detail). This makes the observation that the bacterial flora of the sample of Ardtoe enriched *Artemia* water was mainly dominated by presumptive *Pseudoalteromonas* spp. PCR-RFLP group noteworthy (Figure 5). The TCBS-culturable *V. splendidus* organisms are present, but at a lower density to the non *Vibrio* spp. in the water samples. It may be that these *Vibrio* species selectively associate in some way with the *Artemia* themselves.

It was found in this study that a combination of phenotypic and genotypic tests were best employed to classify the isolates. Recent reports indicate that colony morphology allied to PCR-RFLP may be the best way to classify large numbers of environmental isolates quickly (Lebaron *et al.* 1998). To some extent this was found in this study. The potential discriminatory power of this technique was demonstrated by the clear separation of phenotypically close organisms, such as *V. splendidus* group 1 & 2 (Table 2). This technique was particularly good at separating Gram-negative, motile, non fermentative isolates, which are notoriously difficult to separate using conventional biochemical tests. This study only routinely used one digestive enzyme, *Hae* III. Other studies have suggested using a range of different digestive enzymes, particularly for separating closely related *Vibrio* species, which are not readily separated using this enzyme (Urakawa *et al.* 1997). Some organisms with similar PCR-RFLP digestion patterns were subsequently shown to be different species, for example the *Photo. phosphoreum*, *Photo. profundum* and *V. fischeri*-like organisms, as was shown by subsequent partial 16S rRNA gene sequencing of these isolates.

Some organisms, notably the *V. alginolyticus*-like organisms, did not PCR amplify readily. The fastest way of characterising these was to use BIOLOG GN allied to colony morphology, particularly as this group swarmed on complex media making them readily distinguishable. Antibiotic resistance profile was another useful criterion. Genotypically similar organisms, particularly *V. splendidus* gp 1, were shown to have differing antibiotic resistance profiles in the different hatcheries.

Unfortunately, it was hard to classify *Cytophaga/Flexibacter* like isolates using BIOLOG GN. All the yellow pigmented, Gram-negative rods showing gliding motility tested failed to produce enough positive tests to allow a reliable identification to be made, bar one *Flexibacter ovolyticus*-like isolate.

However, as will be discussed in more detail in Chapter 3, BIOLOG GN appeared to be a quick and reliable method of separating *Pseudoalteromonas* and other *Pseudomonas* type organisms, as was found in another study (Grisez *et al.* 1996). It was also good at identifying metabolically active *Vibrio* species, such as *V. alginolyticus* and *V. splendidus* although it was not particularly discriminatory at separating the metabolically less active *V. salmonicida* and *P. phosphoreum* groups. BIOLOG GN provides potentially useful functional information about the biochemical properties of some of the different groups of organisms, which are not provided by molecular identification techniques such as PCR-RFLP and 16S rDNA gene sequencing.

It may be significant that late successional species, such as *V. salmonicida*-like organisms and *Photobacterium* spp. were generally strongly catalase positive. The production of catalase may aid in the survival of these organisms in the larval and adult gut.

It is appreciated that culture-based identification strategies, such as this study, may be deficient because many of the marine bacteria are probably not culturable using conventional techniques. Accordingly, it is unwise to draw too many conclusions about apparent correlations between the presence and absence of particular bacterial phenotypes and larval survival and performance. It is possible that other bacteria were present in the different samples analysed but were either not recovered, or overgrown by other organisms, on the MA and TCBS plates used.

Other techniques, in particular Denaturing Gradient Gel Electrophoresis (DGGE) may be more applicable for simple identification purposes and bacterial community profiling (Muyzer *et al.* 1993; Muyzer and Smalla, 1998). It should be appreciated that there are inherent biases associated with these techniques as well. Also, an important secondary aim of this study was the collection of pure strains that

Future studies may consider combining both DGGE and conventional culture-based techniques to give a more complete picture of the bacterial population.

Chapter 3. Effect of different rearing conditions on the health and development of an aerobic microflora in Atlantic halibut larvae

3.1. Introduction

As well as characterising the types of bacteria associated with Atlantic halibut larvae of different developmental stages, another project aim was to see how varying the conditions under which halibut larvae were reared microbially affected survival and the development of the gut flora.

As discussed in Chapter 1, it is presently unclear to what extent the variable survivals reported by hatcheries are caused by adverse interactions between the developing larvae and the types of bacteria to which they are normally exposed.

Many UK hatcheries have reported high mortalities towards the end of the yolk-sac development stage, so initially it was decided to concentrate on this key developmental period. Currently there is debate as to whether this is due to faults in the design of the rearing systems, fading out of generally poor quality larvae, changes in the behaviour of the larvae making them more susceptible to mechanical damage, or the presence of larvae with low bioenergetic reserves (Finn *et al.* 1995) and minimal immunological competence being adversely affected by bacteria during the later stages of yolk-sac incubation. For the final factor, the influence of bacteria on survival, this may involve direct invasion of the larvae by opportunistic pathogens or water quality problems associated with toxic microbial metabolites (Ringø and Birkbeck, 1999).

As discussed in the main introduction, direct evidence that bacteria cause the reported poor survivals is lacking. However, it is reported that it is necessary to add antibiotics, such as oxytetracycline, to ensure survival of yolk-sac larvae reared in small-scale static systems (Lein *et al.* 1997; Ottesen and Bolla, 1998).

Also, direct addition of the pathogens *V. anguillarum*, *F. ovolyticus* and *A. salmonicida* subs. *salmonicida* to yolk-sac larvae reared in 6 well tissue culture dishes

caused a significant decrease in survival (Bergh *et al.* 1992; 1997). Although these observations from different laboratory model systems are interesting, it is questionable how relevant such results are to observed variations in survival in full-scale commercial incubators. Flow dynamics, other water quality parameters and, as a consequence, the microfloras to which the larvae are exposed, are likely to be very different. Two studies have investigated the effect of varying the flow rates into yolk-sac incubators and their effect on yolk-sac larval survival, and numbers of culturable bacteria (Opstad and Bergh, 1993; Opstad *et al.* 1998). Interestingly, although increasing the flow rate decreased survival, no survivals were reported after day 12 when the flows were turned off completely. This correlated with an increase in water bacterial numbers compared to those found when flows were maintained.

Therefore, a trial was initiated whereby the rearing conditions of the yolk-sac larvae were varied under full-scale commercial conditions. As well as comparing general performance under recirculated water rearing conditions, as opposed to using conventional flow-through incubators, an attempt was made to reduce bacterial levels in the incubators by application of antibiotics. A comparison was also made between the performance of larvae hatched from surface-disinfected as opposed to non-surface-disinfected eggs, under recirculated water rearing conditions.

3.2. Materials and Methods

3.2.1. Maintenance of yolk-sac larvae

Batches of halibut eggs were reared in upwelling 100L Paxton-style incubators until approximately 100 day-degrees post-fertilisation (eggs from one female fertilised with the milt from two males). At this point approximately 10,000 eggs were transferred to each of four 450L upwelling yolk-sac incubators where they were left to hatch *in situ*. As they were moved, three of the batches of eggs were surface disinfected, the fourth incubator being stocked with non-surface-disinfected eggs, as detailed below in Table 1. Each of the incubators represented a different treatment; treatment replication was achieved by repeating the experiment with four separate batches of eggs, to take account of possible inter-batch variation. The temperature of the incubators was maintained between 5.7 — 6.3 ^{O}C and salinity between 33.8 and 34.5 $^{0}/_{00}$. Flow rates into the incubators were 1.5 L min^{-1}, apart from shortly after the

eggs hatched, at which point the flows were raised to 2 L min^{-1} to remove the hatched egg shells.

3.2.2. Treatments

The treatments are summarised in Table 16. Surface disinfection was achieved by immersion of the eggs in a 1:250 solution of the peracetic acid and hydrogen peroxide based surface disinfectant 'Kick Start' (R. S. Hygeine Ltd) for one minute prior to transferring them into the yolk-sac incubators.

A schematic diagram of the recirculation system is shown in Figure 10; flow-to-waste tanks were fed with a continuous flow of filtered, UV-sterilised, chilled seawater and salinity was maintained by a salt-dosing system. For the treatment involving the addition of antibiotics, a stock solution of oxolinic acid, amoxycillin and oxytetracycline (1.5 g L^{-1} of each compound) was added to the incubator water inflow at a rate of 18 ml min^{-1} for fifteen h. Total flow rates into the incubators were 1.4 L min^{-1}, this resulted in the concentration of each agent in the incoming water being maintained at 20ppm for this period. Theoretically, fifteen h represents three complete incubator water exchanges (420 L).

Table 16 the four different treatment regimes under which the yolk-sac larevae were reared.

Treatment	Description of treatment
RN	Recirculation; non-surface disinfected eggs
RS	Recirculation; surface-disinfected eggs
FA	Flow-to-waste; surface disinfected eggs; addition of antibiotics at 150 and 200 day-degrees
F	F/W; surface-disinfected eggs

3.2.3. Transfer to first feeding

After 220 day-degrees, 400 larvae from each incubator were transferred to two separate 100 L trial tanks. Temperatures in the trial tanks were maintained at between $8^{O}C$ and $10^{O}C$ and algae (*Pavlova lutheri*) were added on a daily basis. Freshly hatched *Artemia* nauplii (INVE EG grade) were presented to the larvae within 24 h of transfer. The balance from each incubator was pooled into a production tank (apart from the antibiotic-treated larvae that were discarded). An approximate overall count of larvae taken out of each yolk-sac incubator was made; from this the percentage survival to 220 day-degrees of the 10,000 eggs originally stocked into each incubator was calculated. Start feed performance was assessed after a week for the trial tanks (by counting the number of larvae with *Artemia* in the gut) and within twelve days for the production tanks.

3.2.4. Microbial analysis

To monitor the general effectiveness of the antibiotic treatment at removing bacteria from the incubator, levels of Marine Agar (MA) culturable bacteria were monitored for both the flow-to-waste treatments (addition of antibiotics and no addition of antibiotics). At the end of yolk sac incubation (220 day-degrees post-

hatch) larvae from all the treatments were also sampled for the presence of gut-associated bacteria as described in 2.2.2.

Twenty colonies from each treatment sample were chosen at random and subcultured onto Marine Agar until pure. Representatives of the different colony morphologies isolated from each treatment were then subjected the BIOLOG GN characterisation scheme as described in 2.2.10.

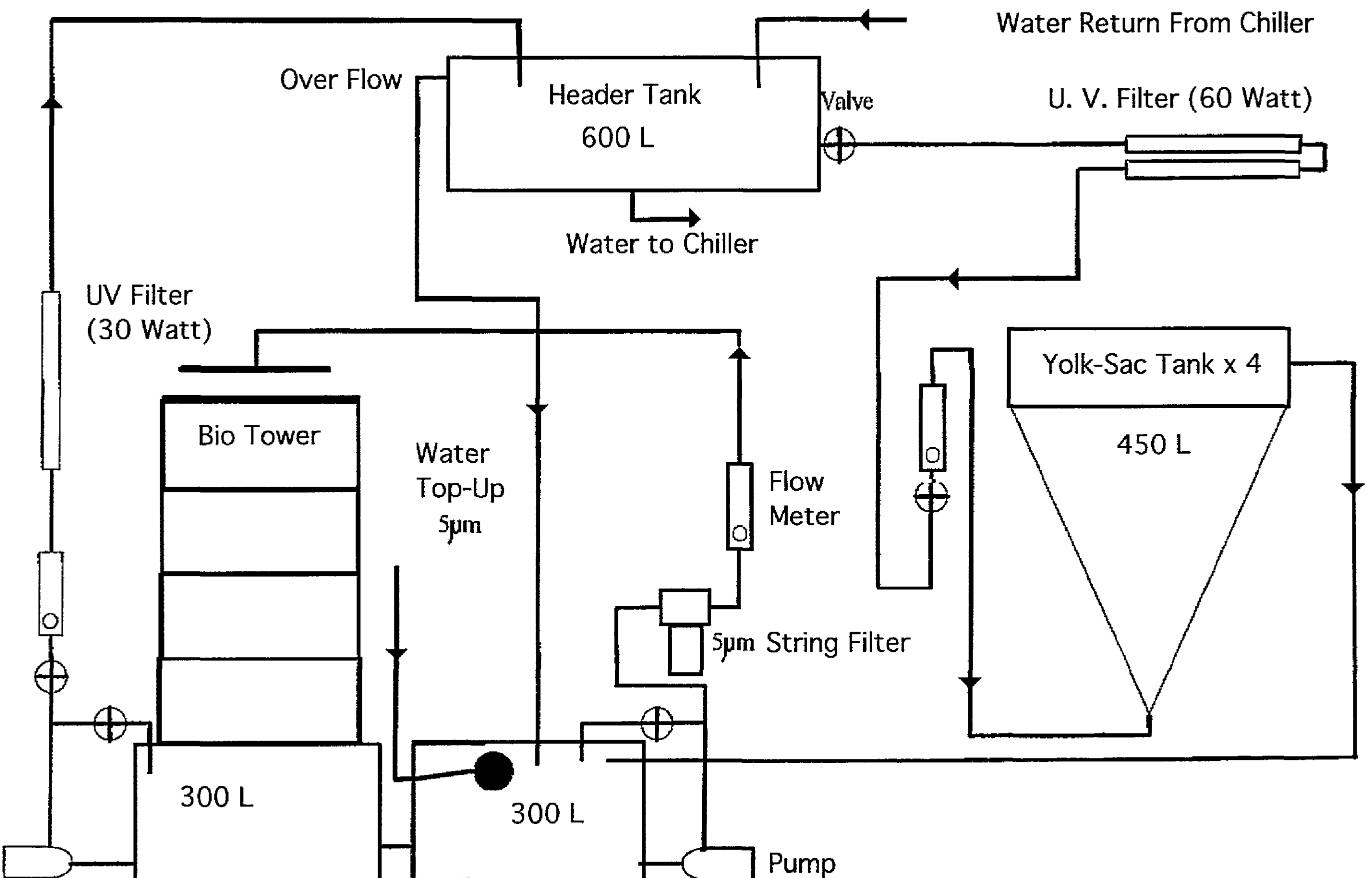
In addition, further biochemical and morphological tests were performed on the isolates to validate the BIOLOG results. Tests done included Gram stain, motility, colony morphology, pigment production, cytochrome oxidase activity, haemolysis of sheep red blood cells, oxidation/fermentation, indole production, Voges-Proskauer, ONPG; argine, lysine and ornithine decarboxylase active and production of acid from arbutin, salicin and sucrose.

Their ability to grow on TCBS and sensitivity to the vibriostat agent 0/129 was assessed to see whether they were presumptive *Vibrio* species. To give an indication of how phenotypic variation of isolates correlated to genotypic variation, the 16S rRNA genes of a number of the isolates from each of the BIOLOG phenons were PCR amplified and compared by both part sequencing and RFLP (see section 2.2.12. Each of the BIOLOG phenons is thus described on the basis of a number of criteria in addition to their panel substrate utilisation.

3.2.5. Statistics

Bacterial counts and survival data are presented using the arithmetic mean and standard error of the mean for the different treatments. Bacterial counts for the different treatments were initially compared using one way analysis of variance following logarithmic transformation of the data (Sokal and Rohlf, 1995). Individual treatments were then compared against each other using Tukey tests. Survival in the different treatment groups were analysed by both parametric (One way analysis of variance) and non parametric methods (Kruskal-Wallis, followed by Mann-Whitley for individual treatment comparisons). Analysis was done using the programme Minitab v. 13. The relative diversity of the isolates recovered from the different treatments was calculated using Shannon's Index.

Figure 10. Schematic diagram of the recirculation system used during the yolk-sac larval rearing trial.



3.3. Results

3.3.1. Survival to the end of yolk-sac incubation

Table 17 shows the survivals to the end of yolk-sac incubation for the different treatments. Average survival under the flow-to-waste rearing regime was markedly lower when antibiotics were not added (28.5% as opposed to 53.0 %). Little discernible difference in survival was apparent for the two recirculation treatments, namely surface-disinfected or non-surface disinfected eggs. The final valid pairwise comparison, rearing of yolk-sac larvae under recirculated water conditions following surface disinfection of the eggs, as opposed to rearing under a flow-to-waste regime, is interesting as the larvae reared under recirculation conditions appeared to perform better (48% survival versus 28.5%).

As can be seen in Figure 11 and Table 18, there is substantial variation in survival in larvae reared from different batches; irrespective of treatment regime. This variation was found to be significant when the survivals for the different treatments were pooled and compared by one way analysis of variance (batch number versus survival; $p<0.01$).

3.3.2. Effect of addition of antibiotics on total viable counts in yolk-sac incubators.

Figure 12 shows that, for the two runs monitored, application of antibiotics to a flow-to-waste incubator resulted in only a marginal suppression of bacterial numbers which were culturable on Marine Agar. In general, levels of bacteria appear to have peaked less than a week after the eggs were transferred into the incubators (first sampling point); this is despite their having been surface disinfected and placed into UV-treated water.

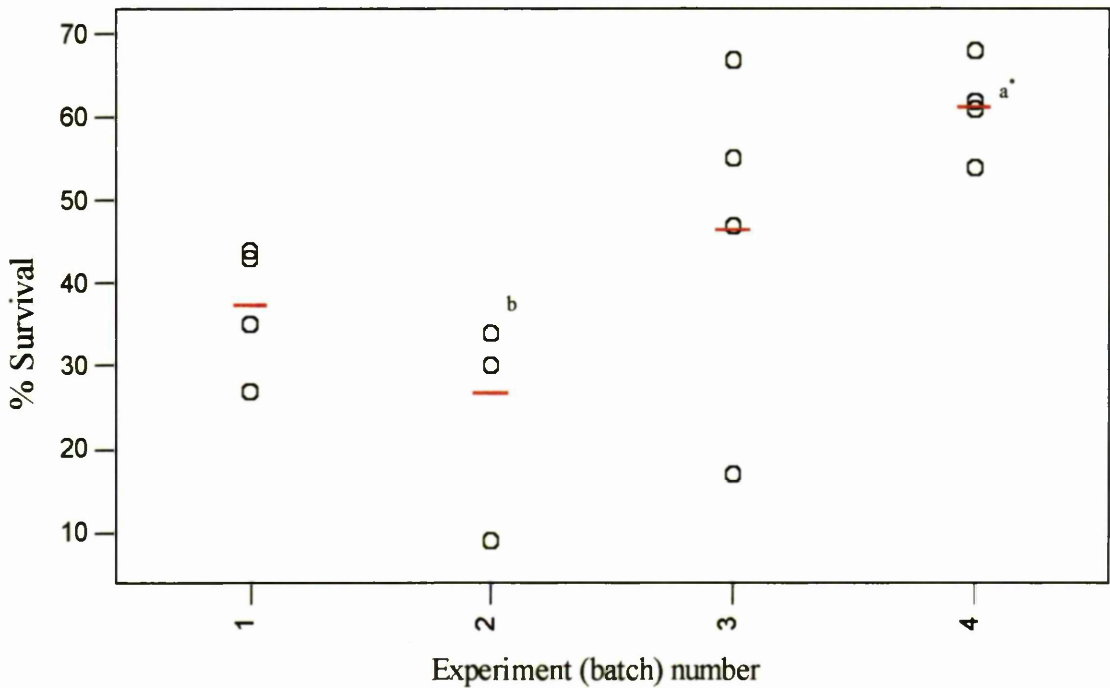
Table 17 Mean percentage survival of yolk-sac larvae to 220 day-degrees and average gut bacterial loading under differing yolk-sac incubator rearing conditions. For each of the experiments, a batch of eggs was split into four separate 450 L yolk sac incubators; each incubator represented a different rearing regime. Means are from four separate runs of the experiment. Each incubator was stocked with 10,000 eggs.

Rearing conditions	Average gut bacterial loading (CFU/ larva ± SEM)	Average survival (%) to 220 day-degrees (± SEM)
RN Recirculated water; non-disinfected eggs	5.78×10^3 ($\pm 5.46 \times 10^3$)	42.5 (8.7)
RS Recirculation; surface-disinfected eggs	7.78×10^3 ($\pm 3.02 \times 10^3$)	48 (8.1)
FA Flow-to-waste; surface disinfected eggs; addition of antibiotics at 150 and 200 day-degrees	[a]< 10	[b]53.0 (9.9)
F Flow-to-waste; surface disinfected eggs (present hatchery practice).	9.85×10^2 ($\pm 7.51 \times 10^2$)	28.5 (11.5)

[a] Bacterial counts significantly different to FA (Tukey; $p < 0.05$)

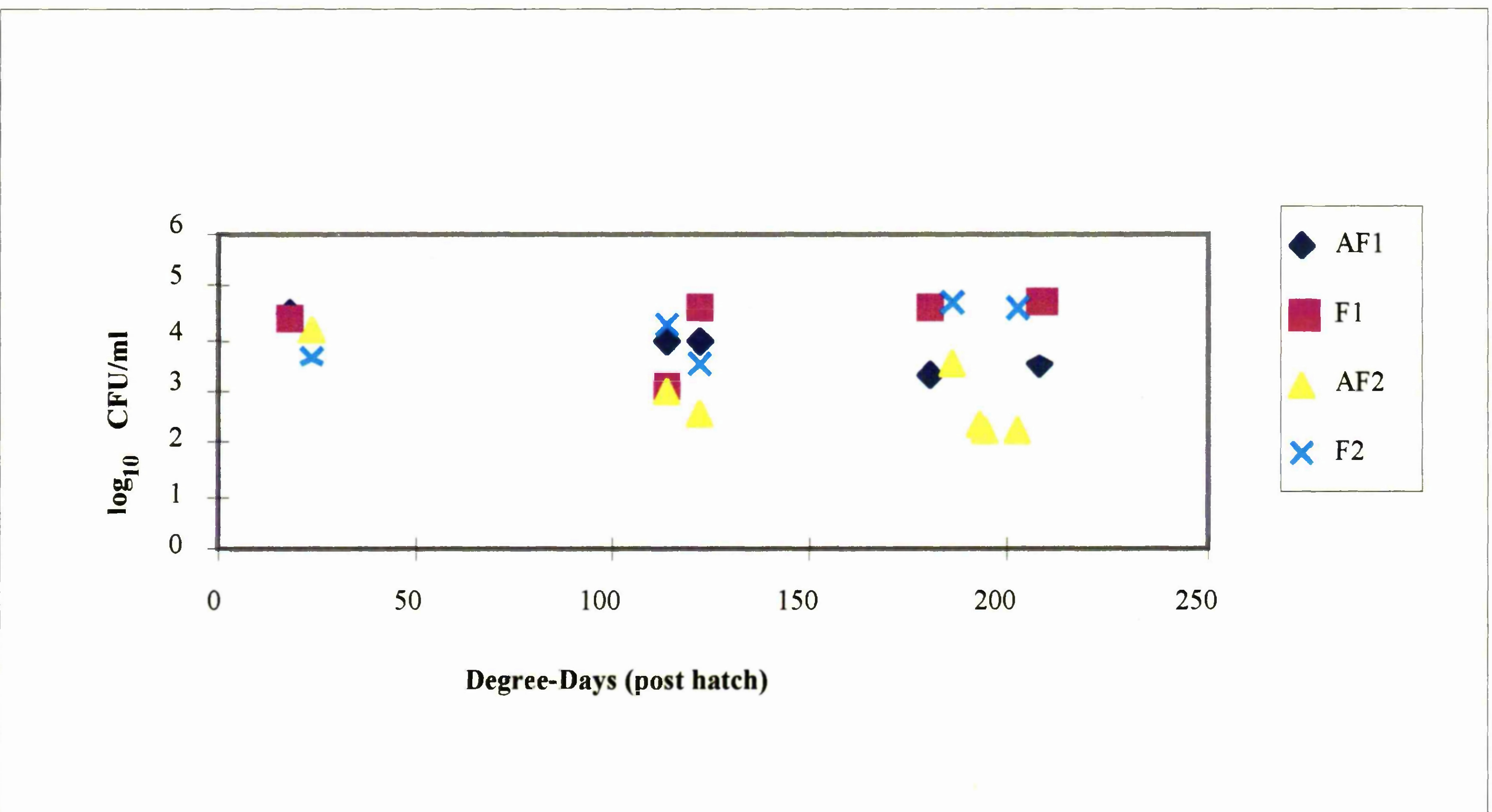
[b] Survival in FA significantly different to other treatments (Kruskal-Wallis; $p < 0.05$)

Figure 11 Dot plots showing the percentage survivals of yolk-sac larvae for the first four runs of the rearing experiment. For each of the experiments, a batch of eggs was split into four separate 450 L yolk sac incubators, each of which represented a different rearing regime (Table 16). The survival in each individual incubator after 220 day-degrees is indicated. Group means are indicated by lines.
Each incubator was stocked with 10,000 halibut eggs.



*Different superscripts indicate means are significantly different to each other (Tukey; $p<0.05$).

Figure 12 Concentration of marine agar culturable bacteria in the flow-to-waste treatments (first two runs only) for the yolk-sac rearing experiment. AF1 and AF2 had antibiotics added at 115 and 180 day-degrees, F1 and F2 were the control flow-to-waste treatments where no antibiotics were added.



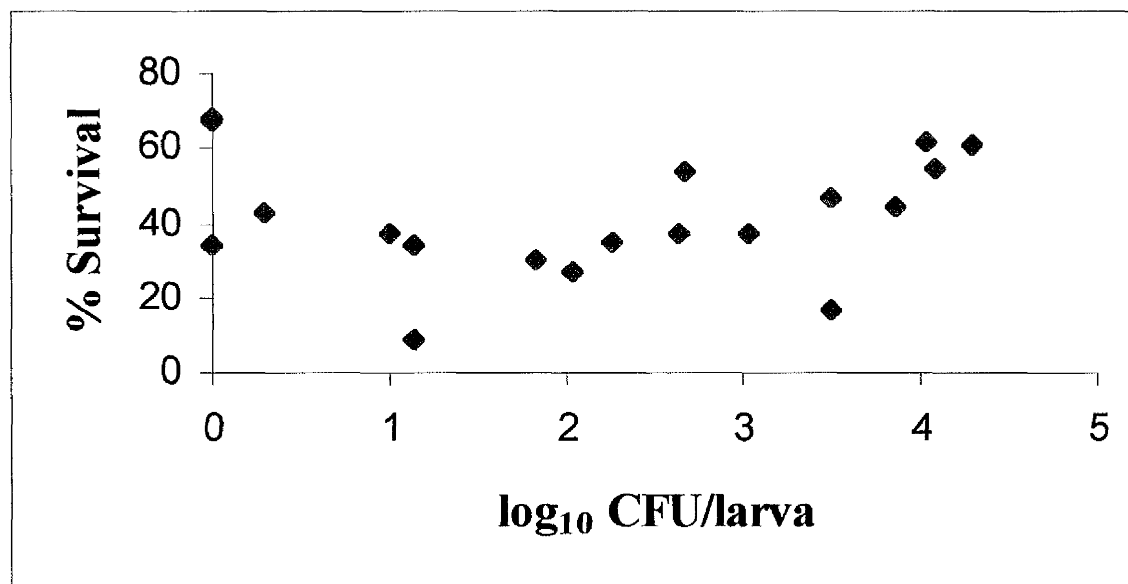
3.3.3. Effect of different rearing conditions on total levels of Marine Agar culturable gut-associated bacteria

Table 17 shows that average levels of gut-associated bacteria vary with different treatments. Concentrations of gut-associated bacteria appear to be generally higher under recirculation conditions than under a flow-to-waste regime, whether or not the eggs have been surface disinfected. Addition of antibiotics appears to be an effective method of preventing the establishment of a microflora in the guts of first-feeding halibut larvae (<10 CFU/larva as opposed to $>10^2$ CFU/ larva. Tukey; $p<0.01$.)

3.3.4. Correlation between total levels of gut-associated bacteria and survival.

There was seemingly no direct correlation between levels of Marine Agar culturable bacteria and survival, as illustrated by Figure 13 below, which shows the combined data for all the different treatments ($R^2 = 0.0871$).

Figure 13. Scatter graph showing percentage survival against average gut bacterial loading for all samples.



3.3.5. Qualitative differences in the types of gut-bacteria isolated under the different rearing regimes.

The effect of different rearing-regime and the types of bacteria that establish themselves within the gut of yolk-sac halibut larvae are summarised in Table 18. Each of the different bacterial phenons that the isolates have been assigned in Table 18 are illustrated in Figure 14 and described in more detail in Table 19 and Table 20.

The phylogenetic affiliations of a number of the bacteria isolates, based on analysis of their partial 16S rRNA gene sequences, are illustrated in Figure 15.

Larvae reared under the recirculated water, disinfected eggs regime (RD) were colonised by a restricted flora dominated by members of Phenon II (*Pseudoalteromonas* species). These were often present at high densities ($> 10^2$ CFU/larva). By contrast, larvae reared under the standard hatchery rearing regime (flow to waste, disnfected eggs) were colonised by a variety of different organisms, this is reflected in the high relative diversity score (0.712). Larvae reared under recirculated water, non-disinfected eggs regime were, similar to those reared under RD conditions, generally colonised by members of Phenon II, although other phenon representatives were also recovered.

Table 18. Summary table showing percentage survival, average number of Marine Agar culturable bacteria per surface-sterilised larva and identity of sample isolates, based on their clustering into BIOLOG GN phenons, of a number of the bacterial isolates from each treatment. The individual phenons are described in Tables 19 and 20 and illustrated in Figure 14. Treatments are as described in Table 16.

[a]Treatment and [b]batch origin.	% survival	CFU/ larva	[b]No ID	I	II	III	IV	V	VI	VII	Total number of isolates analysed	[d]Relative diversity score (J') for each treatment
F1	35	1.78×10^2					4	2		1	7	
F2	9	1.4×10^1							3		3	
F3	17	3.2×10^3			3	1		1			5	0.712
F4	54	4.62×10^2	1		3						4	
F5	37	1.07×10^3	2	1				2	1		6	
FA 1	43	<10										
FA 2	34	<10										
FA 3	67	<10										
FA 4	68	<10										
FA 5	37	10	2			1					3	
RD1	44	7.19×10^3			4						4	
RD2	30	6.6×10^2			3						3	
RD3	55	1.23×10^4			5						5	0.095
RD4	62	1.1×10^4			7				1		8	
RD5	37	4.42×10^2		3	3				2		8	
RN 1	27	1.08×10^2	3	6	2	5					16	
RN 2	34	1.4×10^1	1		4						5	
RN 3	47	3.2×10^3			4						4	0.674
RN 4	61	1.98×10^4			7						7	

[a] F= flow-to-waste, disinfected eggs; FA= Flow-to-waste; addition of antibiotics; RD= Recirculation, disinfected eggs; RN = Recirculation, non-disinfected eggs.
[b] The experiments using batches 1 and 2 were set up in May 1998, batches 3 and 4 in June 1998 and batch 5 was run in May 1999.
[c] No ID = not assigned to any phenon.
[d] Relative diversity scores calculated for F, RD and RN treatments from batches 1-4. Not calculated for FA (too few isolates recovered).

Figure 14 Simplified phenogram showing the BIOLOG GN clusters of the 75 analysed isolates recovered from the guts of yolk-sac larvae sampled during the trial. The phenogram was constructed using the MICROLOG 3.5 software. The distance scale at the bottom is equivalent to 1 test difference per graduation unit. Each of the different phenons is described in more detail in Tables 19 and 20.

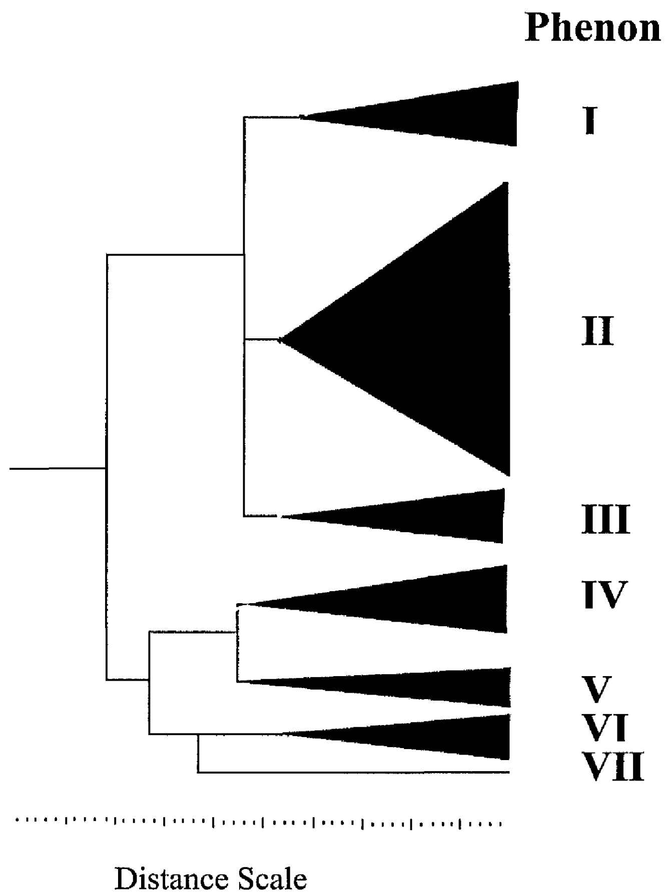


Table 19 Description of the different BIOLOG Microlog-assigned phenons illustrated in Figure 14.

Phenon	Number of isolates	Description
Not assigned	9	Lost on subculture or too few positive results to give reliable BIOLOG identification
I	10	*Vibrio* spp. Most are apparently members of a distinct, previously undescribed, species. On the basis of 16S rRNA gene sequence analysis, the closest species is *V. splendidus*. As well as being clearly distinct genetically (Figure 14) these isolates are mostly unable to utilise sucrose, producing pale cream colonies on TCBS. Arginine decarboxylase and indole positive, haemolytic (SRBC). PCR-RFLP pattern XIb (Figure 1).
II	45	*Pseudoalteromonas* spp. Phenotypically (BIOLOG) and genetically (PCR-RFLP and 16S rRNA gene sequence analysis) the members of this group are closely related. They are all non-fermentative, Gram-negative, oxidase-positive motile rods and comprise at least three distinct species or subspecies. PCR-RFLP patterns Vb and Vc (Figure 1).
III	7	*Pseudoalteromonas* species. Gram-negative motile rods, which are clearly separated phenotypically by BIOLOG GN from Group II, but are highly similar genetically (based on PCR-RFLP and 16S rRNA gene partial sequence analysis). PCR-RFLP pattern Vb.
IV	4	Unidentified Gram negative, fermentative, oxidase negative, catalase postive motile rods. Cream colonies on MA. Phenotypically (BIOLOG GN) and genetically (PCR-RFLP) clearly distinct group.
V	4	Unidentified Gram-negative, fermentative, oxidase-negative, catalase postive motile rods. Cream colonies on MA. Phenotypically (BIOLOG GN) and genetically (PCR-RFLP) clearly distinct group.
VI	7	*Oceanospirillium*-like. Gram-negative, weakly fermentative, oxidase-negative, motile rods. Reduce nitrate. Cream colonies on Marine Agar. Phenotypically (BIOLOG GN) and genetically (PCR-RFLP and 16S rRNA gene sequence analysis) clearly distinct group.
VII	1	Gram negative, weakly fermentative, oxidase-negative, motile rods. Cream colonies on Marine Agar. Phenotypically (BIOLOG) and genetically (PCR-RFLP) clearly distinct organism.

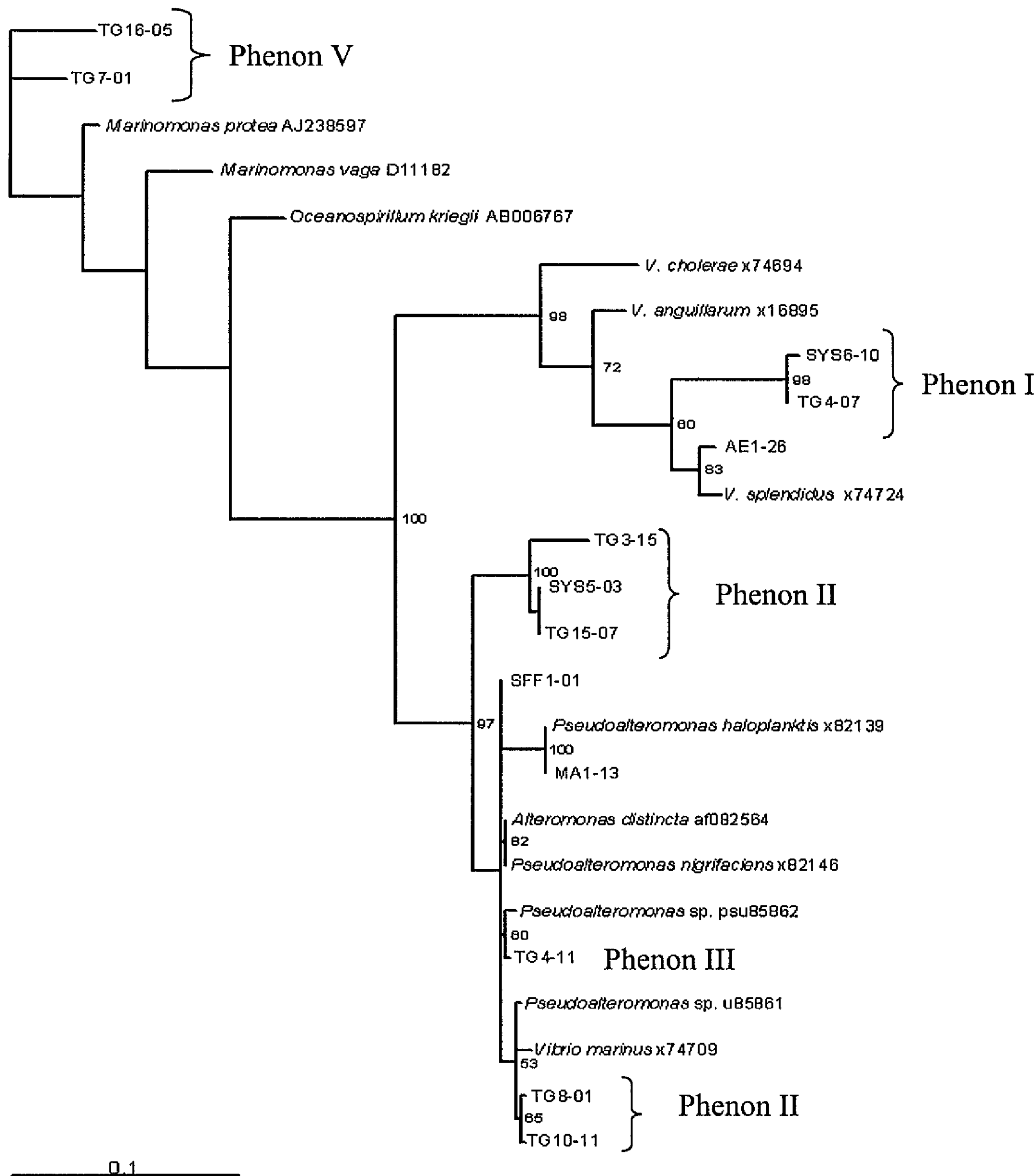
Table 20 Nutritional versatility (% positive) of the BIOLOG GN phenons illustrated in Figure 13 and further described in Table 19. Reactions were scored on a scale of between 0 – 10 and the strains were clustered using the MICROLOG v.3.5 software. n is the number of isolates in each of the phenons.

	Phenon						
Tests	I	II	III	IV	V	VI	VII
	n = 9	n = 46	n=7	n=3	n=4	n = 7	n = 1
Acetic acid	33	59	36	62	56	43	100
N-Acetyl-D-galactosamine	0	50	0	0	0	0	0
N-Acetyl-D-glucosamine	100	90	0	0	0	64	50
cis-Aconitic acid	44	49	21	87	38	100	100
Adonitol	100	1	0	38	13	64	0
Alaninamide	0	16	0	0	13	7	0
L-Alanine	78	86	50	75	88	100	100
D-Alanine	22	2	21	75	44	93	100
L-Alanylglycine	100	86	21	38	81	79	50
2-Aminoethanol	0	1	0	25	13	7	100
Aminobutyric acid	0	0	36	50	63	86	100
L-Arabinose	11	10	0	100	25	71	100
D-Arabitol	0	0	29	100	19	100	100
L-Asparagine	56	33	29	75	75	50	100
L-Aspartic acid	28	22	29	63	50	50	100
Bromosuccinic acid	50	10	57	100	44	64	100
2,3-Butanediol	0	0	0	13	0	7	50
D,L-Carnitine	0	0	0	25	0	0	0
Cellobiose	11	23	0	0	13	86	100
Citric acid	61	64	0	100	88	100	100
α-Cyclodextrin	67	76	0	0	0	7	50
Dextrin	100	93	0	13	63	14	100
meso-Erythritol	0	2	0	100	0	57	100
Formic acid	0	0	21	75	0	0	0
D-Fructose	100	20	7	100	69	93	100
L-Fucose	6	0	0	13	0	64	0
D-Galactonic acid lactone	0	0	0	100	25	14	50
D-Galactose	6	9	0	100	38	100	100
D-Galacturonic acid	0	21	0	63	25	21	100
Gentiobiose	0	4	0	0	13	21	100
D-Gluconic acid	11	0	7	100	88	100	100
D-Glucosamic acid	0	1	7	50	0	14	0
α -D-Glucose	100	52	29	100	88	100	100
Glucose-1-phosphate	11	3	0	0	0	0	50
Glucose-6-phosphate	78	1	0	13	0	0	0
Glucuronamide	0	0	0	25	0	0	50
D-Glucuronic acid	0	1	0	63	13	50	100
L-Glutamic acid	83	48	79	100	63	100	100
Glycerol	89	0	43	100	75	100	100
D.L-α - Glycerol phosphate	78	0	0	13	0	43	0
Glycyl-L-aspartic acid	33	67	0	13	6	43	0
Glycyl-L-glutamic acid	67	96	14	25	44	100	50
Glycogen	78	57	0	25	31	14	50
L-Histidine	17	0	57	50	63	86	0
α -Hydroxybutyric acid	0	3	36	0	0	0	0
γ-Hydroxy butyric acid	0	0	7	0	50	29	0
β-Hydroxybutyric acid	0	0	64	25	100	79	100
Hydroxy-L-proline	0	1	0	25	38	86	50
Hydroxyphenylacetic acid	0	24	21	25	56	29	0
Inosine	100	22	29	75	25	79	0
meso-Inositol	0	0	0	13	31	86	100
Itaconic acid	6	14	29	63	0	7	0
α -Ketobutyric acid	0	8	0	13	25	0	0
α -Ketoglutaric acid	0	8	64	87	38	100	50
α -Ketovaleric acid	0	1	7	0	0	0	0
DL-Lactic acid	89	5	79	100	75	100	100
α -D-Lactose	0	4	0	0	13	14	100
Lactulose	0	3	0	0	0	79	100
L-leucine	0	17	29	50	0	0	50
Malonic acid	0	2	14	75	0	14	100
Maltose	100	98	0	0	50	100	100
D-Mannitol	100	38	29	87	88	100	100
D-Mannose	17	32	0	87	50	100	50
D-melibiose	0	4	0	0	0	86	100

Table 19 (continued)

	Phenon						
	I	II	III	IV	V	VI	VII
Tests							
Methyl pyruvate	39	80	71	88	100	100	100
β -Methyl-D-glucoside	11	2	0	38	44	14	100
Mono-methylsuccinate	0	3	64	38	44	21	0
L-Ornithine	0	3	43	63	25	100	0
L-phenyl alanine	0	13	14	25	63	79	0
Phenyl ethylamine	0	0	0	0	0	21	0
L-proline	61	49	79	100	63	100	100
Propionic acid	6	73	50	37	63	7	50
D-Psicose	78	5	0	50	63	7	50
Putrescine	0	0	0	0	50	7	0
L-Pyroglutamic acid	0	0	29	87	38	100	50
Quinic acid	6	2	36	25	88	71	100
D-Raffinose	6	0	0	13	25	7	100
L-Rhamnose	6	2	0	0	0	36	50
D-Saccharic acid	6	3	14	75	38	0	50
Sebacic acid	0	0	7	25	0	0	0
D-serine	0	1	0	13	13	0	0
L-serine	100	41	71	100	100	100	50
D-sorbitol	11	0	29	63	38	100	100
Succinamic acid	0	2	36	25	38	36	100
Sucrose	17	46	0	13	75	86	100
Succinic acid	89	88	79	100	75	100	100
L-Threonine	94	43	21	13	88	21	50
Thymidine	56	8	7	38	25	29	50
Trehalose	78	27	0	0	25	87	100
Turanose	11	2	0	0	63	93	100
Tween 40	100	99	71	50	38	14	0
Tween 80	72	36	71	38	19	0	0
Uridine	100	41	14	25	0	93	0
Urocanic acid	0	0	29	37	13	14	0
Xylitol	0	1	0	13	0	0	0

Figure 15 Molecular phylogenetic tree constructed by comparing the partial 16S rRNA gene sequences of a number of isolates taken from the guts of yolk-sac larvae over the course of the rearing trial. Also included are a number of reference sequences from the EMBL database and from the survey (Chapter 2). SYS6-10 and SYS5-03 were isolated from Ardtoe yolk-sac larvae, MA1-09 and AE1-26 from Mannin and Ardtoe enriched *Artemia*, respectively and SFF1-01 from first-feeding Ardtoe larvae. Where relevant, the phenons to which particular isolates have been assigned in Figure 14 are also indicated. The unrooted tree was constructed using the Maximum Likelihood-based programme PUZZLE. Puzzling steps, equivalent to bootstrap values, are also shown. The length of the horizontal branches is proportional to the number of nucleotide substitutions



3.3.6. First Feeding Performance

In general, percentage uptake onto first feed was poor in the trial tanks for all treatments (mean 8.6%). This is illustrated graphically in Figure 16. No treatment is significantly worse than any other, although both of the recirculation treatments did better than the flow-to-waste treatments. Large inter-batch and inter-tank start feed success was encountered, illustrated by the large confidence intervals. It appears that the larvae did worse in the trial tanks generally than in the production tanks. Figure 17 shows how larvae split, from the treatment tanks of the same batch, into production tanks do worse than their siblings who were pooled into production tanks.

Figure 16 Average start feed success of yolk-sac larvae transferred to 100 litre trial tanks. Data is presented by yolk-sac rearing treatment (± SEM). RN was recirculated wateer, non-egg-disinfection, RD was recirculated water, egg-disinfection, F was flow to waste, egg -disinfection and FA was the same as F, except antibiotics were added to the yolk-sac rearing incubators at 115 and 180 day-degrees.

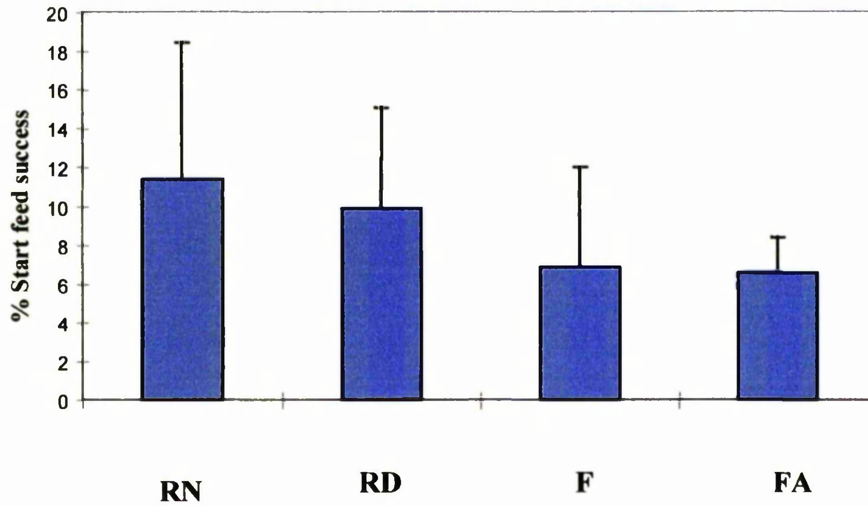
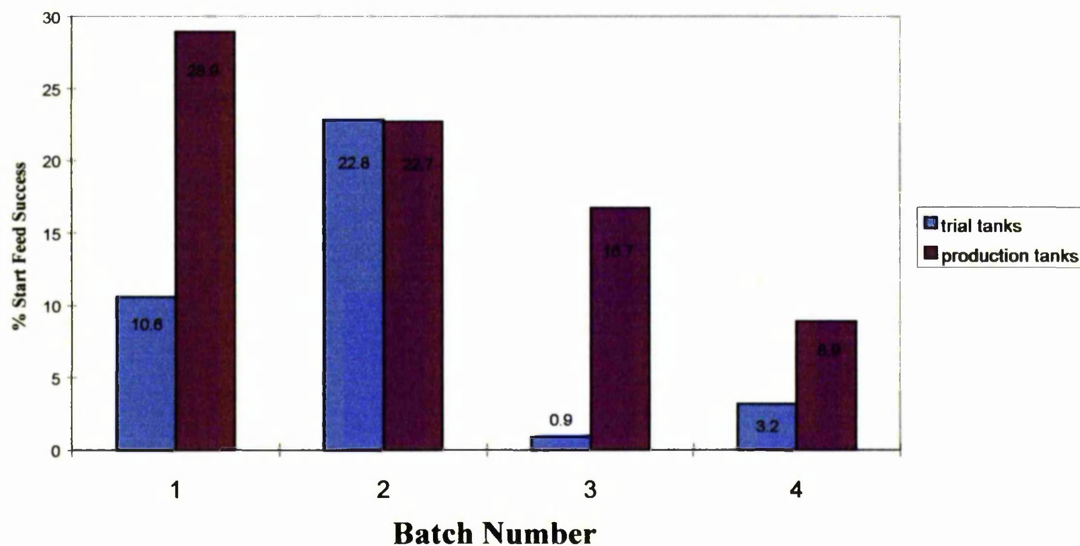


Figure 17 Average start feed success of each of the first four batches of larvae used in the rearing trial. The percentage uptake onto first feed of the larvae transferred to the trial tanks (pooled data, excluding antibiotic-treated larvae) is compared with that of their siblings who were transferred to production tanks.



3.4. Discussion

The results from this study indicate that addition of antibiotics may improve the survival of yolk-sac halibut larvae and also supress the development of a gut microflora. This difference in survival of the antibiotic-treated and non-antibiotic treated flow-to-waste treatments was mariginally significant when non-parametric tests were employed (Mann Whitney; $p<0.046$). There was no significant difference when parametric tests were used.

The results contradict the findings of the few published reports on the effects of addition of antibiotics on larval performance in commercial-scale production systems or on how tankwater and larval gut microfloras develop under recirculation as opposed to flow-through water systems (Holmstrom and Kjelleberg, 1999; Hernández-Cruz *et al.* 1994).

Hansen *et al.* (1992) investigated the effect of rearing herring larvae in the presence of penicillin and streptomycin on the development of a gut microflora. They found that, in contrast to the results of this study, bacteria could be isolated from the antibiotic-treated group. They also visualised the presence of high densities of bacteria in the intestinal lumen of treated and untreated larvae using light, fluoresence and electron microscopy. The bacteria isolated from the antibiotic-treated larvae exhibited multiple antibiotic resistance. It is probable that their choice of antibiotics used was poor. The results from the survey (Chapter 2) illustrate that a high proportion of the bacteria isolated from UK halibut hatcheries exhibit multiple antibiotic resistance, particularly against penicillin and streptomycin (Appendix 2). It is not reported whether the administration of antibiotics increased the survival of the treated larvae.

Hernandez-Cruz *et al.* (1994) looked at the effect of rearing regime on *Sparus aurata* survival, growth and *n*-3 HUFA levels. They found, in contrast to the results from this study, that the best survivals were in their flow-through system and poorest performance was in the recirculation tanks when antibiotics (oxolinic acid and chloramphenicol) were added. Although it was not discussed by the authors, it is

possible that direct addition of antibiotics to a recirculation system may have adversely affected the bacterial components of the biofilter communities, thus adversely affecting water quality.

However, early reports have shown that addition of antibiotics can improve hatching rate and survival of marine fish eggs, so the results are not unexpected. (Oppenheimer, 1955; Shelbourne, 1963). Also, as mentioned in the introduction, the addition of antibiotics is apparently necessary to ensure survival of halibut yolk-sac larvae reared in small-scale static systems (Lein *et al.* 1997; Ottesen and Bolla, 1998).

In this study the recirculation regimes both performed relatively well, despite the high levels of bacteria isolated from yolk-sac larvae reared under these conditions. This, together with the lack of any correlation between numbers of gut-associated bacteria and survival (Figure 13), indicates that it is likely to be the types of bacteria present, rather than numbers *per se*, which is the important factor influencing yolk-sac larval survival. The BIOLOG data further supports this hypothesis, where good survivals are correlated with the presence of bacteria that appear to be different to those found under conditions of poor survival (Table 18). The observation that the best performing flow-to-waste treatment, where antibiotics were not added, showed a high loading of bacteria similar to those found under the recirculation regimes is particularly noteworthy in this context.

In general, larvae reared under recirculation conditions had a culturable aerobic gut flora dominated by members of phenon II (*Pseudoalteromonas* spp.). There was a wider diversity of organisms recovered from the larvae that were raised from non-surface-disinfected as opposed to surface-disinfected eggs during the first four runs of the experiment (relative diversity, J' = 0.67, as opposed to 0.1). Note that these treatments had good overall survival.

Pseudoalteromonas species are commonly isolated from the marine environment and many have been found in association with marine animals (Holmstrom and Kjelleberg, 1999). Some of the *Pseudoalteromonas* species isolated in the trial appeared to be similar to those isolated in the survey, in particular a black pigmented and a brown pigmented species (*Pseudoalteromoas* groups 2 and 4 in the survey; grouped together in phenon II in Table 19).

Pseudoalteromonas species produce a range of different compounds which are active against many different organisms and substances (Holmstrom and Kjelleberg, 1999). The black pigmented organism, which was isolated from both the survey and the trial, produced an extracellular agarase. This organism appears to be similar to another black pigmented *Pseudoalteromonas* organism, originally isolated from the Pacific, for which the extracellular agarase has beencharacterised (Vera *et al.* 1998) As will be discussed later, one of the *Pseudoalteromonas* isolates (TG15-07; Figure 14) was also found to have high levels of activity against other bacteria *in vitro* (Chapter 5).

It is possible that the domination of the gut flora of larvae reared under recirculation conditions by *Pseudoalteromonas* species may confer a level of protection if some these organisms, such as TG15-07, produce inhibitory substances *in vivo* as well as *in vitro*.

The only presumptive *Vibrio* species isolated during the first four runs of the experiment were from the first non-disinfected/ recirculated water treatment (RN1 Table 18). As mentioned in the phenon description (Table 19), it is interesting that this was apparently the so far unidentified *Vibrio* species described in Chapter 3. When some of the treatments were repeated a year later this species was again found, this time in larvae reared under recirculation conditions. It should be noted that the recirculation system was disconnected and restarted in the intervening period.

The poorest performing treatment, disinfected eggs/flow-to-waste, showed a high level of heterogeneity in terms of the types of bacteria that were present from batch to batch (Table 18). This is reflected in a high relative diversity score (J' = 0.72). They appear to be markedly different, in many cases, to the organisms isolated under either of the recirculated water regimes (members of phenons IV, V, VI and VII). However, the best performing batch reared under this regime (run four), was dominated by similar organisms to those found in the generally better performing recirculation treatments (*Pseudoalteromonas* spp, phenon IV).

Very low numbers of culturable bacteria were recovered from the final treatment (addition of antibiotics, flow-to-waste, disinfected eggs), and it could be argued that these may have been accidental environmental contaminants. Few of these

isolates utilised enough of the available carbon sources after 48 hr incubation for BIOLOG to produce an acceptable profile for comparison with other isolates. This, in itself, illustrates that they were very different to the isolates recovered from the other treatments, where the majority of isolates tested produced acceptable BIOLOG GN profiles.

It is possible that there is some selection taking place in terms of the types of bacteria that are able to reside and, possibly, proliferate, in the guts of yolk-sac larvae. This is shown by the observation that, although addition of antibiotics causes a short-term reduction in the total numbers of bacteria in the incubators, levels soon rise back to a high level within 48 h of treatment (Figure 12). The yolk-sac larvae themselves appear to be practically free of culturable bacteria, despite the presence of large numbers of microorganisms ($>10^3$ CFU ml^{-1}) in their immediate environment. This could have been caused by the antimicrobials bioaccumulating within the larvae, making them resistant to later colonisation by bacteria.

As discussed earlier, the high level of heterogeneity in terms of the types of bacteria that appear to be able to colonise the guts of yolk-sac larvae, particularly under a flow-to-waste regime (where antibiotics have not been added) and where the eggs have not been surface-disinfected. Wide variation in the relative performance of different incubators, which have been stocked with eggs from the same batch (inter-batch variation) and between different batches (intra-batch variation) are reported by many of the UK hatcheries (R. J Shields, personal communication). A significant variation between the performances of the different replicates generally, independent of treatment, was also found in this study (Figure 11). Although differences in egg quality and other physical and biological parameters are probably important in explaining some of the observed inter-batch variation, the results from this study do not lessen the likelihood that stochastic variation in the types of bacteria which colonise yolk-sac larvae may also be of some importance.

In terms of possible methods of controlling the microfloras that develop in yolk-sac larvae, with a view to improving performance, it can be said that addition of antibiotics may be of some benefit. However, this route is likely to be short term. The likelihood is that antibiotic-resistant strains will rapidly develop which will both

negate any possible positive effect and also, potentially, cause problems in the future (Ringø and Birkbeck, 1999). This would particularly be the case if this resistance is transferred to pathogens elsewhere in the hatchery rearing system, making their control with convenient antimicrobials problematic. At present only four antibiotics are licensed for use in aquaculture in the UK: oxytetracycline, trimethoprim-sulphadiazine, oxolinic acid and amoxycillin (Hastings, 1997).

Of greater encouragement is the finding that survival under a recycled water regime, where the eggs have been previously surface disinfected, resulted in good survivals and, seemingly, the development of a restricted gut microflora. In general, it was found that other water quality parameters measured (salinity and temperature) were more stable under recirculation than under a flow to waste regime. These results, taken together with the energetic savings to be gained by running a recirculation system, point to a possible way forward for commercial operators.

Why survival and microflora development appear to be less stochastic under a recirculation than under a flow-to-waste regime is hard to explain. It is tempting to say that the data from this trial backs up encouraging results obtained by other workers who have used microbially matured water systems for the rearing of marine fish larvae (Skjermo *et al.* 1997; Salveson *et al.* 1999; Skjermo & Vadstein 1999). However, the recirculation system was closed in that both incoming and outflowing water were UV sterilised. It is possible that what was observed was the difference between two isolated systems which have different communities of micro-organisms colonising the pipework, in the form of biofilms, post-UV. This would result in a difference in the types of organisms flowing into the incubators. It could also be that the generally more stable (physically) conditions in the recirculation system were more conducive to normal development of yolk-sac larvae.

Unfortunately, the generally poor uptake onto first feed for all the treatments does not allow us to say whether the addition of antibiotics merely held up the survival of yolk-sac larvae that were otherwise fatally compromised by the poor physical conditions under which they were held. However, their relatively vigorous movements upon transfer and the fact they did not do noticeably worse than the other treatments does not support this hypothesis.

The better performance of the larvae transferred to the production tanks, as opposed to the trial tanks, is hard to explain (Figure 16). Light levels in the trial room were higher than in the production room, which may have stressed the larvae. It is also possible that differences in tank water flow dynamics and in surface area to volume ratio between the two types of tank may have increased the likelihood of negative interactions between the larvae and the tank walls. Another hypothesis could be that water quality parameters, both physical and biological were more stable in the larger tanks. First feed success was generally assessed later in the production tanks than in the trial tanks so it could be that some of the larvae scored as 'non-viable' in the trial tanks may have started to feed if left in the tanks longer.

The poor performance of otherwise seemingly healthy yolk-sac larvae, following transfer to first feeding tanks, reinforces the conclusions of the industry that this is a key bottleneck restricting production of viable halibut fry.

4. Infection Experiments

4.1. Introduction

As well as identifying the bacteria present within the hatcheries it is important to know whether they are disease-causing agents. Or, alternatively, bacteria may be not only benign but may confer some level of protection on the larvae. As such, some may be potential probiotic candidates.

Initially, to test whether the isolates were virulent it was necessary to develop a challenge model. Ideally, this would involve the larvae being reared under bacteria-free conditions so that the effects of individual bacteria on the health and general performance of larvae, in the absence of other bacteria, could be determined.

In this section of the work a model system for growing halibut larvae in the absence, or limited presence, of MA culturable bacteria was developed and the effects of a number of isolates from the survey and the rearing trial on yolk-sac larval survival was assessed.

4.2. Materials and Methods

4.2.1. Halibut eggs

Apart from an initial pilot trial conducted at SFIA Ardtoe, all the rearing experiments were carried out in the Division of Infection and Immunity, Glasgow University in controlled environment rooms using halibut eggs supplied by either SFIA Ardtoe or Otter Ferry Seafish.

The eggs transported from Ardtoe were double packed in polythene bags containing approximately 10L of 100µm-filtered, UV-sterilised Ardtoe hatchery seawater and 150 ml eggs at 57 day-degrees. The eggs were transported over ice to the laboratory (approximately 4 h journey time), where they were maintained at 5 ^{0}C

for the duration of the experiment. These were used for an experiment to test egg sterilisation methods.

Otter Ferry Seafish eggs, rather than Ardtoe eggs, were used for the majority of the larval rearing experiments. A different transportation protocol to that described above was followed. Eggs were collected after they had been reared at 5 ^{0}C for 13 days (65 day-degrees post-fertilisation) and were transported over ice in 2L stoppered conical flasks containing 1.5 L of seawater and antibiotics at different concentrations (oxolinic acid, kanamycin and erythromycin 0.01g L^{-1}; penicillin G 0.15 g L^{-1} ; streptomycin 0.075 g L^{-1}) to the laboratory. Generally, eggs were transported to the laboratory within 2 h of collection at the hatchery. Collection conditions and treatments were varied as described for the individual experiments.

4.2.2. Egg disinfection procedures.

Different combinations of egg disinfection procedures were tested in the optimisation experiments. For some of the experiments the eggs were surface disinfected with glutaraldehyde just prior to collection by hatchery operators. All the eggs from the conical incubator were removed and treated with a solution of 8ml of 25% glutaraldehyde (Sigma) in 5L of 100μm filtered, UV sterilised hatchery water for 10 min (working concentration of 1:800). The eggs were then rinsed thoroughly in the same source of seawater. Some of these eggs were transferred to the antibiotic solution-containing flasks as earlier described, but the majority were then transferred to upwelling yolk-sac incubators where they were reared on for commercial use. In other experiments, the eggs were surface disinfected after they had been treated with antibiotics immediately prior to stocking into the rearing vessels by a ten second immersion in 1:250 Kick Start. To minimise residual contamination, a combination of disinfection protocols was generally used to treat a batch of eggs.

4.2.3. Disinfection treatments.

To test the effectiveness of different egg disinfection protocols, eggs were treated with two concencentrations of glutalraldehyde (1:800 and 1:2000) and one treatment with Kick Start^{TM}. For the glutaraldehyde treatments, 200 eggs were firstly transferred to a sterile 100μm filter and rinsed twice in SASW (Tropical Marine^{TM} 33 $^{0}/_{00}$). They were then immersed for ten min in 25% glutaraldehyde (Sigma) at either

1:2000 or 1:800 final concentration in 100ml SASW. The eggs were then rinsed a further three times in SASW before assessment for sterility and hatching rate. Other eggs were treated with Kick Start as for the glutaraldehyde treatments, except that they were immersed for 1 minute in 1:250 Kick Start in SASW (≈ 0.02% peracetic acid; Kristjanssen,1995). The 1:2000 glutaraldehyde and 1:250 Kick Start treatments were both performed within an hour of each other. The second (1:800) glutaraldehyde treatment was done the next day. Each treatment was replicated three times.

4.2.4. Assessment of sterility and hatching rate

For assessment of egg sterility following the different treatments, approximately 30 eggs from each treatment replicate were placed individually into holes cut into MA plates with a sterile Pasteur pipette. The MA plates were incubated at 10°C for 18 days, after which they were inspected for signs of bacterial growth around the eggs. From this, the percentage of apparently sterile eggs, with respect to MA culturable organisms, was determined. The remaining treated eggs from each replicate were then transferred to separate 250 ml Erlenmeyer flasks containing 150 ml SASW. The flasks were incubated at 5 ^{O}C for seven days, after which the number of unhatched eggs and hatched larvae were counted. This was used to determine the average percentage hatch rate for each of the treatments.

4.2.5. Infection experiments

Various bacteria isolated during the hatchery bacteriological survey (Chapter 2) and the yolk-sac rearing trial (Chapter 3) were tested for virulence against yolk-sac larvae (Table 21).

All the bacterial isolates tested were incubated overnight in MB shake cultures at 15 ^{O}C , the bacteria were collected by centrifugation and washed three times in 25 $^{0}/_{00}$ ASW. Cultures were diluted in 25 $^{0}/_{00}$ ASW and an OD_{650nm} reading made. Washed cultures were then added to the experimental flasks or tissue culture dishes to give final bacterial cell densities of 1x 10^5 cells ml^{-1} for the multiwell plate experiment and 1 x 10^2 cells ml^{-1} for all subsequent experiments, based on an OD_{650nm} reading of 1.0 being equivalent to 10^9 bacteria ml^{-1}.

Table 21. Bacterial isolates tested in the halibut yolk-sac larval rearing experiments. Isolates, unless otherwise stated, were identified according to the scheme presented in 2.3.1. and are coded as in Table 1.

Isolate	Source	Description
AE1-31	Enriched *Artemia*, Ardtoe.	Haemolytic *V. splendidus* gp 2 organism
TG11-16	Healthy yolk-sac larvae in trial.	*Pseudoalteromonas* gp IV organism
TG15-07	Healthy yolk-sac larvae in trial.	*Pseudoalteromonas* gp IV organism which inhibits the growth of other bacteria *in vitro*.
SYS6-10	Healthy yolk-sac larvae from survey.	Haemolytic *V. splendidus* gp 3 organism.
TG4-07	Healthy yolk-sac larvae from trial reared under recirculation conditions from surface-disinfected eggs.	Haemolytic *V. splendidus* gp 3 organism.
TG7-01	Poorly performing larvae reared under flow-to-waste regime in trial.	*Oceanospiriliuim*-like. Gram negative, weakly fermentative, oxidase negative. Phenon VI in Table 18. See also Figure 14
TG15-08	Healthy yolk-sac larvae in trial reared under recirculation conditions from non-surface disinfected eggs.	Black pigmented *Pseudoalteromonas* gp 2
TG2-12	Poorly performing larvae reared under flow-to-waste regime in trial.	Gram-negative, weakly fermentative, oxidase-negative. Phenon V in Table 18.
TG2-11	Poorly performing larvae reared under flow-to-waste regime in trial.	*Oceanospirillium*-like. Gram negative, weakly fermentative, oxidase negative. Phenon VI in Table 18.

Table 21 (continued)

Isolate	Source	Description
TG4-11	Poorly performing larvae reared under flow-to-waste regime in trial.	*Pseudoalteromonas* species. Phenon III in Table 18. See also Figure 14
Vibrio anguillarum 91079		Pathogen originally isolated from a case of vibriosis in juvenile turbot (Horne *et al.* 1977).
TG10-01	Healthy yolk-sac larvae from rearing trial reared under flow-to-waste regime.	*Pseudoalteromonas*
TG16-05	Healthy yolk-sac larvae in trial reared under recirculation conditions from surface-disinfected eggs.	*Oceanospirillium*-like. Gram negative, weakly fermentative, oxidase negative. Phenon VI in Table 18. See also Figure 14.
TG15-19	Healthy yolk-sac larvae in trial reared under recirculation conditions from non-surface-disinfected eggs.	*Pseudoalteromonas* species. RFLP pattern Vc but different colony morphology to *Pseudoalteromonas* group IV.
TG2-18	Poorly performing larvae reared under flow-to-waste regime in trial.	Gram-negative, oxidase-negative organisms. Phenon VII in Table 18.
TG8-01	Healthy yolk-sac larvae in trial reared under recirculation conditions from surface-disinfected eggs.	*Pseudoalteromonas* species. RFLP pattern Vc but different colony morphology to *Pseudoalteromonas* group IV.
AE1-26	Enriched *Artemia*, Ardtoe.	Haemolytic *V. splendidus* gp 1 organism.

Table 21 (continued)

Isolate	Source	Description
TG2-14	Poorly performing larvae reared under flow-to-waste regime in trial.	Gram-negative, fermentative, oxidase-negative. Phenon IV in Table 18.
TG4-02	Poorly performing larvae reared under flow-to-waste regime in trial.	*Vibrio* species.
V. viscosus 236	A. Laidler, Marine Harvest	
OFE1-19	Otter Ferry seafish eggs	RFLP group VII
E1-03	Freshly fertilised Ardtoe eggs	*Photobacterium phosphoreum.*
OFF1-05	Healthy first-feeding Otter Ferry halibut maintained on copepod diet.	*V. salmonicida*-like organism

4.2.6. Six- well tissue culture tray yolk-sac larvae challenge model.

This was based on the work of Bergh *et al.* (1995); 77-day-degree halibut eggs were disinfected using Kick Start (1:250) and individually transferred to separate wells of 6-well tissue culture trays. Prior to transfer, each well was filled with 11 ml 25 $^0/_{00}$ SASW (Instant Ocean™). Transfer of eggs into the trays was done in a temperature-controlled room at 5 ^{O}C under red light. After 5 days the plates were examined and the water exchanged in wells that contained hatched larvae. For this procedure, 10 ml water was drawn off from each well, using a 10 ml glass pipette. The end of the graduated pipette was flamed in ethanol between each well transfer and the water was replaced with 10ml fresh 25 $^0/_{00}$ sterile ASW.

4.2.7. Yolk-sac rearing trials in glass flasks

4.2.7.1.Five-litre round bottomed glass flasks.

Three 5 L round flasks were filled with 2L of hatchery seawater (100 μm filtered, 32 $^0/_{00}$, UV sterilised), cotton wool plugged and autoclaved. For each flask, fifty 65 day-degree halibut eggs were then transferred to a sterile 100 μm filter, surface disinfected in 1:250 Kick Start for 1 minute, rinsed twice in SSW and washed into the flask. Flasks were then left in a cold box set to 6 ^{O}C in the dark to incubate until 220 day-degrees post-hatch (37 days).

4.2.7.2. Experiment 1: Larvae reared in artificial seawater following disinfection of eggs with a combination of antibiotics, and reared in artificial seawater

For the first experiment, eggs were collected as described above. The eggs were incubated for 24 h in the antibiotic solution before being rinsed and transferred to 24 separate 2L stoppered conical flasks, all containing 1.5 L 25 $^0/_{00}$ sterile Instant Ocean; 25 eggs were stocked into each of the flasks. After seven days, all flasks were monitored for survival of larvae and sterility by plating 100 μl water samples onto MA, and by adding 5ml from each flask to 20 ml MB.

4.2.7.3. Experiment 2. Larvae reared in matured seawater following disinfection of eggs with a combination of antibiotics.

Another experiment was performed, this time using matured seawater collected from UMBS, Millport two years previously instead of ASSW. Firstly six flasks were set up with 25 eggs each. These eggs were disinfected with glutaraldehyde, treated with antibiotics as in the previous experiment, and finally rinsed in sterile seawater, after being quickly rinsed in 1:250 Kick Start. Six further flasks were treated as above, except they were not first surface-disinfected with glutaraldehyde. All flasks were reared at 6 ^{O}C and at 37 days post-hatch (approximately 220 day-degrees), the number of surviving larvae was counted and the sterility of the flasks assessed as above.

4.2.7.4. Experiment 3. Comparison between larvae reared in matured Millport seawater versus Glasgow University aquarium water in the presence and absence of selected bacterial isolates.

The remains of the original stock of matured seawater used in Experiment 2 were used to stock 6 flasks. 17 further flasks were filled with autoclaved, filtered 32 $^{0}/_{00}$ seawater taken from the Glasgow University aquarium. The 23 flasks were then filled with 25 halibut eggs that had originally been surface-disinfected with glutaraldehyde, treated with antibiotics as in the previous experiments, quickly rinsed in 1:250 Kick Start and then rinsed in sterile seawater.

4.2.7.4.1.Infection experiment

The 23 flasks were infected 3 days after the majority of the eggs had appeared to hatch (approximately 18 day-degrees post-hatch). Six flasks were then inoculated with each isolate, with the exception of TG11-16, which was only added to 5 flasks. The 6 remaining flasks were left as non-infected negative controls. Sterility was assessed at this point for all flasks by plating 100 μl water samples onto MA. Flasks were randomly assigned to each treatment and a note made of seawater origin. Twenty six days later the experiment was terminated and a note made of the number of surviving larvae in each flask.

4.2.7.5. Experiment 4. Comparison of survival of yolk-sac larvae reared in different types of seawater.

Twenty four stoppered sterile flasks were set up, each containing 1.5L of one of three different types of seawater and equilibrated to 6 ^{O}C in the controlled environment room. These were: 0.22 μm filtered freshly collected GU aquarium water (pH 7.27; 32 $^{0}/_{00}$), six-week old 0.22 μm-filtered GU aquarium water (pH 7.0; 32 $^{0}/_{00}$) which had been allowed to mature in the dark, and the final treatment was 0.22 μm filtered artificial seawater prepared with Divisional dH_2O (Instant Ocean; 32 $^{0}/_{00}$; pH adjusted to 7.28 per treatment). Two 25 ml samples of 65 day-degree halibut eggs were collected and treated with antibiotics as described previously, except they were not disinfected with glutaraldehyde immediately prior to collection. Following rinsing and treatment for 30 sec in Kick Start, 25 eggs were randomly distributed into each of the 24 flasks. 28 days later (approximately 162 day-degrees post-hatch) all flasks were examined for surviving larvae.

4.2.7.6. Experiment 5. Effect of bacterial isolates, and stocking density during antibiotic treatment, on larval hatch rate and survival

Forty five conical flasks were stocked with 1.5 L of 100μm filtered 32 $^{0}/_{00}$ Otter Ferry Seafish hatchery seawater collected two weeks previously. The flasks were stoppered, autoclaved and allowed to equilibrate to 6 ^{O}C in the controlled environment room.

Two samples of 65 day-degree halibut eggs were collected immediately following glutaraldehyde disinfection and transferred to separate antibiotic-containing flasks as described previously. The first of these samples was approximately 25 ml of eggs (approximately 1000), the second was inadvertently stocked with considerably more eggs (>50ml or more than 2000). The flasks were transported back to the laboratory and incubated overnight as previously described. Following rinsing and treatment for 30 sec in Kick Start, 25 eggs were then distributed into each of the 45 flasks. The egg source for each flask was recorded. Four days later (approximately 18 day-degrees post hatch) flasks were inoculated with 5 x 10^2 ml^{-1} of the different bacterial isolates, as described in experiment 4. Six flasks were assigned to each treatment, with the exception of the untreated control of five flasks. Five otherwise

untreated flasks were also sampled at this time to assess hatch rate, as differences in the performance of flasks stocked with larvae that originated from the high density antibiotic treatment flask were already evident. Sterility was assessed at this point. Thirty days later (approximately 200 day-degrees post-hatch) all flasks were assessed for larval survival.

4.2.7.7. Experiments 6 and 7 Effect of bacterial isolates on larval hatch rate and survival

Two litre conical flasks were stocked with 1.5 L of 100μm filtered 32 $^{0}/_{00}$ Otter Ferry Seafish hatchery seawater which had been collected and allowed to mature for a minimum of two weeks prior to use. The flasks were stoppered, autoclaved and allowed to equilibrate to 6 ^{O}C in the controlled environment room used for the experiment.

As an apparent density-dependent effect was uncovered in the previous experiment, samples no greater than 10ml of 65 day-degree halibut eggs were collected immediately following glutaraldehyde disinfection and transferred to separate antibiotic-containing flasks, transported back to the laboratory and incubated overnight as previously described. Following rinsing in SSW, treatment for 20 sec in 1:250 Kick Start and a further SSW rinse, 25 eggs were then distributed into each of 39 flasks in Experiment 6 and 44 in Experiment 7. Four to seven days later (approximately 18-30 day-degrees post hatch) flasks were inoculated with 5×10^{2} ml^{-1} of the different bacterial isolates to be tested, as described in Experiment 4. Four flasks were assigned to each treatment, with the exception of the untreated controls, which were 6 flasks for Experiment 6 and 8 for Experiment 7; 30 days later (approximately 200 day-degrees post-hatch) all flasks were assessed for larval survival, levels of ammonia and nitrite (Aquarium testing kits; Tetra Werke W. Germany) and levels of culturable bacteria by direct plating onto MA of SSW-diluted flask water samples. Control flasks were also assessed for apparent sterility by direct plating of 100 μl water samples onto MA and addition of 5ml water to 5 ml MB.

4.2.8. Statistics

Differences between treatments were analysed using non parametric tests as the data was not normally distributed. The Kruskal Wallis test was employed where

more than two treatments were being compared. For the infection experiments, survivals for the different treatments were compared in each case against the controls. For the infection experiments, survivals in the different treatments were analysed by comparing numbers of larvae present in the control, uninfected flasks, and the infected flasks using Mann Whitney tests.

4.3. Results

4.3.1. Egg disinfection experiment

As a first step towards the design of a bacteria-free yolk-sac larvae challenge model the effectiveness of different published halibut egg disinfection methods was assessed. The first of these was treatment with two concentrations of glutaraldehyde (1:800 for 10 min and 1:2000 for 10 min). The effectiveness of Kick Start was also investigated as this is routinely used in some UK hatcheries to disinfect eggs before moving them into the yolk-sac incubators (Kristianjssen 1995 & yolk-sac rearing trial, Chapter 3).

As shown in Table 22, all three treatments achieved an apparent egg disinfection efficiency of close to 90%. The apparent differences in hatching rate were probably unrelated to the treatments used. The low (3.53%) rate seen in the 1:800 glutaraldehyde treatment was probably caused by differences in handling of the eggs prior to disinfection. The eggs that were surface disinfected with Kick Start and 1:2000 glutaraldehyde were both treated immediately upon arrival at the laboratory, whilst the eggs treated for ten min with a 1:800 solution of glutaraldehyde were not processed until the following day. In the meantime, they were left in a flask at high density, which probably affected their later development.

Table 22 Effect of 3 different treatments on egg disinfection efficiency and hatch rate. Results for 3 replicate experiments are shown.

Treatment	Number hatched/ initial number (%)	Average hatch rate for each treatment (%± SEM).	Proportion (and %) of eggs tested which were sterile.	Average sterility for each treatment (% ± SEM)
1:250 Kick Start	3/ 21 (14.3)	65.8 (± 6.13)	13/15 (86.7)	90 (±1.67)
1:250 Kick Start	42/43 (97.7)		28/30 (93.3)	
1:250 Kick Start	41/48 (85.4)		27/30 (90)	
1:2000 glutaraldehyde	18/91 (19.8)	43.6 (± 7.32)	24/30 (80)	87.8 (±1.67)
1:2000 glutaraldehyde	22/35 (62.9)		27/30 (90)	
1:2000 glutaraldehyde	54/112 (48.2)		28/30 (93.3)	
1:800 glutaraldehyde	3/39 (7.7)	3.53 (±1.45)[1]	27/30 (90)	90 (± 3.33)
1:800 glutaraldehyde	0/ 100 (0)		26/30 (86.7)	
1:800 glutaraldehyde	3/103 (2.9)		14/15 (93.3)	

[1]Eggs from this treatment had been allowed to sit in a flask containing antibiotics for 24 hours longer than in the other two treatments.

4.3.2. Infection trial using 6-well tissue culture tray yolk-sac larvae challenge model.

4.3.2.1.Infection Experiment

Four different isolates were selected; TG7-01 (isolated from a batch of larvae that had performed poorly under a flow-to-waste regime in the trial); TG15-07 (isolated from a of larvae which performed well under recirculation conditions and which inhibited the growth of other bacteria *in vitro*); TG11-16 (taken from yolk-sac larvae performing well under recirculation conditions); and TG10-01 (taken from poorly performing larvae reared under a flow-to-waste regime. A fifth, control treatment, was also included. For this 100μl SASW was added to each well.

4.3.2.2.Monitoring

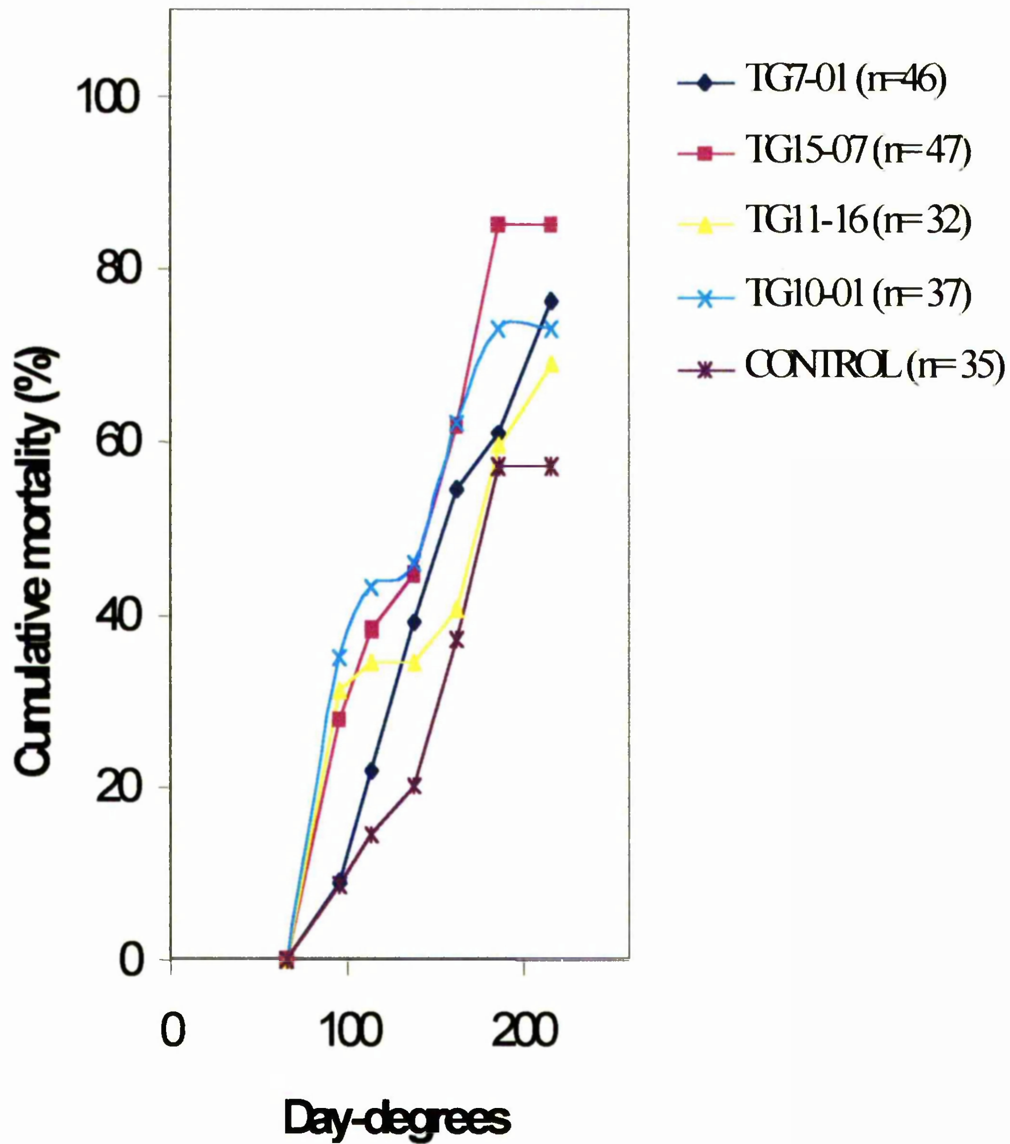
Plates were checked every 2-3 days for mortalities until 220 day-degrees post-hatch when the experiment was terminated. Each well was checked for sterility by plating out 10 μl onto MBA, just prior to addition of the challenge inocula.

Cumulative mortality in all groups (Figure 18) was high (57 – 85 %). No statistically significant between-treatment differences were discernible, although it was interesting to note that the best cumulative survival was in the control group.

4.3.2.3.Sterility

Only 21/342 (6.1%) of the wells tested were bacteriologically sterile by 70 day-degrees, the point at which the challenge inocula were added. Previous experiments had indicated that 1-minute treatment of the eggs in 1:250 Kick Start™ should have resulted in over 90% of the eggs being sterile when placed into the wells, indicating that the degree of sterility had not been achieved or that contamination has occurred after transfer. The most likely time at which this could have occurred was during water exchange after the eggs had hatched, as this was not done in a laminar flow hood.

Figure 18 Cumulative mortality of yolk-sac larvae in the infection trial using 6-well tissue culture trays. All the bacteria used, TG7-01, TG15-07, TG11-16 and TG10-01, were originally isolated from the rearing trial (Chapter 3).



4.3.3. Experiments using 5L round-bottomed flasks

As problems were encountered with the tissue culture dish rearing model other methods were investigated which would require less operator maintenance and a higher success rate of healthy larvae free of culturable bacteria by 220 day-degrees post-hatch.

In the 5 L flasks, approximately 40% of the eggs did not appear to have hatched and larvae, after hatching, generally settled to the base of the flasks. Observations, under red light , were kept to a minimum during larval development to prevent excessive disturbance and stress to the larvae. Larvae appeared to develop normally and for the greater part of their development displayed very little locomotory activity. By 160 day-degrees they showed a greater range of responses to external stimuli (e.g. stereotypic 'C' posture).

Larvae survived to the end of 220 day-degrees in all flasks (Table 23) and one flask did not appear to contain any culturable bacteria. However, survival in this flask was less than in the contaminated flasks. The highest survival (approximately 70%), occurred in one of the contaminated flasks.

Nitrate and ammonia concentrations were measured at the end of the experiment (Table 24). Ammonia concentrations were elevated in all the flasks, presumably from excretion of nitrogenous waste products by the larvae during their development. From the very low nitrite levels there was apparently little conversion of the ammonia produced, presumably, due to the absence of denitrifying bacteria in the culture flasks.

Table 23. Survival of yolk-sac larvae reared to 220 day-degrees post-hatch in 5L round-bottomed flasks.

Flask	Culturable bacteria present	Number of survivors/ 50 (%)	[a]Number of gapers	Number of dead larvae
1	Yes, orange/yellow pigmented	20 (40)	4	[b]Autolysed
2	No	16 (32)	5	24
3	Yes, orange/yellow pigmented	35 (70)	8	2

[a]Deformed larvae with open jaws.

[b]Dead larvae could not be distinguished due to autolysis or proteolysis.

Table 24 Nitrite and ammonia concentration in 5L round-bottomed flask rearing of halibut larvae.

Flask	NO_2/N mg/L	NH_3 /N mg/L
1	0.004	3.136
2	0.003	3.232
3	0.006	4.416

4.3.4. Experiments using 2L conical flasks

Further experiments were carried out in 2L cotton wool-stoppered conical flasks as rearing vessels, instead of 5L round-bottomed flasks as these would take up less space and allow a greater number of replicates to be established. The model was optimised by running a series of experiments which utilised different sources of sterile seawater and egg disinfection methods, until a method was found that provided both satisfactory survivals to the end of the yolk-sac absorption phase (220 day-degrees) and a high apparent rate of flask sterility.

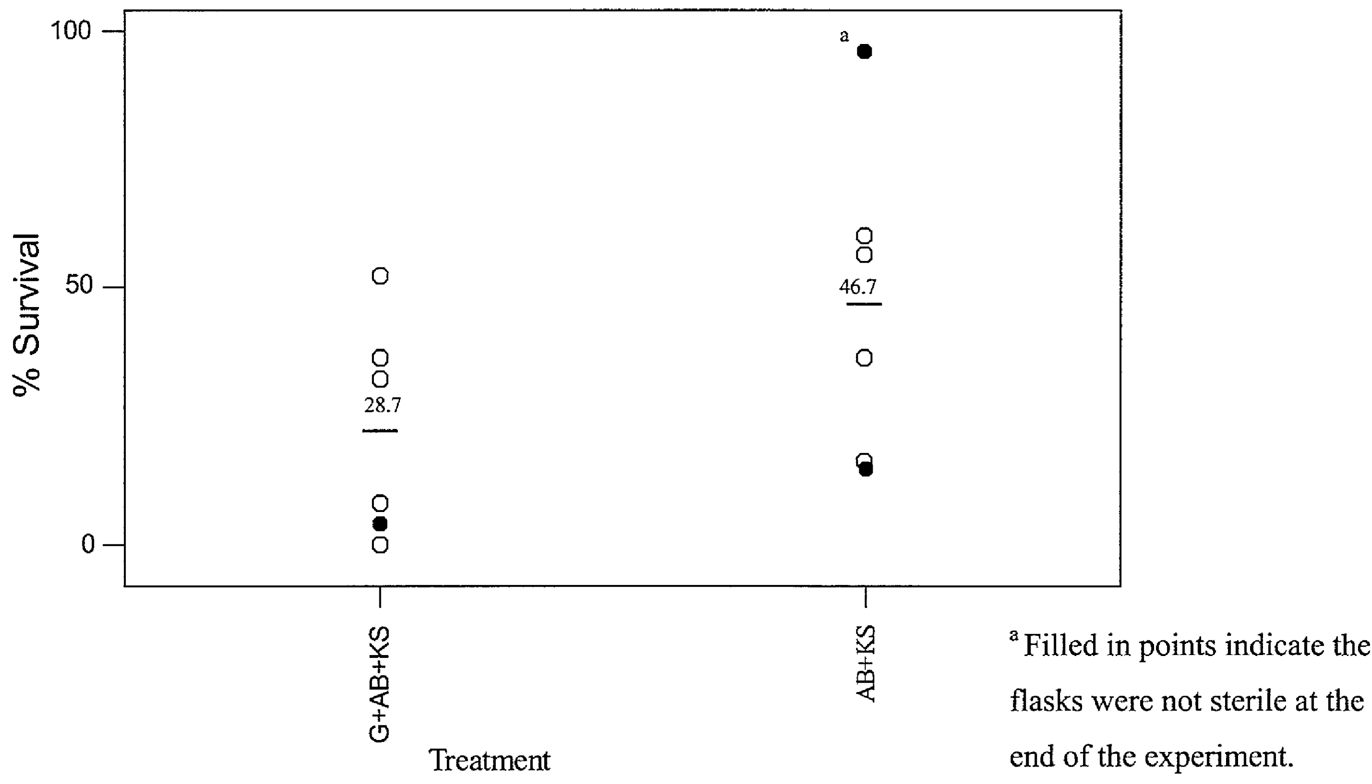
4.3.4.1. Experiment 1. Rearing of larvae in 2L flasks in artificial seawater following treatment of eggs with a combination of antibiotics.

All 24 flasks were contaminated within a week of egg transfer; in most cases the colony morphology of the contaminating organisms closely resembled those previously isolated from Otter Ferry eggs, suggesting that the decontamination procedure used was ineffective. In addition, all larvae died.

4.3.4.2. Experiment 2. Survival of larvae reared in 2L flasks in matured seawater following disinfection of eggs with a combination of antibiotics.

In this experiment the effect of treatment of eggs with glutaraldehyde prior to antibiotic/Kick Start treatment was tested; the source of the water was 2-year matured UMBS Millport sea water. Larvae survived to 220 day degrees under both treatment regimes, including the aggressive glutaraldehyde/antibiotic / Kick Start treatment (Figure 19). Higher survival was seen in the antibiotic /Kick Start treatment, including one flask where 24 larvae survived (out of an original 25 eggs stocked) to the end of the incubation period. Hatch rate under both treatments appeared, based on visual observations, to be very good. It should be noted that these survival figures are the product of both hatch rate and subsequent larval survival.

Figure 19 Survival of yolk-sac larvae to 220 day-degrees post-hatch in Experiment 2. Twelve 2L conical flasks were each stocked with 25 eggs which had been treated with either one of two egg disinfection procedures (six flasks per treatment). The first treatment, G + AB + KS, was immersion in gluteraldehyde, followed by incubation for 12h in a solution of antibiotics, followed by immersion in Kick Start. The second treatment, AB + KS, was the antibiotic treatment, followed by immersion in Kick Start. Individual survivals for the six flasks in each treatment are shown, the lines indicate the group averages. 25 eggs were stocked into each flask.



[a] Filled in points indicate the flasks were not sterile at the end of the experiment.

4.3.4.3. Experiment 3. Rearing of larvae in matured Millport seawater or Glasgow University aquarium water in the presence or absence of selected bacterial isolates.

An experiment was set up to investigate the effects of three different bacterial isolates on yolk-sac larval survival. The first of these was AE1-31 a haemolytic *V. splendidus* group 2 organism originally isolated from Ardtoe enriched *Artemia*. The other two were *Pseudoalteromonas* species group 4 organisms: TG15-07, which inhibited the growth of other bacterial species *in vitro* (see Chapter 5), and TG11-16. Both organisms were recovered from apparently healthy larvae reared under recirculation conditions in the yolk-sac rearing trial (Chapter 3).

It can be seen in Table 25 that although larvae survived in some flasks for 26 days (approximately 156 day-degrees) after hatching, in many of the flasks there were no surviving larvae by this time. Also, many of those that were alive were deformed, independent of whether the flasks had been infected or were apparently sterile at the time of sampling. From Table 25 it appears that water quality has an important influence on yolk-sac larval survival. Larvae reared in aged Millport-collected seawater performed better than those reared in seawater collected from the aquarium The difference in survival was highly significant (Mann-Whitney; $p< 0.01$).

From the limited data, it was not possible to determine whether any of the bacterial isolates tested were harmful, although it did appear that AE1-31 was not harmful. Two flasks containing the optimal aged Millport seawater were inoculated with this isolate and in both cases appreciable numbers of larvae survived (80% and 16%), relative to the non-inoculated controls. Apparently pure cultures of AE1-31 were recovered from these flasks in high quantities (greater than 10^5 ml^{-1} sucrose-positive TCBS-culturable organisms of morphology similar to AE1-31). It should be noted that all the larvae reared in the flask containing aged Millport seawater and infected with TG15-07 died.

Table 25 Individual survivals for the 23 flasks at 26 days post-hatch in Experiment 3. The flasks were inoculated with three different bacteria strains, except for the control treatments, four days after they were stocked with twenty five 70 day-degree halibut larvae and 1.5L of filtered, autoclaved seawater from two different sources, aged seawater from UMBS, Millport and freshly-collected seawater from the DEEB aquarium.

		Bacterial strain inoculated			
Water treatment	Control (survivors/25)	TG15-07 (survivors/25)	AE1-31 (survivors/25)	TG11-16 (survivors/25)	Average percentage survival for each water treatment (pooled data ± SEM).
Matured seawater	[a]20, 8, 6	0	4, 20		[b]38.7 (±15.1)
Aquarium seawater	0, 0, 6	0, 0,0, 0, 2	0, 2, 0, 0	0, 6, 0, 0, 0,	3.76 (±1.95)

[a]With the exception of the first flask, controls were all sterile at the point at which they were sampled as assessed by plating 100µl water samples on MA and inoculating 5ml water samples into MB.

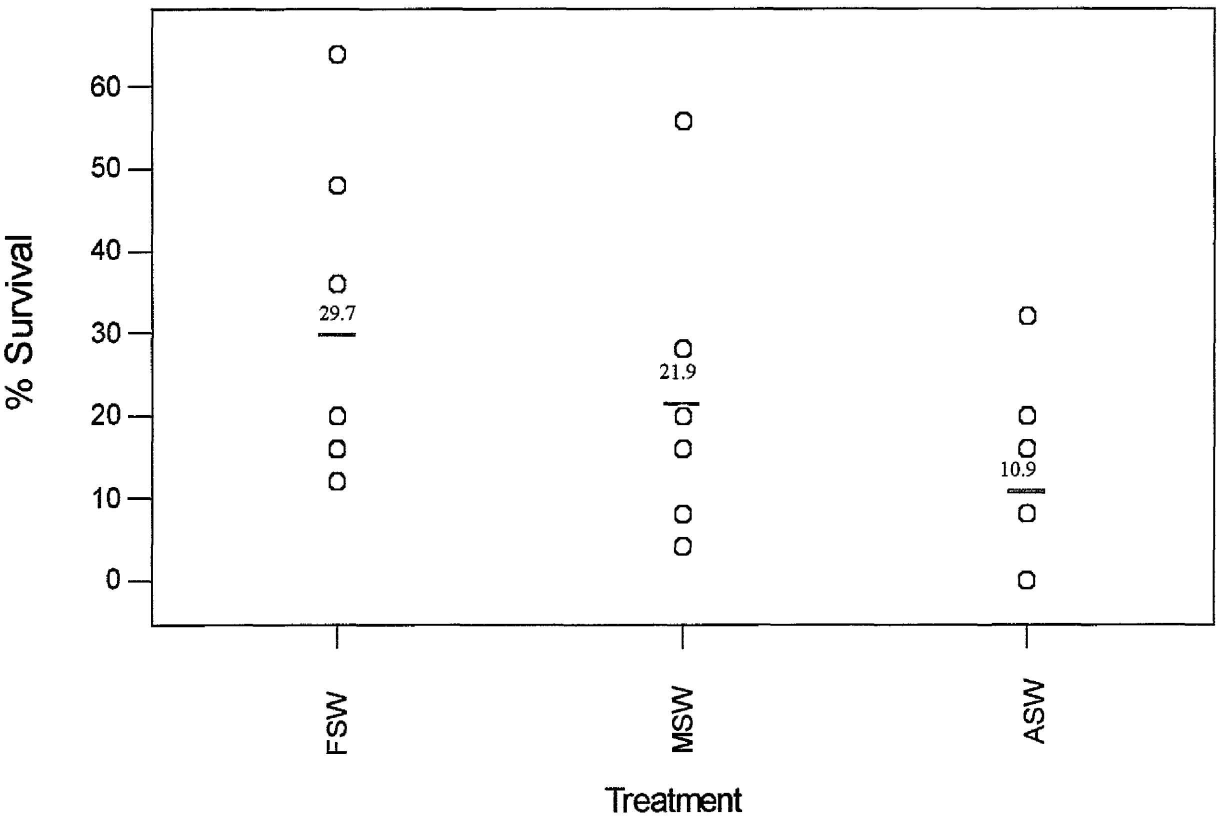
[b]Survival of larvae reared in matured seawater was highly significantly different to those reared in aquarium seawater (Mann-Whitney; $p < 0.0013$)

4.3.4.4. Experiment 4. Comparison of survival of yolk-sac larvae reared in different types of seawater.

To help control the apparent effects of seawater source and quality on yolk-sac larval survival, a flask-rearing experiment was carried out which compared the effects of using three different seawater sources. The waters compared were freshly collected or aged DEEB aquarium water and artificial seawater. It was intended to include freshly collected seawater from a high quality natural source (Otter Ferry Seafarms hatchery water) but this treatment was unfortunately lost during flask preparation.

Survivals in all three treatments were poor and non parametric tests failed to uncover significant differences in treatment survivals (Figure 20). It was noticeble that survival of larvae reared in the artificial sewater treatments was a lot worse than those reared in the two DEEB aquarium seawater treatments. When these were pooled and compared with the artificial seawater treatment the differencewas nearly significant (Mann Whitney; $p<0.056$). The aged DEEB treatment was the same batch of water used in Experiment 3. Although average survival of larvae was 3.76% in experiment 3 and 21.5% in this experiment, it cannot at this stage be concluded that ageing the water was the cause of this improved performance, which could be due to normal batch-to-batch variation in survival and differences in the ways the eggs were handled after collection. Also, the eggs in Experiment 3 were surface-disinfected with glutaraldehyde.

Figure 20 Survival of yolk sac larvae to 28 days post-hatch reared in different sources of 32 $^{0}/_{00}$ seawater in 2L flasks (Experiment 4). Twenty two flasks, each containing one of three types of sea water, were each randomly stocked with 25 halibut eggs, which were then allowed to hatch *in situ*. FSW was freshly-collected sea water from the DEEB aquarium, MSW was aged (6 weeks) DEEB water and ASW was artificial seawater made up using Instant Ocean™ and dH_2O. Individual survivals for all the treatment flasks are shown, the lines indicate the treatment averages.



4.3.4.5. Experiment 5. Effect of bacterial isolates and stocking density during antibiotic treatment on larval hatch rate and survival

Having determined that matured seawater from a clean natural source was likely to be the best medium to use in flask-based yolk-sac rearing experiments, an experiment was set up to investigate the effects of six different bacterial isolates on yolk-sac survival. The first three were those used in Experiment 3 (AE1-31, TG15-07 and TG11-16). A further two isolates from the trial were also tested; one, TG7-01, was isolated from poorly performing larvae reared under flow-to-waste conditions, and the other, TG4-07 was isolated from apparently healthy larvae that had been reared under recirculated water conditions. TG4-07 was one of the *Vibrio splendidus* group 3 organisms described in Chapter 3, another member of this group, SYS6-10, isolated from healthy halibut larvae during the original survey, was also included.

Inadvertently, another variable, subsequently shown to be of importance, was also tested. This was the effect of original stocking density during the antibiotic treatment part of the egg disinfection protocol prior to stocking the rearing flasks (Table 26). Larvae hatched from eggs from the high density antibiotic treatment flask did highly significantly worse than those hatched from the low density flask (Mann-Whitney; $p < 0.001$). This was apparently independent of whether culturable bacteria were detected in the flasks. Accordingly, only flasks that contained eggs originating from the low-density flask were included in subsequent analysis (Figure 21).

None of the bacteria tested appeared to be harmful, (Figure 21), although the controls flasks had the highest average survival (84%). There were no statistically significant differences between the different treatments, however. All 3 controls appeared to be sterile, as determined by plating 100 μl water samples onto MA and inoculating 5ml samples into 10ml MB.

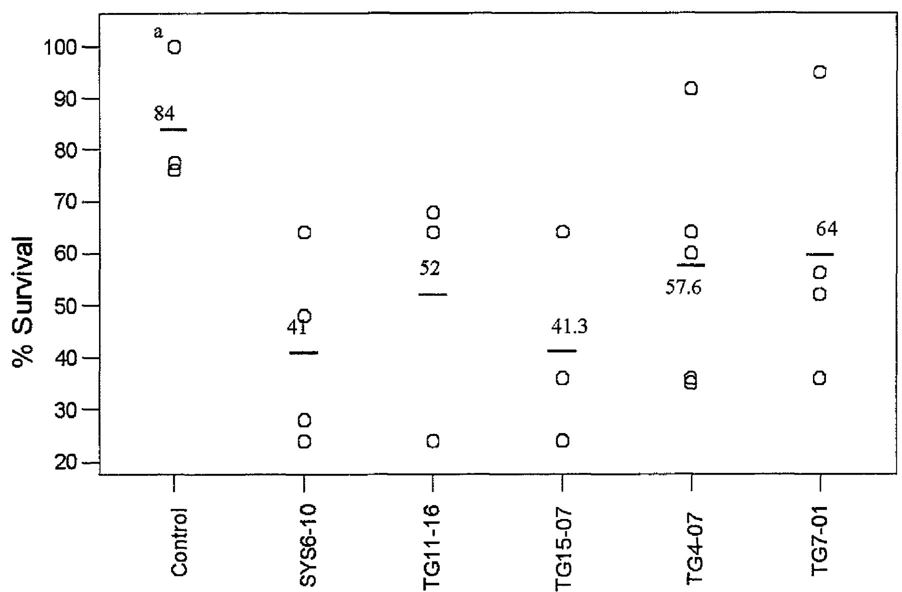
All the inoculated flasks appeared to have high numbers of the bacteria under test (> 10^6 CFU ml^{-1}) at the end of the incubation period, showing that all isolates were able to grow in the larvae-containing flasks and retain viability during the course of the experiment. The estimated inoculum of 5×10^2 CFU ml^{-1}, was confirmed by plating out dilutions of the inoculate onto MA.

Table 26 Effect of concentration of eggs during antibiotic treatment on survival of flask-reared larvae in Experiment 5 according to whether the eggs used to stock the flasks had been pre-treated with antibiotics in a densely stocked (>2000 eggs) or less densely stocked (approximately 1000 eggs) 1.5 L flask.

Treatment	Number of flasks stocked from antibiotic treatment flask	Average % survival (± SEM)
Low concentration flask (1000 eggs/ 1.5L)	23	[a]58 (± 5.48)
High density flask (>2000 eggs/ 1.5L)	18	6.22 (± 2.47)

[a]The survival of larvae that were hatched from eggs that originated from the low concentration flask was very highly significantly different to those that originated from the high density flasks (Mann-Whitney; $p<0.001$).

Figure 21 Average survival to 34 days post-hatch (approximately 200 day-degrees) of bacterially-infected flask-reared yolk-sac larvae in Experiment 5. All flasks were stocked with twenty five 70 day-degree eggs that had been treated with a combination of glutaraldehyde surface disinfection, antibiotic treatment (low stocking density antibiotic-treatment) and rinsing in 1:250 Kick Start and sterile seawater. Flasks were inoculated, 5 days after the flasks had been stocked (approximately 18 day-degrees post-hatch), with 5×10^2 $CFUml^{-1}$ of a range of bacteria isolated from both the survey and the yolk-sac rearing trial. Individual survivals for all the treatment flasks are shown, the lines indicate the treatment averages. No significant differences in survival between the different treatments were uncovered.



[a]No culturable bacteria were detected in any of the control flasks at the end of the experiment, as assessed by plating 100 µl of water onto MA plates and 5ml of water into 10ml MB. The plate and broth cultures were inspected for growth after 30 days incubation at 10 °C.

4.3.4.6. Experiments 6 and 7 Effect of different bacterial isolates on yolk-sac larval survival in optimised 2L flask-rearing system.

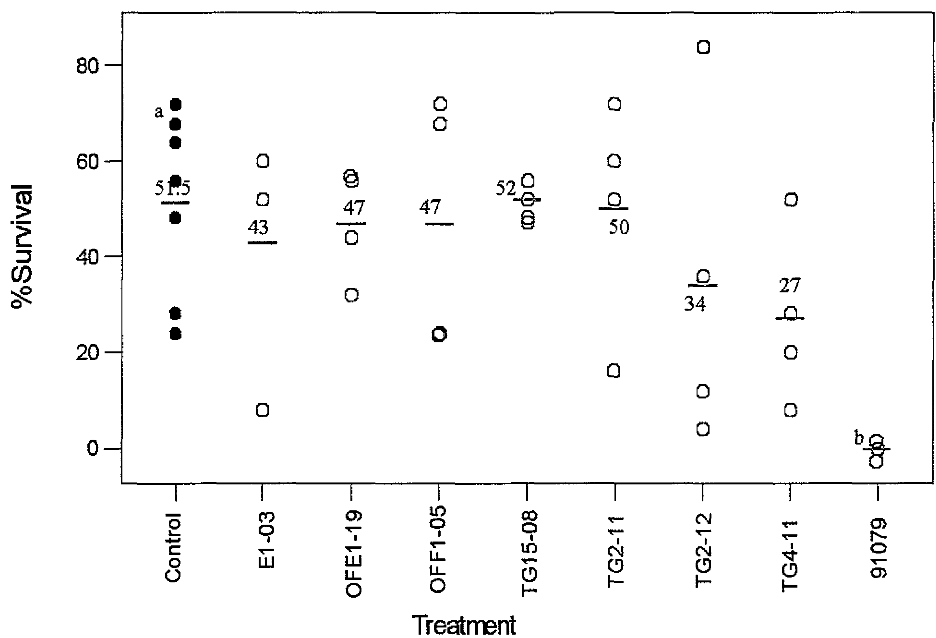
Having established the best apparent larval rearing conditions in the previous experiments, two further trials were run to investigate the effects of seventeen bacterial isolates on larval halibut survival in the absence or limited presence of other bacteria (Figures 22 and 23).

High numbers of bacteria were found in all the control flasks at the end of Experiment 6 (Figure 22 and Appendix 4). Thus it can be seen that there were problems in this experiment indicating a high apparent rate of flask contamination independently of whether the flasks had been infected (Figure 22). This was surprising, as 43% of flasks appeared to have less than 1 culturable bacterium per 5 ml prior to inoculation (as assessed by inoculating MB prior to inoculation). As flasks were randomly assigned to the different treatments, it would appear an unfortunate coincidence that all the controls were contaminated. At least four different contaminating bacteria appeared to be present in the control flasks, and presumably in the other flasks as well, as assessed by a limited range of tests on the isolates (colony morphology, ability to grow on TCBS and sensitivity to a range of antibiotics).

It is clear that *V. anguillarum* 91079 is highly pathogenic to halibut yolk-sac larvae at the low infection level used in this experiment (5×10^2 ml^{-1}). No larvae survived in any of the flasks infected with *V. anguillarum*, whether other contaminating bacteria were present or not; the difference in survival in *V. anguillarum* infected flasks was significant, compared to the non-infected controls (Mann-Whitney; $p< 0.05$). Levels of *V. anguillarum* free-living in the flask water were apparently less than the levels of other contaminating organisms in three of the flasks. In one of the flasks no *V. anguillarum* could be detected at all. Living yolk-sac larvae were present in all of the *V. anguillarum* infected flasks 29 days after they hatched (approximately 150 day degrees post infection) so it took a considerable period of time for all of the larvae to be killed.

None of the other isolates tested appeared to be particularly pathogenic, including a luminescent *Photo. phosphoreum* isolate E1-03, originally isolated from halibut eggs or a haemolytic *V. splendidus* strain, AE1-26, or a *V. viscosus* strain, 236.

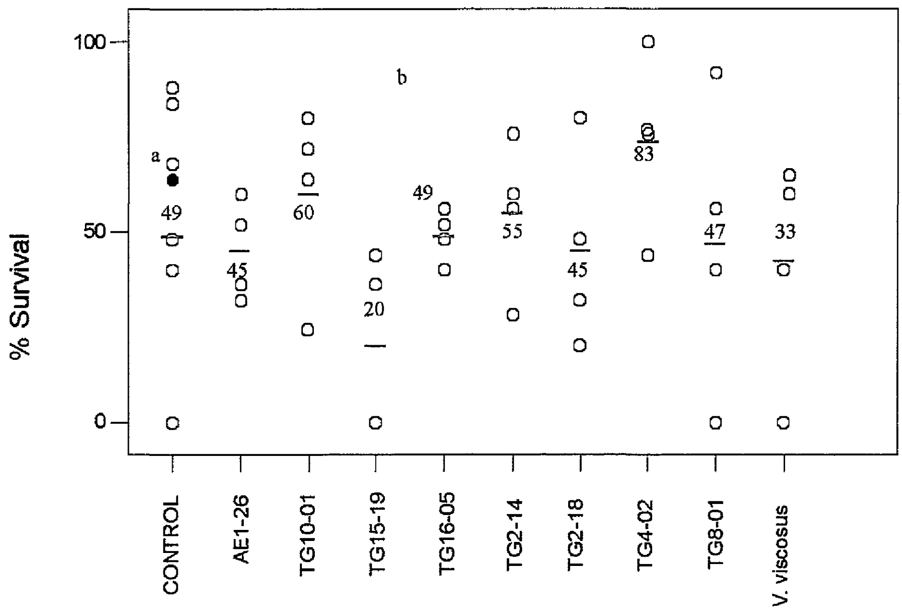
Figure 22 Average survival to 34 days post-hatch (approximately 220 day-degrees) of bacterially-infected flask-reared yolk-sac larvae in Experiment 6. All flasks were stocked with twenty five 70 day-degree eggs that had been treated with a combination of glutaraldehyde surface disinfection, antibiotic treatment (low stocking density antibiotic-treatment) and rinsing in 1:250 Kick Start and sterile seawater. Flasks were inoculated, 4 -7 days after the flasks had been stocked (approximately 18- 30 day-degrees post-hatch), with 5 x 10^2 $CFUml^{-1}$ of a range of bacteria isolated from both the survey and the yolk-sac rearing trial. Also included was the turbot pathogen, *V. anguillarum* 91079. Individual survivals for all the treatment flasks are shown, the lines indicate the treatment averages.



[a]Culturable bacteria (> 10^5 CFU ml^{-1}) were detected in all of the control flasks at the end of the experiment, as assessed by plating 100 µl of water onto MA plates and 5ml of water into 10ml MB.

[b]Survival of larvae in flasks inoculated with *V. anguillarum* 91079 was significantly lower than in the controls (Mann-Whitney; $p<0.05$)

Figure 23 Average survival to 34 days post-hatch (approximately 220 day-degrees) of bacterially-infected flask-reared yolk-sac larvae in Experiment 7. All flasks were stocked with twenty five 70 day-degree eggs that had been treated with a combination of glutaraldehyde surface-disinfection, antibiotic treatment (low stocking density antibiotic-treatment) and rinsing in 1:250 Kick Start and sterile seawater. Flasks were inoculated, 4 -7 days after the flasks had been stocked (approximately 18- 30 day-degrees post-hatch), with 5 x 10^2 $CFUml^{-1}$ of a range of bacteria isolated from both the survey and the yolk-sac rearing trial. Also included was the salmon pathogen, *V. viscosus* 236. Individual survivals for all the treatment flasks are shown, the lines indicate the treatment averages.



[a]Culturable bacteria (> 10^5 CFU ml^{-1}) were detected in only one of the control flasks at the end of the experiment, as assessed by plating 100 µl of water onto MA plates and 5ml of water into 10ml MB.

[b]No significant differences in survival between the different treatments were uncovered.

4.4. Discussion

Although the original aim of the work described in this chapter was to analyse the effects of individual bacterial isolates on halibut yolk-sac larval survival under controlled conditions, a number of other interesting observations were made during the development of an appropriate challenge model.

Firstly, it appears that water quality is a critical parameter affecting larval survival in small-scale static systems. Poor survivals were found when seawater from a non-optimal source (the GU aquarium) or artificial seawater was used. Acceptable survivals to the end of the yolk-sac absorption period (> 50%) were only found when matured seawater, originally collected from the West Coast of Scotland, was used.

The reasons for this are not clear, as water quality analysis was not done on the different water samples. It is quite possible that the GU aquarium water was contaminated with dissolved organic and or non-organic (e.g. metals) material. However, the reasons for the poor performance of larvae reared in the artificial seawater are not so clear. All the water used was made up using freshly-prepared Divisional dH_20 and Instant Ocean, which is routinely used in seawater aquaria to raise a variety of species. pH and salinity was adjusted to be similar to the optimal seawater treatments. It is possible that a trace organic contaminant came across when the water was distilled. A future experiment may wish to examine the effect of using artificial seawaters prepared with different sources of distilled water.

Stocking density was also found to be important during the initial antibiotic-treatment of the eggs. Survival of larvae hatched from eggs that originated from a flask containing a high density of eggs was significantly lower that those that originated from a low density flask.

The use of artificial, rather than matured natural seawater from a pristine source probably explains why survivals in the multiwell dish system were considerably worse than those reported by Norwegian workers (Bergh *et al.* 1992).

In general, halibut eggs were resistant to an aggressive disinfection protocol and, with the exception of Experiment 6, high apparent rates of sterility could be obtained at the end of the yolk-sac absorption phase. If the eggs were infected intra-ovularly, this did not appear to be at a high prevalence, in contrast to an earlier report

(Hansen and Olafsen, 1999). Future work should investigate whether there are unculturable bacteria present in control flasks. One such method would be direct visualisation of water samples, following treatment with nucleic acid stains, such as 4', 6-diamidino-2-phenylindole (DAPI) (Hoff, 1988).

The large size and resistance of their eggs to a high degree of mechanical and chemical handling stress makes halibut a good model for analysing the effects of bacteria on larval development and survival under microbially -controlled conditions. It is also possible that studies investigating other parameters, such as water quality, temperature and salinity, may wish to utilise the model as it removes, or controls, the micobiological variable.

There was a high rate of deformities in all the treatments, whether bacteria were apparently present or not. It has been speculated that the lock jaw deformity, commonly observed in halibut hatcheries, may be caused by bacterial infection (Morrison and Macdonald, 1995). Larvae presenting this condition were found in apparently sterile flasks, making it unlikely that there was a bacterial cause. Also, another condition where larvae have opaque patches in the intestinal cavity at the end of their development was also observed in control flasks. These observations reinforce the value of such model systems in helping to isolate the cause of specific conditions.

In general, as was found by Munro *et al* (1995) when they challenged turbot larvae with a range of bacterial isolates under monoxenic conditions, none of the bacteria tested appeared to be very pathogenic, with the exception of *V. anguillarum* 91079. As can be seen in Appendix 4, most of the bacteria introduced into flasks at a low initial inoculum were present at levels in excess of 10^5 CFU ml^{-1} by the end of the experiments. Thus, for the most part, yolk-sac larvae appeared to be unaffected by high densities of culturable bacteria which were originally isolated from different rearing systems. In the previous chapter it was noted that there were apparent correlations between the survival of yolk-sac larvae and the presence or absence of particular bacterial isolates. However, none of these appeared to be very pathogenic when the larvae were exposed to them in the model rearing system, when compared to *V. anguillarum*-infected flasks. Although it is interesting to note that levels of survival in the TG4-11 treated larvae were lower than in any other of the tested

groups (apart from the *V. anguillarum*-infected larvae). TG4-11 was originally isolated from poorly performing larvae reared under flow to waste in the yolk-sac rearing trial. However, the difference is not quite significant and in one of the flasks survival was high (13/25).

Also, in Experiment 5, average survival in the control flasks was higher than in any of the other treatments (Figure 21, average 84 % survival). Survival in the SYS6-10 treatment, an example of a *V. splendidus* group 3 organism (Chpater 2), was markedly lower (41%), this difference was significant when individually comapred with the controls (Mann-Whitney; $p<0.05$). However, there were only three control flasks, and when group differences were explored by both parametric (ANOVA) and non-parametric (Kruskal-Wallis) means, there were apparently no significant group effects. It may be worth repeating the experiments involving SYS6-10 and TG4-11 to see if lower survivals, relative to controls, are consistently found.

It is possible that the larvae may have been compromised by some of the tested bacteria, making them less competent at feeding and this should be tested by attempting to rear larvae on further than was attempted in this study in future experiments.

It is also possible that some of the isolates may not be pathogenic at the low rearing temperatures (< 6 ^{O}C). AE1-26 and OFF1-05 were isolated from first feeding larvae reared at temperatures in excess of 10 ^{O}C. In two of the four flasks inoculated with the haemolytic *V. splendidus* type 1 organism AE1-26 it could not be recovered at the end of the experiment, possibly indicating that the environmental conditions were not optimal for this strain (Appendix 4). This organism was originally recovered from an Ardtoe enriched-*Artemia* culture. *Artemia* are cultured in excess of 20 ^{O}C, which may represent the optimum thermal range for this particular organism.

It was not surprising that *Vibrio anguillarum* was pathogenic to halibut yolk-sac larvae. As discussed in Chapter 1 (section 1.7.1.1), previous studies have shown that halibut yolk sac larvae fry and juveniles are susceptible to this pathogen (Bergh *et al.*, 1992; Bricknell *et al.*, 2000). There were two main differences between the study by Bergh and this one. Firstly, different challenge strains were used (a turbot pathgogen, 91079 as opposed to NCIMB 6, which was originally isolated from cod).

Secondly, the inoculum was at a much lower level (5×10^2 two days prior to hatch as opposed to $2 - 3 \times 10^6$ organisms ml^{-1} 4 days before the eggs hatched). It was interesting that, although the larvae had presumably been invaded and killed by *V. anguillarum*, it was not readily isolated from the different flask water at the termination at the termination of this and other, unreported, experiments.

Munro *et al.* (1995) showed that *V. anguillarum* was present at very low levels in inoculated flasks containing turbot larvae until periods of high mortality, which presumably coincide with infected larvae shedding the pathogen into the water. These results together reinforce speculation that certain *V. anguillarum* serotypes are specialised invasive pathogens that are not well suited to a free-living aquatic existence. It was interesting to note that larval mortalities were not noticeable until later on during development (after 150 day-degrees). This could be either be a temperature-related effect, with the pathogen multiplying slowly inside the larvae at the low rearing temperature ($6^{O}C$) used in this study. Alternatively, the larvae may be resistant to invasion early on during their development, as their maternally-derived host defences are exhausted they may then become more susceptible.

It should be noted that it has been proposed that the gut is the route of invasion of body tissues by *V. anguillarum* in larval turbot (Grisez *et al.* 1996). Recent work (unpublished observations) indicate that it is difficult to establish a *V. anguillarum* infection if very low levels (<2 CFU ml^{-1}) are added to flasks containing halibut yolk-sac larvae. If the route of entry is the gut, it is possible that below a certain concentration threshold larvae are unlikely to ingest *V. anguillarum* through drinking. In the present experiment the larvae were not feeding and it would be of interest to carry out histology to determine the sequence of infection of *V. anguillarum* in the halibut larva.

Problems were encountered in Experiment 6 where a number of the experimental flasks were contaminated with organisms other than the bacteria being tested. Two of these contaminants could easily be distinguished from the test isolates because they were orange pigmented morphotypes with a very different antibiotic resistance profile to any of the tested organisms (Appendix 4). The other three organisms were much harder to differentiate because one was a TCBS culturable

organism with a similar antibiotic resistance profile to E1-03. However, E1-03 is a luminous *P. phosphoreum* isolate. So, provided the culture was fresh and it had not lost its bioluminescent properties since it was inoculated into the flask, it should have been readily distinguishable.

Pseudoalteromonas-like isolates present in the controls were very similar to certain of the test isolates in terms of colony morphology and antibiotic resistance profile (OFE1-19, TG2-11 and TG2-12). This made it hard to ascertain whether these test organisms were present in the treatment flasks at the end of the experimental period, particularly if they had lost or exchanged antibiotic resistance determinants.

It is not possible to say why there was such a wide range in survivals in the individual flasks, irrespective of treatment. This was particularly noticeable in Experiment 7, where survivals in the control flasks varied between 0 and 88% (Figure 23). Culturable bacteria were only isolated from one of these flasks so a bacterial etiology is unlikely, although it is possible the poor survivals were caused by an unculturable bacterium. There are various other possible causes; as has been shown, yolk-sac larvae appear to be very susceptible to poor water water quality. It is possible that some of the flasks may have contained trace amounts of some toxic substance. Future experimrents should consider steeping flasks in distilled water following normal cleaning and rinsing to remove traces of posible toxic compounds.

It is also possible that the mortalities were caused by a viral agent that was present in some of the eggs, despite the aggressive surface-disinfection procedure. In future experiments, electronmicroscropy later may reveal the presence of viral particles in moribund larvae taken from affected flasks. It was not possible to do this in these experiments because by the time the experiment was terminated the dead larvae had autolysed.

5. Chapter 5 Screening bacterial isolates for inhibitory properties *in vitro*

5.1. Introduction

A classic approach in the identification of potential probiotic candidates is to screen them *in vitro* for the production of bacteriocins. The hypothesis is that such bacteria may produce substances that will inhibit the growth of potentially pathogenic bacteria *in vivo* as well as *in vitro*. By so doing, they may confer some protection on the host animal when administered as probiotics. Obviously it is also important that these bacteria are harmless to the host animal.

5.2. Materials and methods

A range of isolates was screened for their ability to produce bacteriocins by a standard cross-streaking approach. All the isolates were tested against each other, by streaking onto a MA plate and then, at right angles to the streak, the organism under test was also streaked. If growth of one organism was inhibited by another there was a zone of inhibition. 35 BIOLOG cluster representatives from the initial characterisation (first feeding, weaning and gut bacteria, plus *Artemia* isolates) and 36 isolates from the yolk-sac rearing trial were streaked against each other.

The following isolates from the survey (Chapter 2) were tested for inhibitory activity *in vitro*. These included: representatives of *V. splendidus* group 1 (SFF4-01, SFF4-03, SFF4-06, SFF4-12, SFF4-14, SFF4-17, SFF4-19), *V. splendidus* group 2 (SFF1-19, AE1-31), *Photobacterium phosphoreum* (SFF3-02, E1-02, S1-01, S1-04), *Pseudoalteromonas* group Vb (SFF1-01, SFF1-14, SF1-20), *V. alginolyticus* (AE1-24, SFF6-02, SFF6-03, SFF6-09, SFF6-19), *V. salmonicida*-like(W1-08) and a *Pseudomonas veronii*-like isolate (W1-03).

A number of isolates from the rearing trial (Chapter 3) were also tested (Table 27).

5.3. Results

Of the 68 isolates tested, only one, TG15-07, a *Pseudoalteromonas* species, showed inhibitory activity *in vitro* (Table 27). This isolate was obtained from one of the Ardtoe recirculation, disinfected egg treatments. Survival for this group was good, so this organism appeared to be a potential probiotic candidate.

5.4. Discussion

Using the cross-streaking method for assessing the ability of isolates, originally isolated from the guts of larval and adult halibut, to inhibit the growth of other bacteria *in vitro*, only one organism, TG15-07, inhibited the growth of other gut bacterial isolates. TG15-07 is a *Pseudoalteromonas* species and, as discussed in Chapter 4, many *Pseudoalteromonas* species produce bioactive compounds *in vitro* and *in vivo* (Holmstrom and Kjelleberg, 1999).

It is presently unclear by what mechanism TG15-07 inhibited the growth of the other test organisms. Other work, (not reported), shows that this inhibition also takes place when other screening protocols are used than the simple cross-streaking approach employed in this study. Indeed, zones of inhibition against *V. anguillarum* 91079 were very large; indicating that the extracellular inhibitory-factor(s) produced by TG15-07 are potentially highly potent.

Although TG15-07 was originaly isolated from healthy halibut larvae reared under recirculation conditions, it is not clear whether the larvae were protected by the presence of this organism. Other organisms were also recovered in high density from the same larval gut sample as TG15-07, showing that other organisms may be able to grow in its presence *in vivo*, if not *in vitro*. Other closely related *Pseudoalteromonas* isolates were also recovered in high numbers from larvae reared under recirculation conditions but none of those tested showed the levels of inhibitory activity seen in TG15-07.

It was surprising that none of the other bacteria tested appeared to inhibit the growth of other organisms *in vitro*. Bergh (1995) showed that a high proportion of the organisms he tested from first feeding halibut larvae reared on wild collected copepods inhibited the growth of pathogenic *Vibrio* species *in vitro*. None of the *V.*

salmonicida, Photobacterium phosphoreum or *V. fisheri* like organisms tested showed inhibitory properties using the cross-streaking assay on MA.

Table 27. Inhibition test matrix for bacterial isolates from the rearing trial (Chapter 4). All isolates were cross-streaked against each other on Marine Agar. + indicates that the isolate on the horizontal was inhibited by the isolate in the vertical column. ∔ indicates weak inhibition. An empty space indicates the test was not performed. [a]TG15-07 also inhibited growth of *V. anguillarum* 91079.

	TG1-02	TG15-20	TG4-19	TG10-06	TG4-02	TG10-01	TG10-11	TG12-20	TG4-08	TG3-15	TG15-08	[a]TG15-07	TG13-14	TG12-14	TG6-02	TG4-19	TG6-09	TG13-10	TG15-01	TG15-05	TG15-19	TG4-11	TG4-03	TG2-14	TG2-13	TG7-01	TG7-11	TG7-05	TG16-05	TG2-11	TG2-18
TG1-02	x	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG15-20	-	x	-	-	-	-	-	-	-	-	-	∔	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG4-19	-	-	x	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG10-06	-	-	-	x	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG4-02	-	-	-	-	x								-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG10-01	-	-	-	-	-	x	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG10-11	-	-	-	-	-	-	x	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG12-20	-	-	-	-	-	-	-	x	-	-	-	∔	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	∔
TG4-08	-	-	-	-	-	-	-	-	x	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG3-15	-	-	-	-	-	-	-	-	-	x	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG15-08	-	-	-	-	-	-	-	-	-	-	x	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG15-07	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG13-14	-	-	-	-	-	-	-	-	-	-	-	+	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG12-14	-	-	-	-	-	-	-	-	-	-	-	+	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG6-02	-	-	-	-	-	-	-	-	-	-	-	+	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TG4-19	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
TG6-09	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-	+
TG13-10	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-
TG15-01	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
TG15-05	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-
TG15-19	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-
TG4-11	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-
TG4-03	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-
TG2-14	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-
TG2-13	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-
TG7-01	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-
TG7-11	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-
TG7-05	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-
TG16-05	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-
TG2-11	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-
TG2-18	-	-	-	-	-	-	-	-	-	-	-	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x

6. General Discussion

The main aim of this thesis was to investigate the influences of bacteria on larval halibut health and development. Accordingly, a survey of the types of bacteria present in UK hatcheries was undertaken and trials were run whereby the conditions that larvae were reared under microbially were varied. In addition, a series of experiments were performed where selected bacterial isolates were added to larvae reared in small-scale static systems.

One obvious conclusion to be drawn, particularly from the infection experiments, is that many of the bacteria present at high quantities in association with larval halibut of different developmental stages do not appear to be obviously pathogenic. High survivals, relative to uninfected controls reared in the absence of culturable bacteria, were found when yolk sac larave were reared in the presence of high concentrations (> 10^6 CFU ml^{-1}) of bacteria isolated from different halibut rearing systems.

Bacteria appear to have a role in larval performance, however, as was seen by the improval in yolk sac survivals seen when antibiotics were added to the incubators. Also, they were shown to be highly susceptible to a turbot pathogen, *V. anguillarum* 91079.

It is not possible to say that the generally good performance of larvae reared under recirculated water conditions was influenced by bacteria. However, it was noteworthy that there was an apparent stabilisation in terms of the types of bacteria isolated from larvae reared under these conditions.

The types of bacteria isolated from larval halibut before they started to feed were heterogenous, although *Pseudoalteromonas* species were common components. In the case of intensively-reared halibut fed enriched *Artemia*, the primary source of the gut microflora was the live feed. There was some evidence that only a certain fraction of the bacteria associated with the enriched *Artemia* were able to colonise the guts of first-feeding halibut, in particular *V. splendidus* and *V. alginolyticus* organisms. Later on during their development larvae appeared to be colonised by

other less metabolically active *Vibrio* species, such as *V. salmonicida* and *V. fischeri*-type bacteria. These did not appear to originate from the live feed.

6.1. Future work

As mentioned at the end of Chapter 2, it is possible that unculturable bacteria may have a role to play in larval halibut performance. It would be interesting to investigate this further by the application of appropriate techniques, such as DGGE.

It also remains to see how the presence or absence of bacteria affects first-feeding performance. Work is presently underway to grow on yolk-sac larvae which have been reared under bacteria-free conditions. If a suitable model can be developed whereby larvae are fed bacteria-free *Artemia* in the presence of axenic algae, as has been done with larval turbot (Munro *et al.* 1995) selected bacterial species can then be added to see how they affect first-feeding performance. Although the isolates tested using the model system described in Chapter 4 did not appear to be pathogenic, with the exception of *V. anguillarum* 91079, it is possible that they may be harmful at the higher temperatures currently employed during first feeding protocols. If this is the case, suitable ways of controlling microflora development in *Artemia* rearing systems should then be investigated. As mentioned previously, it is evident that only certain *Vibrio* species associated with the live feed appear to establish themselves in larval halibut guts. If these species are harmful it may be possible to establish rearing protocols that exclude such organisms.

Another potentially useful line of research could be testing potential probiotic candidates, such as TG15-07. Initially this would probably be best done by using model systems. If an isolate, or group of strains, shows promise, large-scale rearing trials can then be attempted.

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Appendix 1

Identification Tests

Kovac's Oxidase
For making 10ml

0.1g Tetramethyl -p- phenyldiamine dihydrochloride (0.1%)

0.01g Ascorbic Acid (0.01%)

The solution was made up to 10ml in dH_20 and use within 12 h of manufacture. It was possible to prepare a number of universals with the required amounts of dry reagents in advance. 10mls of dH_20 was then added to a universal when required. Universals should be stored in the dark.

Test

A piece of filter paper was moistened with dH_20 and inoculated with a fresh growth of the organism to be tested from a MA plate using a glass or wood applicator. Nichrome wire should not be used as there is a possible risk of false positives. A drop of the oxidase reagent was then added. A deep purple colouration almost immediately indicated a positive reaction. Positive (eg *V. anguillarum*) and negative (eg *E. coli*) controls were included.

Growth on TCBS

TCBS (Oxoid) agar plates were prepared according to the manufacturer's instructions. Fresh MA cultures of the test isolates were spotted onto the prepared plates and incubated for upwards of two weeks at $10^{O}C$ or $20^{O}C$. Growth after this time was scored as a positive character. It was also noted whether the isolate was able to utilise

sucrose (indicated by an indicator dye change from green to yellow in the area surrounding the colony).

Full Characterisation

General Recipes

Electrolyte supplement

NaCl	10g
MgCl2.6H20	4g
KCl	4g
$dH_2 0$	to 100ml

The solution was distributed in 5 ml bottles and sterilised by autoclaving. The supplement was added to the appropriate medium in the proportions of 0.1ml supplement to 1ml medium

Acid from carbohydrates

Phenol Red Broth:

Peptone water (Oxoid)	10gm
NaCl	10gm
Phenol Red	0.018gm
Distilled Water	1 litre

The pH was adjusted to 7.1 and the medium autoclaved at 121 ^{0}C for 15 minutes. Filter-sterilised carbohydrate solution was added aseptically to a final concentration of 1% for arbutin and sucrose, or 0.5% for salicin. The medium was aseptically distributed in 1ml amounts into sterile tubes, inoculated and incubated at 20^{0}C for up to 14 days. Acid production was indicated by a yellow colour.

Aesculin Hydrolysis (Lee and Donovan, 1985)

Aesculin agar:

Tryptone (Oxoid)	10gm
Aesculin	1gm
Ferric citrate	0.5gm
NaCl	10gm
Technical agar No. 3 (Oxoid)	15gm
Distilled water	

The agar, tryptone and NaCl were dissolved by heating, then the aesculin and ferric citrate were added. The medium was autoclaved at 115 ^{0}C for 10 minutes and poured into petri dishes. Plates were spot inoculated with no more than 12 isolates per plate and incubated at 20 ^{0}C for 5 days. Blackening indicated aesculin hydrolysis.

Oxidation/Fermentation

Hugh and Leifson (1953) medium with 1.5% NaCl was prepared in 5 ml amounts and sterilised by autoclaving at 121 ^{O}C for 15 min. To this medium 10% filtered sterilised D-glucose solution was added. The tubes were prepared in duplicate, one set with liquid paraffin and the other without. The tubes were inoculated and incubated at 20°C for 48h.

Decarboxylase tests (Furniss *et al.* 1979)

Decarboxylase medium (Difco) was prepared with the addition of 1% NaCl, 0.4% $MgCl_2.H_20$ and 0-.4% KCl. For convenience, the medium was dispensed into 4 x 100 ml volumes and the amino acids L-lysine, L-arginine and L-ornithine added to a final concentration of 1%. The three amino acid media and the basal medium were distributed into 5ml screw-capped bottles and covered with a layer of liquid paraffin before autoclaving at 121 ^{0}C for 15 min.

The four bottles were inoculated through the liquid paraffin, with a growth from MA, and incubated at 20^0C for 4 days. Vibrios should show acid (yellow colour) in the blank. A positive result was indicated by an alkaline reaction (purple).

Voges – Proskauer (Furniss *et al.*, 1979)

Reagents

Yeast Extract	1g
Bacteriological peptone	12g
Glucose	10g
NaCl	10g
Agar	3g
dH_20	900 ml
E supplement - see general recipes	100 ml

Adjust pH to 7.0 and distribute in 3ml amounts in tubes. Stab inoculate and incubate for 24 h. 0.2 ml solution A and 0.1 ml solution B were then added.

Solution A: 5% α-naphthol in absolute alcohol.
Store in the dark

Solution B:	KOH	40g
	Creatine	0.3g
	dH_20 to	100ml

Solutions A and B have a limited shelf life and should be stored at 4 ^{O}C. A red ring indicates a positive result. Incubation for 15 minutes may be necessary.

Indole Production (Smibert and Krieg, 1994)

Kovac Reagent:

150 ml isoamyl alcohol

10g 4-dimethyl amino benzaldehyde

50 ml cc HCl

Slowly dissolve aldehyde in alcohol at 37°C (place in water bath) then add HCl. Gives yellow soln. A few drops of the agent are added to an incubated peptone culture after 48 hours. A positive result is indicated by a red ring on the surface.

Nitrate Reduction

KNO_3 0.1%

NaCl 1%

Nutrient Broth No. 2

E salts supplement

dH_20

2ml aliquots were distributed into culture tubes. Tubes were then inoculated and incubated for four days at 20 °C. 1ml of reagents A and B were then added.

Reagent A:	Sulphuric acid	0.5 g (0.51 ml 98% H_2SO_4)
	Glacial acetic acid	30 ml
	dH_20	120 ml
Reagent B:	Cleeve's acid	0.2g Inaphthyl 4 amino- 7 sulphonic acid.
	Glacial acetic acid	30 ml
	dH_20	120 ml

Add H_20 to Cleeves acid and warm to dissolve. Filter, cool then add glacial acetic acid.

A red colour indicated reduction of nitrate to nitrite. If the result was negative, zinc powder was added. A purple colour change is negative for nitrate reduction (zinc reduces nitrate). If the solution stayed colourless, this was indicative of production of N_2 gas. ($NO_3 > NO_2 > N_2$ complete reduction).

Luminescence

The culture organism inoculated onto a marine agar plate. After 24 (20 °C) or 48 h (10 °C) incubation the plate was examined in the dark for luminescence, following time for eyes to become dark-adapted. Include positive control strain. Luminescence is most marked in young cultures and may be lost on further incubation. For slow growing strains a longer period of time may be required than indicated.

Haemolysis of sheep red blood cells *(Furniss et al., 1979)*

Brain Heart Thioglycolate Cysteine Agar:

Brain heart infusion broth (Oxoid)	500ml
10% sodium thioglycolate solution	2.5ml
10% L-cysteine in 10% HCl	2.5ml
Technical agar No. 3(Oxoid)	5g

The medium was autoclaved at 121°C for 15 minutes, cooled to 52°C and 20ml of a fresh saline suspension of washed sheep red blood cells was added. Plates were spot inoculated (not more than 12 isolates per plate) and incubated at 20°C for 24 hours. A positive result was indicated by a clear zone around the colony.

ONPG Test (Smibert and Krieg, 1994)

ONPG Solution:

ONPG (Koch-Light Laboratories Ltd) 6gm
0.01 M Na_2HPO_4 1 Litre

ONPG (O-Nitrophenyl-β-D-Galactopyranoside) was dissolved in the phosphate solution at pH 7.5 at room temperature, sterilised by filtration and stored in the dark. ONPG solution (250ml) was added to 750 ml sterile peptone water (Oxoid) supplemented with 1% NaCl and distributed into 1ml amounts. The inoculated medium was incubated at 20^0C for 24 hours. A positive result, i.e. β-galactosidase activity, was indicated by a deep yellow colour.

Appendix 2a Biochemical, physiological and antibiotic susceptibility test results for isolates classified as *Flexibacter ovolyticus*-like, *Cytophaga/Flexibacter* and *Pseudomonas veronii*-like. Each test was scored on a scale of between 0 –10 for a strongly positive reaction. Isolates are coded as in Table 1.

	Flexibacter ovolyticus isolates					*Cytophaga / Flexibacter* isolates										*Pseudomonas veronii* isolates							
Character	F.ov-like A	F.ov-like B	O2-06	O-11	**Group average**	O2-09	SYS6-20	SYS4-16	E1-09	YSW4-06	YSW4-08	YSW4-11	SYS5-15	YSW5-18	**Group average**	W1-03	W1-04	W2-17	AH-01	AC1-19	W1-05	AC1-26	**Group average**
PCR-RFLP pattern	IA		IA	IA		IB	II								**1B/ II**	III	III	III		III		III	**III**
Biolog[a]	G	G	[c]		**G**		NG	NG	NG	NG	NG	NG		NG	**NG**		G	G		G	G		**G**
Gram stain	0	0			**0**		0	0	0			0		0	**0**	0	0	0	0	0	0		**0.0**
Motility	10	10			**10**		10	10	10			10		10	**10**	10	10	5	10				**9.0**
Pigment[b]	Y	Y			**Y**		Y	Y	Y	Y	Y	Y	Y	Y	**Y**	0	0	0	0				**0**
Morphology	10	10			**10**		10	10	10	10	10	10		10	**Y**	10	10	10	10				**10.0**
Oxidase	5	5			**5**		10	5	0	0	0	0		0	**0**		10		10				**10.0**
TCBS	0	0			**0**		0	0	0	0	0	0		0	**0**	5	10		0				**5.0**
TCBS - sucrose +ve	0	0			**0**		0	0	0	0	0	0		0	**0**	0	0		0				**0.0**
Oxidation	0	0			**0**		0								**0**	10	10						**10.0**
Fermentation	10				**10**		0									10	5						**7.5**
Swarming	0				**0**		0	0	0	0	0	0		0	**0**	0	0				0		**0.0**
Luminescence	0				**0**		0	0	0	0	0	0		0	**0**	0	0				0		**0.0**
Growth 4 °C	10				**10**		10	10	10	10	10	10		10	**10**	10	10						**10.0**
Growth 20 °C	10				**10**		10	10				10		10	**10**	10	10				10		**10.0**
Growth 37 °C	0				**0**		0								**0**	5	0						**2.5**

[a] G more than 3 positive reactions in BIOLOG GN microplate; NG not enough positive reactions for identification using BIOLOG
[b] Pigment production. Y, yellow; B, black; Br, brown.
[c] Where no data is shown tests were not done.

Appendix 2a (continued)

Character	*Flexibacter ovolyticus* isolates					*Cytophaga / Flexibacter* isolates										*Pseudomonas veronii* isolates							
	F.ov-like A	F.ov-like B	O2-06	O-11	**Group average**	O2-09	SYS6-20	SYS4-16	E1-09	YSW4-06	YSW4-08	YSW4-11	SYS5-15	YSW5-18	**Group average**	W1-03	W1-04	W2-17	AH-01	AC1-19	W1-05	AC1-26	**Group average**
Growth on CLED medium	0				**0**		0								**0**	5	10						**7.5**
Arginine decarboxylase[a]	NG				**NG**		NG								**NG**	NG	NG						**NG**
Lysine decarboxylase[a]	NG				**NG**		NG								**NG**	NG	NG						**NG**
Ornithine decarboxylase[a]	NG				**NG**		NG								**NG**	NG	NG						**NG**
Nitrate reduction	10				**10**		0									N2	N2						
Indole	0				**0**		0									0	5						**2.5**
ONPG	0				**0**		0									0	0						**0**
Voges Proskauer	0				**0**		0									0	0						**0**
0/129 10 μg							0	0								0	0	5					**1.6**
0/129 150 μg							0	0								5		10					**7.5**
Agar digestion (agarase)							0	0								0	0						**0**
Aesculin hydrolysis							0										0						**0**
Haemolysis srbc							0									0	0						**0**
Acid arbutin	0						0									0	0						**0**
Acid salicin	0						0									0	10						**5**
Acid sucrose	0						0									5	5						**5**
Catalase	0						0									10	10						**10**
Utilisation of single carbon sources (BIOLOG GN):																							
Acetic acid	10	10			**10**							10	0		**5**	5	0	0	10	10	5		**5.0**
n-Acetyl-D-galactosamine	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
n-Acetyl-D-glucosamine	0	0			**0**							0	0		**0**	0	10	10	0	0	0		**3.3**
cis-Aconitic acid	0	0			**0**							0	10		**5**	10	0	0	0	0	0		**1.7**
Adonitol	0	0			**0**							0	0		**0**	0	0	0	5	0	0		**0.8**

[a] NG in decarboxylase tests indicates no acid reaction in blank tube.

Appendix 2a (continued)

Utilisation of single carbon sources (BIOLOG GN):	*Flexibacter ovolyticus* isolates					*Cytophaga / Flexibacter* isolates										*Pseudomonas veronii* isolates							
	F.ov-like A	F.ov-like B	O2-06	O-11	**Group average**	O2-09	SYS6-20	SYS4-16	E1-09	YSW4-06	YSW4-08	YSW4-11	SYS5-15	YSW5-18	**Group average**	W1-03	W1-04	W2-17	AH-01	AC1-19	W1-05	AC1-26	**Group average**
D-Alaninamide	5	5			**5**							0	0		**0**	0	0	0	10	0	10		**3.3**
Alanine	0	0			**0**							0	0		**0**	5	0	0	0	10	0		**2.5**
L-alanine	5	5			**5**							0	0		**0**	5	0	5	5	5	10		**5.0**
L-Alanyl glycine	10	10			**10**							0	0		**0**	5	0	5	5	0	10		**4.2**
2- Amino ethanol	0	0			**0**							0	0		**0**	0	0	0	5	0	0		**0.8**
γ-Amino butyric acid	0	0			**0**							0	0		**0**	10	0	0	0	0	0		**1.7**
D-L-arabinose	0	0			**0**							0	0		**0**	10	0	0	0	0	0		**1.7**
Arabitol	0	0			**0**							0	0		**0**	10	0	0	0	0	0		**1.7**
L-Asparagine	5	5			**5**							0	5		**2.5**	10	10	10	0	0	0		**5.0**
L-Aspartic acid	10	10			**10**							0	0		**0**	10	5	10	5	0	10		**6.7**
Bromo-succinic acid	0	0			**0**							0	0		**0**	5	0	0	0	0	0		**0.8**
2,3 Butanediol	0	0			**0**							0	0		**0**	5	0	0	0	0	0		**0.8**
D,L-carnitine	0	0			**0**							0	0		**0**	5	0	0	0	0	0		**0.8**
Cellobiose	0	0			**0**							0	0		**0**	0	0	10	10	0	0		**3.3**
Citric acid	0	0			**0**							0	0		**0**	10	0	0	0	0	0		**1.7**
α-Cyclodextrin	0	0			**0**							0	0		**0**	0	0	0	10	0	10		**3.3**
Dextrin	0	0			**0**							0	5		**2.5**	0	10	10	10	0	10		**6.7**
I- Erythritol	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
Formic acid	0	0			**0**							0	0		**0**	5	0	0	0	0	0		**0.8**
D-Fructose	0	0			**0**							0	0		**0**	10	10	10	10	0	0		**6.7**
L-Fucose	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
D- Galactonic acid lactone	0	0			**0**							0	0		**0**	5	0	0	5	0	0		**1.7**
D-Galactose	0	0			**0**							0	0		**0**	10	0	0	10	0	0		**3.3**
D-Galacturonic acid	0	0			**0**							0	0		**0**	10	0	0	0	0	0		**1.7**
Gentiobiose	0	0			**0**							0	0		**0**	0	0	10	10	0	0		**3.3**

Appendix 2a (continued)

Utilisation of single carbon sources (BIOLOG GN):	*Flexibacter ovolyticus* isolates					*Cytophaga / Flexibacter* isolates										*Pseudomonas veronii* isolates							
	F.ov-like A	F.ov-like B	O2-06	O-11	**Group average**	O2-09	SYS6-20	SYS4-16	E1-09	YSW4-06	YSW4-08	YSW4-11	SYS5-15	YSW5-18	**Group average**	W1-03	W1-04	W2-17	AH-01	AC1-19	W1-05	AC1-26	**Group average**
D-Gluconic acid	0	0			**0**							0	0		**0**	10	5	10	5	0	0		**5.0**
D-Glucosamic acid	0	0			**0**							0	0		**0**	5	0	0	0	0	0		**0.8**
α-D-Glucose	0	0			**0**							0	0		**0**	10	10	10	10	0	10		**8.3**
Glucose-1-phosphate	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
Glucose-6-phosphate	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
Glucuronamide	0	0			**0**							10	5		**7.5**	0	0	0	0	0	0		**0.0**
D-Glucuronic acid	0	0			**0**							0	0		**0**	5	0	0	10	0	0		**2.5**
L-Glutamic acid	10	10			**10**							0	0		**0**	10	10	10	10	0	10		**8.3**
Glycerol	0	0			**0**							0	0		**0**	10	10	10	10	0	0		**6.7**
D-L-α-Glycerol phosphate	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
Glycyl-L-aspartic acid	10	10			**10**							0	0		**0**	0	0	10	5	0	10		**4.2**
Glycyl-L-glutamic acid	10	10			**10**							0	0		**0**	0	5	5	10	0	10		**5.0**
Glycogen	0	0			**0**							0	0		**0**	0	0	5	10	0	10		**4.2**
L-histidine	10	10			**10**							0	0		**0**	0	0	0	0	10	0		**1.7**
α-Hydroxy butyric acid	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
β-Hydroxy butyric acid	0	0			**0**							0	0		**0**	10	0	0	10	10	0		**5.0**
γ-Hydroxy butyric acid	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
Hydroxy L-proline	0	0			**0**							0	0		**0**	0	0	0	0	0	5		**0.8**
p-Hydroxy phenylacetic acid	0	10			**5**							0	0		**0**	0	0	0	0	0	0		**0.0**
Inosine	0	0			**0**							0	0		**0**	10	10	10	0	0	0		**5.0**
meso-inositol	0	0			**0**							0	0		**0**	0	0	0	10	0	0		**1.7**
Itaconic acid	0	0			**0**							0	0		**0**	10	0	0	0	0	0		**1.7**
α-Ketobutyric acid	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
α-Ketoglutaric acid	0	0			**0**							0	0		**0**	10	0	0	0	5	0		**2.5**
α-Ketovaleric acid	0	0			**0**							5	0		**2.5**	0	0	0	0	0	5		**0.8**

Appendix 2a (continued)

	Flexibacter ovolyticus isolates					*Cytophaga / Flexibacter* isolates										*Pseudomonas veronii* isolates							
Utilisation of single carbon sources (BIOLOG GN):	F.ov-like A	F.ov-like B	O2-06	O-11	**Group average**	O2-09	SYS6-20	SYS4-16	E1-09	YSW4-06	YSW4-08	YSW4-11	SYS5-15	YSW5-18	**Group average**	W1-03	W1-04	W2-17	AH-01	AC1-19	W1-05	AC1-26	**Group average**
D,L-lactic acid	0	0			**0**							0	0		**0**	10	0	0	0	0	0		**1.7**
α-lactose	0	0			**0**							0	0		**0**	0	0	0	10	0	0		**1.7**
α-D-lactose-lactulose	0	0			**0**							0	0		**0**	0	0	0	10	0	0		**1.7**
L-Leucine	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
Malonic acid	0	0			**0**							0	0		**0**	5	0	0	5	0	0		**1.7**
Maltose	0	0			**0**							0	0		**0**	10	10	10	10	0	10		**8.3**
D-Mannitol	0	0			**0**							0	0		**0**	10	10	10	10	0	10		**8.3**
D-Mannose	0	0			**0**							0	0		**0**	0	0	10	0	0	10		**3.3**
D-Melibiose	0	0			**0**							0	0		**0**	0	0	0	10	0	0		**1.7**
Methyl pyruvate	0	0			**0**							0	0		**0**	5	0	0	0	10	0		**2.5**
β-methyl-d-glucoside	0	0			**0**							0	0		**0**	0	0	10	10	0	5		**4.2**
Mono-methyl succinate	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
L-Ornithine	10	10			**10**							0	5		**2.5**	5	0	0	0	0	10		**2.5**
L-Phenylalanine	0	0			**0**							0	10		**5**	0	0	0	0	0	0		**0.0**
Phenylethylamine	0	0			**0**							0	5		**2.5**	0	0	0	0	0	0		**0.0**
L-proline	5	5			**5**							0	5		**2.5**	5	0	5	0	10	10		**5.0**
Propionic acid	0	0			**0**							0	0		**0**	10	0	0	10	5	5		**5.0**
Psicose	0	0			**0**							0	0		**0**	0	0	0	5	0	0		**0.8**
Putrescine	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
L-Pyroglutamic acid	0	0			**0**							0	0		**0**	5	0	0	0	0	0		**0.8**
Quinic acid	0	0			**0**							0	0		**0**	10	0	0	5	0	0		**2.5**
D-Raffinose	0	0			**0**							0	0		**0**	0	0	0	5	0	0		**0.8**
L-Rhamnose	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
D-Saccharic acid	0	0			**0**							0	0		**0**	10	0	0	0	0	0		**1.7**
Sebacic acid	0	0			**0**							0	0		**0**	0	0	0	5	0	0		**0.8**

Appendix 2a (continued)

Utilisation of single carbon sources (BIOLOG GN):	*Flexibacter ovolyticus* isolates					*Cytophaga / Flexibacter* isolates										*Pseudomonas veronii* isolates							
	F.ov-like A	F.ov-like B	O2-06	O-11	**Group average**	O2-09	SYS6-20	SYS4-16	E1-09	YSW4-06	YSW4-08	YSW4-11	SYS5-15	YSW5-18	**Group average**	W1-03	W1-04	W2-17	AH-01	AC1-19	W1-05	AC1-26	**Group average**
D-Serine	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**
L-Serine	5	5			**5**							0	0		**0**	0	0	10	5	0	10		**4.2**
D-Sorbitol	0	0			**0**							0	0		**0**	10	0	0	5	0	0		**2.5**
Succinamic acid	0	0			**0**							0	0		**0**	5	0	0	0	0	0		**0.8**
Sucrose	0	0			**0**							0	0		**0**	10	10	0	10	0	0		**5.0**
Succinic acid	0	0			**0**							0	0		**0**	10	0	10	10	5	0		**5.8**
L-Threonine	0	0			**0**							0	0		**0**	0	0	5	0	0	5		**1.7**
Thymidine	0	0			**0**							0	0		**0**	0	10	10	5	0	0		**4.2**
D-Trehalose	0	0			**0**							0	0		**0**	0	0	10	10	0	0		**3.3**
Turanose	0	0			**0**							0	0		**0**	0	0	5	10	0	0		**2.5**
Tween 40	10	10			**10**							5	0		**3**	5	0	5	10	10	10		**6.7**
Tween 80	0	0			**0**							0	0		**0**	5	0	0	10	10	0		**4.2**
Uridine	0	0			**0**							0	0		**0**	5	10	10	10	0	5		**6.7**
Urocanic acid	0	0			**0**							0	0		**0**	5	0	0	0	0	0		**0.8**
Xylitol	0	0			**0**							0	0		**0**	0	0	0	0	0	0		**0.0**

Appendix 2a (continued) Antibiotic sensitivity and resistance of *Flexibacter ovolyticus-like, Cytophaga/Flexibacter* and *Pseudomonas veronii.*

	Flexibacter ovolyticus isolates					*Cytophaga / Flexibacter* isolates										*Pseudomonas veronii* isolates							
Antibiotic	F.ov-like A	F.ov-like B	O2-06	O-11	**Group average**	O2-09	SYS6-20	SYS4-16	E1-09	YSW4-06	YSW4-08	YSW4-11	SYS5-15	YSW5-18	**Group average**	W1-03	W1-04	W2-17	AH-01	AC1-19	W1-05	AC1-26	**Group average**
Chloramphenicol 25 µg							0	0	0			10	10	10	**5.0**	0	0	10	10				**3.3**
Erythromycin 5 µg							10	0	0			0	10	10	**5.0**	0	0	3	10				**2.1**
Fusidic acid 10 µg							10	10	10			10	5	10	**9.2**	0	0	3	0				**4**
Methicillin 10 µg							0	5	0			0	5	10	**3.3**	0	0	10	0				**1.7**
Novobiocin 5 µg							10	0	0			0	3	10	**3.3**	0	0	10	10				**3.3**
Penicillin G 1 unit							0	0	0			0	0	10	**1.7**	0	0	0	0				**0**
Streptomycin 10 µg							0	0	0			0	0	0	**0.0**	10	0	0	10				**3.3**
Tetracycline 25 µg							10	10	10			10	0	10	**8.3**	0	0	10	10				**3.3**
Tetracycline 100 µg							10	10	10			10	5	10	**9.2**	0	0	10	10				**3.3**
Ampicillin 10 µg							0	10	0			10	10	10	**6.7**	0	0	10	0				**1.7**
Ampicillin 25 µg							0	10	10			10	10	10	**8.3**	0	0	10	5				**2.5**
Cephalothin 5 µg							0		0			0	0	10	**1.7**	0	0	10	0				**1.7**
Colistin sulphate 25 µg							0		0			0	0	10	**1.7**	0	0	10	10				**3.3**
Gentamycin 10 µg							0	0	0			0	10	0	**1.7**	10	10	10	10				**6.7**
Sulphatriad 200 µg							10		10			0	0	10	**5.0**	0	0	0	0				**0.0**
Cotrimoxazole 25 µg							0		10			10	3	10	**5.0**	0	0	10	10				**3.3**
Nitrofurantoin 50µg							0	0	10			10	0	10	**5.0**	0	0	10	3				**2.1**
Ticarcillin 75 µg							0	10	0			10	3	10	**5.0**	0	0	10	0				**1.7**
Nalidixic acid 30 µg							10	3	0			10	10	10	**7.1**	0	0	10	10				**3.3**
Trimethoprim 2.5 µg							0	10	10			10	0	0	**5.0**	0	0	0	0				**0.0**
Sulphamethoxazole 50µg							0	3	5			5	0	0	**2.1**	0	0	0	3				**4.2**

10 = > 2mm zone of clearing outside of test disc, 5 = < 2mm zone of clearing, 2.5 = disruption of growth, 0 = resistant.

Appendix 2b Biochemical, physiological and antibiotic susceptibility test results for isolates classified as *Pseudoalteromonas* groups 1 and 2. Each test was scored on a scale of between 0 –10 for a strongly positive reaction. Isolates are coded as in Table 1.

	Pseudoalteromonas group1					*Pseudoalteromonas* group 2																
Isolate	AE1-19	AE1-01	AE1-16	AE1-03	**Group average**	SYS4-22	SYS4-24	SYS4-26	SYS4-27	SYS4-28	SYS4-29	SYS5-21	SYS5-22	SYS5-23	SYS5-30	SYS3-02*	TG8-02	TG8-01*	TG4-19	T15-08	TG4-08	Group average
PCR-RFLP pattern	Va	Va			**Va**		Vb	Vb	Vb	Vb	Vb	Vb	Vb	Vb	Vb	Vb		Vb	Vb	Vb	Vb	**Vb**
Biolog[a]	G	[c]	G	G	**G**	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	**G**
Gram stain	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0			0	0	0	**0**
Motility	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10			10	10	10	**10**
Pigment[b]	0	0	0	0	**0**	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	**B**
Morphology	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10			10	10	10	**10**
Oxidase	5	5	10	10	**5**	10	10	10	10	10	10	10	10	10	10	10			10	10	10	**10**
TCBS	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0			0	0	0	**0**
TCBS - sucrose +ve	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0			0	0	0	**0**
Oxidation of glucose	10	10			**10**											0			0	0	0	**0**
Fermentation of glucose	10	10			**10**											0			0	0	5	**0**
Swarming	0	0			**0**	0	0	0	0	0	0	0	0	0	0	0			0	0	0	**0**
Luminesence	0	0			**0**	0	0	0	0	0	0	0	0	0	0	0			0	0	0	**0**
Growth 4 °C	10	10			**10**		10	10	10	10		10	10	10	10	10			10	10	10	**10**
Growth 20 °C	10	10			**10**		10	10	10	10		10	10	10	10	10			10	10	10	**10**
Growth 37 °C	0	0			**0**											0			0	0	0	**0**
Growth on CLED medium	0	10			**5**											0			0	0	0	**0**
Arginine decarboxylase	NG	NG			**NG**											NG			NG	NG	NG	**NG**
Lysine decarboxylase	NG	NG			**NG**											NG			NG	NG	NG	**NG**
Ornithine decarboxylase	NG	NG			**NG**											NG			NG	NG	NG	**NG**
Nitrate reduction	0	0			**0**											0			0	0	0	**0**
Indole	0	0			**0**											NG			0	0	0	**0**
ONPG	0	0			**0**											0			0	0	0	**0**

[a] G more than 3 positive reactions in BIOLOG GN microplate; NG not enough positive reactions for identification using BIOLOG.
[b] Pigment production. Y, yellow; B, black; Br, brown.
[c] NG no growth in blank for decarboxylase tests.
[d] Where no data is shown tests were not done.
*Denotes isolate 16S rDNA gene partially sequenced- refer to Figure 6 for phylogenetic affiliation.

Appendix 2b (continued)

Character		AE1-19	AE1-01	AE1-16	AE1-03	Group average	SYS4-22	SYS4-24	SYS4-26	SYS4-27	SYS4-28	SYS4-29	SYS5-21	SYS5-22	SYS5-23	SYS5-30	SYS3-02	TG8-02	TG8-01	TG4-19	T15-08	TG4-08	Group average
		Pseudoalteromonas group1					*Pseudoalteromonas* group 2																
Voges Proskauer		0	0			**0**											0			0	0	0	**0**
0/129 10 μg		0	0	0	0	**0**	0	0	0	0	0		0	0	0	0	0						**0**
0/129 150 μg		10	10	5	5	**10**	0	0	0	0	0		0	0	0	0	0						**0**
Agar digestion (agarase)		0	0	0	0	**0**	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10**
Aesculin hydrolysis		0	0			**0**											10			0	10	10	**7.5**
Haemolysis srbc		0	0			**0**											0			0	0	0	**0**
Acid arbutin		0	0			**0**											0			0	0	0	**0**
Acid salicin		0	0			**0**											0			0	0	0	**0**
Acid sucrose		0	0			**0**											5						
Catalase		0	5			**25**											0			0	0	0	**0**
Utilisation of single carbon sources (BIOLOG GN):																							
	Acetic acid	5		10	10	**5**	5					0					10	0	10	0	5		**4.3**
	n- Acetyl-D-galatosamine	0		10	10	**0**	0					0					0	0	0	0	0		**0.0**
	n- Acetyl-D-glucosamine	5		10	10	**5**	0					0					0	10	10	0	0		**2.9**
	cis-Aconitic acid	5		10	10	**5**	10					0					0	0	0	5	5		**2.9**
	Adonitol	10		5	0	**10**	0					0					0	0	0	0	0		**0.0**
	D-Alaninamide	0		5	0	**0**	0					0					0	0	5	0	5		**1.4**
	Alanine	10		0	0	**10**	0					0					0	0	0	0	0		**0.0**
	L-alanine	10		5	5	**10**	10					10					10	10	10	5	5		**8.6**
	L-Alanyl glycine	5		5	5	**5**	10					10					10	10	10	10	10		**10.0**
	2- Amino ethanol	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
	γ-Amino butyric acid	10		0	0	**10**	0					0					0	0	0	0	0		**0.0**
	D-L-arabinose	0		10	10	**0**	10					10					10	0	0	0	0		**4.3**
	Arabitol	10		0	0	**10**	0					0					0	0	0	0	0		**0.0**
	L-Asparagine	10		5	5	**10**	0					5					0	5	5	0	5		**2.9**
	L-Aspartic acid	10		5	5	**10**	0					10					0	5	0	5	5		**3.6**

Appendix 2b (continued)

Utilisation of single carbon sources (BIOLOG GN):	*Pseudoalteromonas* group1					*Pseudoalteromonas* group 2																
	AE1-19	AE1-01	AE1-16	AE1-03	**Group average**	SYS4-22	SYS4-24	SYS4-26	SYS4-27	SYS4-28	SYS4-29	SYS5-21	SYS5-22	SYS5-23	SYS5-30	SYS3-02	TG8-02	TG8-01	TG4-19	T15-08	TG4-08	**Group average**
Bromo-succinic acid	10		0	0	**10**	0					0					0	0	5	0	0		**0.7**
2,3 Butanediol	0		5	0	**0**	0					0					0	0	0	0	0		**0.0**
D,L-carnitine	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
Cellobiose	10		10	10	**10**	10					10					10	0	0	10	10		**7.1**
Citric acid	10		10	10	**10**	10					10					10	0	0	10	10		**7.1**
α-Cyclodextrin	5		10	10	**5**	10					10					10	0	10	10	10		**8.6**
Dextrin	5		10	10	**5**	10					10					10	10	10	10	10		**10.0**
I- Erythritol	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
Formic acid	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
D-Fructose	10		10	10	**10**	0					0					0	0	5	0	5		**1.4**
L-Fucose	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
D- Galactonic acid lactone	0		0	0	**0**	10					0					0	0	0	0	0		**1.4**
D-Galactose	0		0	0	**0**	10					10					10	0	0	10	10		**7.1**
D-Galacturonic acid	0		0	0	**0**	0					10					10	0	0	10	10		**5.7**
Gentiobiose	0		5	5	**0**	0					0					0	0	0	0	0		**0.0**
D-Gluconic acid	10		10	0	**10**	0					0					0	0	0	0	0		**0.0**
D-Glucosamic acid	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
α-D-Glucose	10		10	10	**10**	10					10					10	0	5	10	10		**7.9**
Glucose-1-phosphate	0		0	0	**0**	0					5					0	0	0	0	5		**1.4**
Glucose-6-phosphate	0		0	0	**0**	0					5					0	0	0	0	5		**1.4**
Glucuronamide	0		5	0	**0**	0					0					0	0	0	0	0		**0.0**
D-Glucuronic acid	10		0	0	**10**	0					0					0	5	0	0	0		**0.7**
L-Glutamic acid	10		10	5	**10**	5					5					0	0	10	0	5		**3.6**
Glycerol	10		0	0	**10**	0					0					0	0	0	0	0		**0.0**
D-L-α-Glycerol phosphate	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
Glycyl-L-aspartic acid	0		0	5	**0**	0					5					0	5	10	5	5		**4.3**
Glycyl-L-glutamic acid	5		10	10	**5**	10					10					10	10	10	5	10		**9.3**

Appendix 2b (continued)

Utilisation of single carbon sources (BIOLOG GN):	*Pseudoalteromonas* group1					*Pseudoalteromonas* group 2																
	AE1-19	AE1-01	AE1-16	AE1-03	**Group average**	SYS4-22	SYS4-24	SYS4-26	SYS4-27	SYS4-28	SYS4-29	SYS5-21	SYS5-22	SYS5-23	SYS5-30	SYS3-02	TG8-02	TG8-01	TG4-19	T15-08	TG4-08	**Group average**
Glycogen	5		10	10	**5**	10					10					10	10	10	10	10		**10.0**
L-histidine	10		0	0	**10**	0					0					0	0	0	0	0		**0.0**
α-Hydroxy butyric acid	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
β-Hydroxy butyric acid	0		10	10	**0**	0					0					0	0	0	0	0		**0.0**
γ-Hydroxy butyric acid	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
Hydroxy L-proline	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
p-Hydroxy phenylacetic acid	0		0	0	**0**	0					0					0	0	5	0	0		**0.7**
Inosine	5		5	0	**5**	0					0					0	0	0	0	5		**0.7**
m-inositol	10		0	0	**10**	0					0					0	0	0	0	0		**0.0**
Itaconic acid	0		0	0	**0**	0					0					0	5	0	0	0		**0.7**
α-Ketobutyric acid	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
α-Ketoglutaric acid	10		0	0	**10**	0					0					0	0	0	0	5		**0.7**
α-Ketovaleric acid	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
D,L-lactic acid	10		0	0	**10**	0					0					0	0	0	0	0		**0.0**
α-lactose	0		0	0	**0**	10					10					0	0	0	5	10		**5.0**
α-D-lactose-lactulose	0		0	0	**0**	0					5					0	0	0	0	0		**0.7**
L-Leucine	0		0	5	**0**	0					0					0	0	0	0	0		**0.0**
Malonic acid	0		0	0	**0**	0					5					0	0	5	0	5		**2.1**
Maltose	10		10	10	**10**	10					10					10	10	10	10	10		**10.0**
D-Mannitol	10		5	10	**10**	10					10					10	10	10	10	10		**10.0**
D-Mannose	10		10	10	**10**	10					10					5	0	0	0	5		**4.3**
D-Melibiose	0		0	5	**0**	10					10					10	0	0	5	5		**5.7**
Methyl pyruvate	10		10	10	**10**	10					10					10	10	5	0	5		**7.1**
β-methyl-D-glucoside	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
Mono-methyl succinate	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
L-Ornithine	10		0	0	**10**	0					0					0	0	0	0	0		**0.0**

Appendix 2b (continued)

Utilisation of single carbon sources (BIOLOG GN):	*Pseudoalteromonas* group 1					*Pseudoalteromonas* group 2																
	AE1-19	AE1-01	AE1-16	AE1-03	**Group average**	SYS4-22	SYS4-24	SYS4-26	SYS4-27	SYS4-28	SYS4-29	SYS5-21	SYS5-22	SYS5-23	SYS5-30	SYS3-02	TG8-02	TG8-01	TG4-19	T15-08	TG4-08	**Group average**
L-Phenylalanine	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
Phenyl ethylamine	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
L-Proline	10		10	10	**10**	10					10					10	0	10	10	10		**8.6**
Propionic acid	0		10	10	**6.7**	5					10					10	10	10	5	5		**7.9**
Psicose	0		10	5	**5**	0					0					0	0	0	0	0		**0.0**
Putrescine	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
L-Pyro glutamic acid	10		0	0	**3.4**	0					0					0	0	0	0	0		**0.0**
Quinic acid	10		0	0	**3.4**	0					0					0	5	0	0	0		**0.7**
D-Raffinose	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
L-Rhamnose	10		0	0	**3.4**	0					0					0	0	0	0	0		**0.0**
D-Saccharic acid	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
Sebacic acid	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**
D-Serine	0		5	5	**3.3**	0					0					0	0	5	0	0		**0.7**
L-Serine	5		10	10	**8.3**	0					5					0	5	10	0	5		**3.6**
D-Sorbitol	10		0	0	**3.3**	0					0					0	0	0	0	0		**0.0**
Succinamic acid	0		5	0	**1.7**	0					0					0	0	0	0	0		**0.0**
Sucrose	10		10	10	**10**	10					10					10	0	0	10	10		**7.1**
Succinic acid	10		10	10	**10**	10					10					10	10	10	10	10		**10.0**
L-Threonine	5		5	5	**5**	5					5					0	5	10	0	5		**4.3**
Thymidine	0		0	0	**0**	0					0					0	0	0	0	5		**0.7**
D-Trehalose	10		10	10	**10**	10					10					10	0	5	10	10		**7.9**
Turanose	5		0	0	**1.7**	0					0					0	0	0	0	0		**0.0**
Tween 40	0		10	10	**6.7**	10					10					10	10	10	10	10		**10.0**
Tween 80	0		10	10	**6.7**	10					10					10	0	10	0	5		**6.4**
Uridine	10		0	0	**3.3**	10					5					0	0	10	5	5		**5.0**
Urocanic acid	5		0	0	**1.7**	0					0					0	0	0	0	0		**0.0**
Xylitol	0		0	0	**0**	0					0					0	0	0	0	0		**0.0**

Appendix 2b (continued) Antibiotic sensitivity and resistance of *Pseudoalteromonas* groups 1 and 2

	Pseudoalteromonas group1					*Pseudoalteromonas* group 2																
	AE1-19	AE1-01	AE1-16	AE1-03	**Group average**	SYS4-22	SYS4-24	SYS4-26	SYS4-27	SYS4-28	SYS4-29	SYS5-21	SYS5-22	SYS5-23	SYS5-30	SYS3-02	TG8-02	TG8-01	TG4-19	T15-08	TG4-08	**Group average**
Antibiotic sensitivity:																						
Chloramphenicol 25 μg	10	10	10	10	**10**	10	10	10	10	5	10	10	10	5	10	10						**9.1**
Erythromycin 5 μg	0	0	10	10	**5**	10	10	10	10	10	10	10	10	10	10	10						**10.0**
Fusidic acid 10 μg	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0						**0.0**
Methicillin 10 μg	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0						**0.0**
Novobiocin 5 μg	0	0	0	0	**0**	0	0	5	0	0	0	0	0	0	0	0						**0.5**
Penicillin G 1 unit	10	0	0	0	**2.5**	0	0	0	0	0	0	0	0	0	0	0						**0.0**
Streptomycin 10 μg	10	10	10	5	**8.8**	0	0	0	0	0	0	0	0	0	0	0						**0.0**
Tetracycline 25 μg	10	10	10	10	**10**	10	10	10	10	10	10	10	10	5	10	10						**9.5**
Tetracycline 100 μg	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10						**10.0**
Ampicillin 10 μg	10	10	10	10	**10**	10	10	10	10	10	10	10	10	5	10	10						**9.5**
Ampicillin 25 μg	10	10	10	10	**10**	10	10	10	10	10	10	10	10	5	10	10						**9.5**
Cephalothin 5 μg	10	0	0	0	**2.5**	10	0	0	0	0	5	0	0	0	0	0						**1.4**
Colistin sulphate 25 μg	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10						**10.0**
Gentamycin 10 μg	10	10	10	10	**10**	10	5	0	0	0	0	10	10	0	10	5						**4.5**
Sulphatriad 200 μg	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0						**0.0**
Cotrimoxazole 25 μg	5	5	10	10	**7.5**	0	5	0	0	0	10	10	10	10	10	10						**5.9**
Nitrofurantoin 50μg	10	10	3	5	**7**	10	0	0	0	0	10	0	0	0	0	0						**1.8**
Ticarcillin 75 μg	10	0	3	0	**3.3**		10	10	10	10	10	10	10	0	10	10						**8.2**
Nalidixic acid 30 μg	10	10	10	10	**10**		10	10	10	10	3	10	10	10	10	10						**8.4**
Trimethoprim 2.5 μg	10	10	0	0	**5**		0	0	0	0	0	0	0	0	0	0						**0.0**
Sulphamethoxazole 50μg	0	0	0	0	**0**		0	0	0	0	3	0	0	0	0	0						**0.2**

10 = >2mm zone of clearing outside of test disc, 5 = < 2mm zone of clearing, 2.5 = disruption of growth, 0 = resistant.

Appendix 2c Biochemical, physiological and antibiotic susceptibility test results for isolates classified as *Pseudoalteromonas* groups 3-5. Each test was scored on a scale of between 0 –10 for a strongly positive reaction. Isolates are coded as in Table 1.

	Pseudoalteromonas group 3																							
Character	OFF2-11	SYS5-15	E1-18	OFC1-16	YSW1-07	SYS5-16	SYS5-17	SYS3-01	YSW1-15	SYS5-27	YS2-19	YSW1-05	E1-06	E1-20	MA1-13*	E1-12	E1-11	SFF1-10	SFF1-04	SFF1-01*	SFF1-20	SFF1-14	SFF4-12	**Group average**
PCR-RFLP pattern	Vb		Vb	Vb				Vb						Vb	Vb	Vb	Vb	Vb	Vb	Vb	Vb	Vb	Vb	**Vb**
Biolog	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G				G	**G**
Gram stain	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.0**
Motility			10		10	10	10	10		10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10.0**
Pigment	0		0			0	0	0		0	0		0	0	0	0	0	0	0	0	0	0	0	**0**
Morphology		10	10		10	10	10	10		10	10		10	10	10	10	10	10	10	10	10	10	10	**10.0**
Oxidase		10	10		0	10	10	10		10		0	10	10		5	10	10	10	10	10	10	10	**7.8**
TCBS	0	0				0	0	0		0	0		0					0	0	0	0	0	0	**0.0**
TCBS - sucrose positive	0	0				0	0	0		0	0		0					0	0	0	0	0	0	**0.0**
Oxidation of glucose								5												0	10	0	5	**4.0**
Fermentation of glucose								5												5	0	5	5	**4.0**
Swarming	0	0						0			0			0				0	0	0	0	0	0	**0.0**
Luminesence		0	10					0			0			0				0	0	0	0	0	0	**0.0**
Growth 4 °C		10	10					10						10		10	10			10	10	10	10	**10.0**
Growth 20 °C	10	10		10				10						10		10	10	10	10	10	10	10	10	**10.0**
Growth 37 °C								0												10	10	10	10	**8.0**
CLED								0												0	0	0	0	**0.0**
Arginine decarboxylase								NG												NG	NG	NG	NG	**ng**
Lysine decarboxylase								NG												NG	NG	NG	NG	**ng**
Ornithine decarboxylase								NG												NG	NG	NG	NG	**ng**
Nitrate reduction								0												0	0	0	0	**0**
Indole								ng												ng	0	0	0	**0**
ONPG								ng												0	ng	0	0	**0**

	Pseudoalteromonas group 4						*Pseudoalteromonas* Group 5			
Character	SYS5-07	SYS5-03	SYS5-12	TG15-06	TG15-07*	**Group average**	TG3-13	TG6-02	TG13-06	**Group average**
PCR-RFLP pattern	Vc	Vc		Vc	Vc	**Vc**	Vc	Vc	Vc	**Vc**
Biolog	G	G	G	G	G	**G**				**G**
Gram stain	0	0	0			**0.0**	0	0	0	**0**
Motility	10	10	10			**10.0**	10	10	10	**10**
Pigment	Br	Br	Br			**Br**	0	0	0	**0**
Morphology	10	10	10			**10.0**	10	10	10	**10**
Oxidase	10	10	10			**10.0**	10	10	10	**10**
TCBS	0	0	0			**0.0**	0	0	0	**0**
TCBS - sucrose positive	0	0	0			**0.0**	0	0	0	**0**
Oxidation of glucose	0	0				**0.0**				
Fermentation of glucose	0	0				**0.0**				
Swarming	0	0				**0.0**				
Luminesence	0	0				**0.0**				
Growth 4 °C	10	10				**10.0**				
Growth 20 °C	10	10				**10.0**				
Growth 37 °C	0	5				**2.5**				
CLED	0	0				**0.0**				
Arginine decarboxylase	NG	NG				**ng**				
Lysine decarboxylase	NG	NG				**ng**				
Ornithine decarboxylase	NG	NG				**ng**				
Nitrate reduction	0	0				**0.0**				
Indole	0	ng				**0.0**				
ONPG	0	0				**0.0**				

[a] G more than 3 positive reactions in BIOLOG GN microplate; NG not enough positive reactions for identification using BIOLOG
[b] Pigment production. Y, yellow; B, black; Br, brown.
[c] Where no data is shown tests were not done.
*Denotes isolate 16S rDNA gene partially sequenced- refer to Figure 6 for phylogenetic affiliation.

Appendix 2c (continued)

Character	*Pseudoalteromonas* group 3 OFF2-11	SYS5-15	E1-18	OFC1-16	YSW1-07	SYS5-16	SYS5-17	SYS3-01	YSW1-15	SYS5-27	YS2-19	YSW1-05	E1-06	E1-20	MA1-13	E1-12	E1-11	SFF1-10	SFF1-04	SFF1-01*	SFF1-20	SFF1-14	SFF4-12	**Group average**	*Pseudoalteromonas* group 4 SYS5-07	SYS5-03*	SYS5-12	TG15-06	TG15-07*	**Group average**	*Pseudoalteromonas* Group 5 TG3-13	TG6-02	TG13-06	**Group average**
Voges Proskauer								0												0	0	0	0	**0**	0	0				**0.0**				
0/129 10 µg		0	0			0		0		0		0	0	0	0	0	0			0	0	0	0	**0**	0	0				**0.0**				
0/129 150 µg		0	5			0		0		0		0	0	0	5	0	5			0	0	0	0	**0.9**	0	0				**0.0**				
Agar digestion (agarase)	0	0	0			0		0		0		0	0	0						0	0	0	0	**0**	0	0				**0.0**				
Aesculin hydrolysis								0												5	3	8	10	**5**	10	10				**10.0**				
Haemolysis srbc	10			10				0							10					0	0	10	0	**5**	0	0				**0.0**				
Acid arbutin								0												0		0	0	**0**	0	0				**0.0**				
Acid salicin								0												0		0	0	**0**	0	0				**0.0**				
Acid sucrose								10												10		3	0	**5.6**	5	5				**5.0**				
Catalase								10												8		0	3	**5**	0	0				**0.0**				
Utilisation of single carbon sources (BIOLOG GN):																																		
Acetic acid	10	5	0	0	0	5	5	10	5	5	10	5	10	5	5	5	5	10	10				10	**5.5**	0	5	5	10	10	**6.0**	10	10	5	**8.3**
n-Acetyl-D-galactosamine	0	0	5	0	0	10	10	0	0	0	0	0	0	0	0	0	0	0	0				0	**1.1**	0	0	10	10	10	**6.0**	10	10	10	**10.0**
n-Acetyl-D-glucosamine	10	0	0	10	0	10	10	0	0	0	0	0	0	10	0	10	10	10	10				0	**4.1**	10	10	10	10	10	**10.0**	10	0	0	**3.3**
cis-Aconitic acid	0	0	0	0	0	10	10	10	5	10	5	5	0	0	0	0	0	10	5				5	**3.4**	5	5	10	10	10	**8.0**	10	10	10	**10.0**
Adonitol	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	0	0	**0.0**	10	5	0	**5.0**
D-Alaninamide	0	0	0	0	0	0	0	10	5	0	0	0	5	0	0	5	0	5	5				5	**1.8**	0	0	0	10	10	**4.0**	0	10	10	**6.7**
Alanine	5	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0				0	**0.5**	0	0	0	10	10	**4.0**	0	10	10	**6.7**
L-Alanine	10	0	0	10	10	5	10	10	10	10	10	10	10	10	0	10	10	10	10				10	**7.5**	5	5	5	0	0	**3.0**	0	0	0	**0.0**
L-Alanyl glycine	10	0	0	10	10	0	5	10	10	10	10	10	10	10	0	10	10	10	10				10	**7.0**	5	5	10	0	0	**4.0**	0	10	0	**3.3**
2- Amino ethanol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
γ-Amino butyric acid	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	10	10	**4.0**	10	10	0	**6.7**
D-L-Arabinose	0	0	0	0	0	10	10	0	0	0	0	10	0	0	0	10	10	10	10				0	**3.2**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Arabitol	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	5	0				0	**0.7**	0	0	0	5	10	**3.0**	0	10	0	**3.3**
L-Asparagine	5	0	0	0	0	0	0	0	0	0	5	5	5	0	0	10	5	5	5				5	**2.3**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
L-Aspartic acid	0	5	0	0	0	0	0	5	0	0	0	0	10	5	0	0	0	5	5				5	**1.8**	0	0	0	0	0	**0.0**	10	0	0	**3.3**
Bromo-succinic acid	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	5	0	5	5				5	**1.1**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
2,3 Butanediol	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	10	10	**4.0**	10	10	10	**10.0**

Appendix 2c (continued)

Utilisation of single carbon sources (BIOLOG GN):	*Pseudoalteromonas* group 3																								*Pseudoalteromonas* group 4						*Pseudoalteromonas* Group 5			
	OFF2 -11	SYS5-15	E1-18	OFC1-16	YSW1-07	SYS5-16	SYS5-17	SYS3-01	YSW1-15	SYS5-27	YS2-19	YSW1-05	E1-06	E1-20	MA1-13	E1-12	E1-11	SFF1-10	SFF1-04	SFF1-01	SFF1-20	SFF1-14	SFF4-12	**Group average**	SYS5-07	SYS5-03	SYS5-12	TG15-06	TG15-07	**Group average**	TG3-13	TG6-02	TG13-06	**Group average**
D,L-Carnitine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Cellobiose	0	0	0	0	10	10	10	10	10	10	10	10	10	0	0	0	0	0	0				0	**4.1**	5	5	5	0	0	**3.0**	5	0	0	**1.7**
Citric acid	0	0	10	0	10	10	10	10	10	10	10	10	0	0	0	5	5	5	5				0	**5.0**	5	5	10	0	0	**4.0**	0	0	0	**0.0**
α-Cyclodextrin	0	0	5	0	10	10	10	10	10	10	10	10	10	0	0	0	5	10	5				0	**5.2**	10	5	10	10	10	**9.0**	10	10	10	**10.0**
Dextrin	10	0	5	10	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10				10	**8.0**	0	0	10	0	0	**2.0**	10	0	0	**3.3**
meso- Erythritol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0				0	**0.2**	0	0	0	0	5	**1.0**	10	5	10	**8.3**
Formic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	10	0	0	**3.3**
D-Fructose	0	0	0	0	10	10	10	10	10	0	0	0	10	0	0	5	0	5	0				5	**3.4**	0	0	5	0	0	**1.0**	0	0	0	**0.0**
L-Fucose	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	5	5	**2.0**	0	5	0	**1.7**
D- Galactonic acid lactone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
D-Galactose	0	0	0	0	10	0	0	10	10	10	10	0	5	0	0	0	0	0	0				0	**2.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
D-Galacturonic acid	0	0	0	0	0	10	10	0	0	0	0	0	10	0	0	0	0	0	0				0	**1.4**	0	5	10	0	0	**3.0**	0	0	0	**0.0**
Gentiobiose	10	0	5	5	0	0	0	0	0	10	0	0	5	10	10	10	10	10	10				10	**4.8**	0	0	0	10	10	**4.0**	10	10	10	**10.0**
D-Gluconic acid	0	0	0	0	10	0	0	10	10	10	5	10	10	0	0	0	5	0	5				0	**3.4**	0	0	0	10	10	**4.0**	10	10	10	**10.0**
D-Glucosamic acid	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	5	0	0	0	0	**1.0**	0	0	0	**0.0**
α-D-Glucose	10	0	0	10	10	10	10	10	10	10	10	0	10	10	0	10	10	10	10				10	**7.3**	0	0	5	0	0	**1.0**	0	0	0	**0.0**
Glucose-1-phosphate	5	0	0	0	0	0	0	0	0	0	0	0	0	0	5	5	0	5	5				5	**1.4**	0	0	0	10	10	**4.0**	10	10	10	**10.0**
Glucose-6-phosphate	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0				5	**0.7**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Glucuronamide	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	5	0	**1.0**	5	10	5	**6.7**
D-Glucuronic acid	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	5	5	**2.0**	10	10	10	**10.0**
L-Glutamic acid	5	10	0	0	0	10	10	10	5	5	0	5	10	0	5	5	5	10	10				5	**5.0**	0	5	5	10	10	**6.0**	10	10	10	**10.0**
Glycerol	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0				0	**0.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
D-L-α-Glycerol phosphate	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	5	0				0	**0.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Glycyl-L-aspartic acid	5	5	0	0	0	0	5	5	0	0	0	0	10	10	5	5	5	10	5				5	**3.4**	0	5	5	10	10	**6.0**	10	10	0	**6.7**
Glycyl-L-glutamic acid	10	5	5	10	10	10	10	10	10	10	10	10	10	10	0	10	10	10	10				10	**8.2**	5	5	10	0	0	**4.0**	0	0	0	**0.0**
Glycogen	10	0	5	10	0	0	0	5	0	0	5	10	5	10	10	5	10	10	5				10	**5.0**	0	0	10	0	0	**2.0**	0	0	0	**0.0**
L-histidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
α-Hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**

Appendix 2c (continued)

Utilisation of single carbon sources (BIOLOG GN):	*Pseudoalteromonas* group 3																								*Pseudoalteromonas* group 4						*Pseudoalteromonas* Group 5			
	OFF2 -11	SYS5-15	e1-18	OFC1-16	YSW1-07	SYS5-16	SYS5-17	SYS3-01	YSW1-15	SYS5-27	YS2-19	YSW1-05	E1-06	E1-20	MA1-13	E1-12	E1-11	SFF1-10	SFF1-04	SFF1-01	SFF1-20	SFF1-14	SFF4-12	**Group average**	SYS5-07	SYS5-03	SYS5-12	TG15-06	TG15-07	**Group average**	TG3-13	TG6-02	TG13-06	**Group average**
β-Hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	10	5	10	10	10	10				0	**2.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
γ-Hydroxy butyric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Hydroxy-L-proline	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0				0	**0.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
p-Hydroxy phenylacetic acid	5	0	0	0	0	0	10	0	0	0	0	0	0	0	5	0	0	0	0				0	**0.9**	0	0	0	0	0	**0.0**	0	0	5	**1.7**
Inosine	10	0	0	5	0	0	5	10	0	0	0	5	0	10	10	10	10	10	10				10	**4.8**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
meso-Inositol	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	0	0	**0.0**	5	0	0	**1.7**
Itaconic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				5	**0.2**	0	0	0	0	5	**1.0**	0	0	0	**0.0**
α-Ketobutyric acid	5	0	0	0	0	0	0	0	0	0	0	0	5	0	5	5	5	5	5				0	**1.6**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
α-Ketoglutaric acid	0	0	0	0	0	0	0	0	0	5	5	0	0	0	0	0	0	0	5				0	**0.7**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
α-Ketovaleric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	5	5	**2.0**	10	10	5	**8.3**
D,L-lactic acid	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				10	**0.7**	0	0	0	0	0	**0.0**	0	0	5	**1.7**
α-lactose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0				0	**0.2**	0	0	0	0	0	**0.0**	0	0	5	**1.7**
α-D-lactose-lactulose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
L-Leucine	10	0	0	0	0	5	5	0	0	0	0	0	10	5	10	10	5	10	5				5	**3.6**	0	0	0	5	5	**2.0**	10	10	10	**10.0**
Malonic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	5				0	**0.5**	0	0	0	0	5	**1.0**	5	5	0	**3.3**
Maltose	10	0	5	10	10	10	10	10	10	10	10	0	10	10	0	10	10	10	10				10	**7.5**	5	5	10	0	0	**4.0**	0	0	0	**0.0**
D-Mannitol	0	5	0	0	10	0	0	10	10	10	10	0	10	0	0	0	0	0	0				0	**3.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
D-Mannose	5	0	0	0	0	0	5	10	0	0	0	0	0	0	0	0	0	0	0				0	**0.9**	0	0	0	0	5	**1.0**	0	0	0	**0.0**
D-Melibiose	0	0	0	0	0	0	0	10	10	10	10	10	0	0	0	0	0	5	0				0	**2.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Methyl pyruvate	10	0	5	5	0	10	10	10	10	10	10	10	10	10	0	10	10	10	10				10	**7.3**	0	5	10	5	5	**5.0**	10	10	10	**10.0**
β-Methyl-D-glucoside	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	5	0	10	0				5	**1.1**	0	0	0	5	10	**3.0**	10	5	10	**8.3**
Mono-methyl succinate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				5	**0.2**	0	0	0	0	5	**1.0**	5	0	5	**3.3**
L-Ornithine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	5	**1.0**	10	0	5	**5.0**
L-Phenylalanine	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5				5	**0.9**	0	0	0	5	5	**2.0**	0	10	10	**6.7**
Phenyl ethylamine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	5	5	**2.0**	5	5	5	**5.0**
L-Proline	10	0	0	10	0	10	10	10	10	10	10	10	10	10	5	10	10	10	10				10	**7.5**	5	0	5	5	5	**4.0**	10	10	10	**10.0**
Propionic acid	5	0	0	0	0	10	10	10	10	10	5	5	10	5	5	10	5	10	5				10	**5.7**	5	5	5	0	0	**3.0**	0	0	0	**0.0**
Psicose	0	0	0	0	0	0	0	10	5	0	0	0	0	0	0	0	0	0	0				5	**0.9**	0	0	0	0	0	**0.0**	0	0	0	**0.0**

Appendix 2c (Continued)

Utilisation of single carbon sources (BIOLOG GN):	OFF2 -11	SYS5-15	E1-18	OFC1-16	YSW1-07	SYS5-16	SYS5-17	SYS3-01	YSW1-15	SYS5-27	YS2-19	YSW1-05	E1-06	E1-20	MA1-13	E1-12	E1-11	SFF1-10	SFF1-04	SFF1-01	SFF1-20	SFF1-14	SFF4-12	Group average	SYS5-07	SYS5-03	SYS5-12	TG15-06	TG15-07	Group average	TG3-13	TG6-02	TG13-06	Group average
	Pseudoalteromonas group 3																								*Pseudoalteromonas* group 4						*Pseudoalteromonas* Group 5			
Putrescine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	5	5	**2.0**	5	5	0	**3.3**
L-Pyroglutamic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Quinic acid	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
D-Raffinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	5	5	**2.0**	10	10	0	**6.7**
L-Rhamnose	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
D-Saccharic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5	0	5	5				0	**0.9**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Sebacic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				10	**0.5**	0	0	0	0	5	**1.0**	0	5	5	**3.3**
D-Serine	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.5**	0	0	0	5	5	**2.0**	5	5	5	**5.0**
L-Serine	10	0	0	10	0	5	10	10	10	5	5	5	10	10	0	10	10	10	10				10	**6.4**	5	5	5	0	0	**3.0**	0	0	0	**0.0**
D-Sorbitol	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.0**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Succinamic acid	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	5	0				5	**0.7**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Sucrose	10	0	5	10	10	10	10	10	10	0	0	0	10	0	0	10	10	10	10				10	**6.1**	10	5	10	5	5	**7.0**	0	0	5	**1.7**
Succinic acid	10	0	0	10	0	10	10	10	20	10	10	10	10	10	0	10	10	10	10				0	**7.3**	5	0	5	5	5	**4.0**	10	0	5	**5.0**
L-Threonine	5	0	0	0	0	5	5	10	10	0	0	5	10	5	5	5	5	10	10				5	**4.3**	0	5	5	0	0	**2.0**	0	0	5	**1.7**
Thymidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0				5	**0.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
D-Trehalose	0	0	0	0	10	10	10	10	10	0	0	0	0	10	0	10	10	10	10				0	**4.5**	5	5	10	0	0	**4.0**	0	0	0	**0.0**
Turanose	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	5				0	**0.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Tween 40	10	10	5	10	0	10	10	10	5	10	10	0	10	10	10	10	10	10	10				10	**7.7**	0	5	10	0	0	**3.0**	0	0	0	**0.0**
Tween 80	5	0	0	0	0	10	10	10	5	0	10	10	10	5	0	5	5	10	10				10	**5.2**	0	5	5	0	0	**2.0**	0	0	0	**0.0**
Uridine	10	0	0	5	10	0	5	5	10	5	0	0	10	5	10	5	10	10	10				10	**5.5**	0	0	0	0	0	**0.0**	0	0	0	**0.0**
Urocanic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0				0	**0.2**	0	0	0	0	0	**0.0**	0	5	0	**1.7**
Xylitol	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	**0.2**	0	0	0	0	0	**0.0**	0	0	0	**0.0**

Appendix 2c (continued). Antibiotic sensitivity and resistance of *Pseudoalteromonas* groups 3 and 4.

	Pseudoalteromonas group 3																							*Pseudoalteromonas* group 4						*Pseudoalteromonas* Group 5			
	OFF2 -11	SYS5-15	E1-18	OFC1-16	YSW1-07	SYS5-16	SYS5-17	SYS3-01	YSW1-15	SYS5-27	YS2-19	YSW1-05	E1-06	E1-20	MA1-13	E1-12	E1-11	SFF1-10	SFF1-04	SFF1-01	SFF1-20	SFF1-14	SFF4-12	**Group average**	SYS5-07	SYS5-03	SYS5-12	TG15-06	TG15-07	Group average	TG3-13	TG6-02	TG13-06	Group average
Antibiotic sensitivity:																																		
Chloramphenicol 25 µg		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	3	10	10	10	5	10	**8.6**	10	10	10			**10.0**				
Erythromycin 5 µg		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**9.2**	10	10	10			**10.0**				
Fusidic acid 10 µg		5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.2**	0	0	0			**0.0**				
Methicillin 10 µg		5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	**0.3**	0	0	0			**0.0**				
Novobiocin 5 µg		3	0	0	0	0	10	0	0	0	0	0	0	5	10	10	10	3	3	5	5	0	0	**2.6**	10	10	0			**6.7**				
Penicillin G 1 unit		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.0**	0	0	0			**0.0**				
Streptomycin 10 µg		0	0	0	0	5	0	5	0	0	3	0	0	0	10	0	0	0	0	0	0	0	10	**1.4**	0	0	0			**0.0**				
Tetracycline 25 µg		0	10	0	10	5	10	10	10	3	10	10	10	10	0	10	10	10	5	10	10	3	5	**6.7**	10	10	3			**7.5**				
Tetracycline 100 µg		5	10	10	10	5	10	10	10	10	10	10	10	10	0	10	10	10	10	10	10	10	10	**8.3**	10	10	5			**8.3**				
Ampicillin 10 µg		10	10	5	10	5	10	10	10	5	5	10	10	5	0	5	5	5	10	0	5	0	5	**5.8**	10	10	10			**10.0**				
Ampicillin 25 µg		10	10	5	10	10	10	10	10	5	5	10	10	10	0	5	5	5	10	5	3	0	3	**6.3**	10	10	10			**10.0**				
Cephalothin 5 µg		0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.4**	0	0	0			**0.0**				
Colistin sulphate 25 µg		0	10	10	10	10	0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**8.3**	0	0	0			**0.0**				
Gentamycin 10 µg		10	10	5	0	10	10	10	10	10	10	0	10	10	10	5	0	10	10	5	5	5	3	**6.6**	5	5	10			**6.7**				
Sulphatriad 200 µg		0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	10	0	0	0	0	0	**0.5**	0	0	0			**0.0**				
Cotrimoxazole 25 µg		3	10	0	10	3	10	5	10	10	10	10	10	3	0	10	0	0	0	10	10	0	5	**5.3**	5	5	5			**5.0**				
Nitrofurantoin 50µg		0	10	5	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1.0**	0	0	0			**0.0**				
Ticarcillin 75 µg		3	10	0	10	0	0	10	10	10	0	0	10	10	0	0	10	0	0	0	3	3	0	**3.6**	10	10	10			**10.0**				
Nalidixic acid 30 µg		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	5	10	10	10	10	10	**9.0**	10	10	10			**10.0**				
Trimethoprim 2.5 µg		0	10	0	0	0	0	0	0	0	0	5	0	0	0	10	0	0	0	0	0	0	0	**1.0**	0	0	0			**0.0**				
Sulphamethoxazole 50µg		0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.2**	0	0	0			**0.0**				

10 = >2mm zone of clearing outside of test disc, 5 = < 2mm zone of clearing, 2.5 = disruption of growth, 0 = resistant.

Appendix 2d Biochemical, physiological and antibiotic susceptibility test results for isolates classified as *Vibrio splendidus* groups 1 and 2. Each test was scored on a scale of between 0 –10 for a strongly positive reaction. Isolates are coded as in Table 1.

	V. splendidus group 1																									*V. splendidus* group 2											
Isolate	OFA1-15	OFA1-17	OFA1-19	OFF2-14	OFF2-16	OFF2-18	SFF4-01	SFF4-03*	SFF4-05	SFF4-07	SFF4-10	SFF4-14*	SFF4-17	SFF4-19*	AE1-26*	FFW2-16	MFF1-03	MFF1-10	MFF1-14	MFF2-03*	MFF2-05	MFF2-12	MA1-03*	MA1-09*	**Group average**	SFF2-13	SFF2-20	AE1-31*	SFF1-19*	MFF1-13	SYS4-13	SYS4-17	SYS4-23	SYS4-30	SYS6-17	SYS6-19	**Group average**
PCR-RFLP pattern	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	XIa	**XIa**	XIb	XIb	XIb	XIb	XIb	XIb	XIb	XIb	XIb	XIb	XIb	**XIb**
[a]Biolog	[b]	G	G	G	G	G	G	G	G		G				G	G		G	G	G	G	G	G	G	**G**	G	G	G	G	G	G	G	G	G	G	G	**G**
Gram stain							0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
Motility							10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
Pigment							0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
Morphology							10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
Oxidase							10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10		**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
Growth on TCBS		10		10		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
TCBS - sucrose positive		10		10		10	10	10	10	10	10	10	10	10	10	0	10	10	10	10	10	10	10	10	**10**	10	10	10	10	0	?	?	10	10	0	10	**7.8**
Oxidation of glucose							5	5		5	5	0	5	5	5											5	5	5	5								**5.0**
Fermentation of glucose							10	10		10	10	10	10	10	10										**10**	10	10	10	10								**10.0**
Swarming							0	0		0	0	0	0	0	0	0		0		0		0			**0**	0	0	0	0		0	0	0	0	0		**0.0**
Luminesence							0	0		0	0	0	0	0	0	0		0		0		0			**0**	0	0	0	0	0	0	0	0	0	0		**0.0**
growth 4 °C							10	10		10	10	10	10	10	10										**10**	10	10	10	10								**10.0**
Growth 20 °C							10	10		10	10	10	10	10	10										**10**	10	10	10	10								**10.0**
Growth 37 °C							5	5		10	0	10	10	5	10										**7**	5	0	0	0								**1.3**
Growth on CLED medium							0	0		0	0	0	0	0											**0**	0	0	0	0								**0.0**
Arginine decarboxylase							10	10		10	10	10	10	10	10										**10**	10	10	10	10								**10.0**
Lysine decarboxylase							5	0		0	5	0	10	0	5										**3**	0	5	0	10								**3.8**
Ornithine decarboxylase							0	0		0	0	0	0	0	0										**0**	0	0	0	0								**0.0**
Indole							0	0		5	5	0	5	5	0										**3**	0	0	5	10								**3.8**
ONPG							0	0		5	0	10	5	10	NG										**4**	0	NG	0	NG								**0.0**
Nitrate reduction							10	10		10	10	10	10	10	10										**10**	10	10	10	10								**10.0**

[a] G more than 3 positive reactions in BIOLOG GN microplate; NG not enough positive reactions for identification using BIOLOG
[b] Where no data is shown tests were not done.
*Denotes isolate 16S rDNA gene partially sequenced- refer to Figure 6 for phylogenetic affiliation.

Appendix 2d (continued)

V. splendidus group 1

Charcater	OFA1-15	OFA1-17	OFA1-19	OFF2-14	OFF2-16	OFF2-18	SFF4-01	SFF4-03	SFF4-05	SFF4-07	SFF4-10	SFF4-14	SFF4-17	SFF4-19	AE1-26	FFW2-16	MFF1-03	MFF1-10	MFF1-14	MFF2-03	MFF2-05	MFF2-12	MA1-03	MA1-09	**Group average**
Voges Proskauer							0	0		0	0	0	0	0	0										**0**
0/129 10 µg		0	50		0		0	0	0		0	0	0	0	0	0	0	0	0	0	0	0		0	**3**
0/129 150 µg		8	8		0		0	0	8		8	8	3	8	8	0	8	8	8	3	8	0		3	**5**
Agar digestion (agarase)		0					0	0			0	0			0	0			0	0				0	**0**
Aesculin hydrolysis								5			0	10													**5**
Haemolysis srbc	0	10	10		10		?	0		10	?	10	10	5	?		0	10	0	10	10		0	5	**6**
Acid arbutin							10	10		0	0	10	10	0	0										**5**
Acid salicin							10	10		0	0	10	10	0	0										**5**
Acid sucrose							10	0		0	10	10	10	10	10										**8**
Catalase							10	10		10	10	10	10	10	10										**10**
Utilisation of single carbon sources (BIOLOG GN):																									
Acetic acid	10	5	10	10	10	5	10	10	10		10				5	0		5	5	5	10	10	5	10	**8**
n- Acetyl-d-galatosamine	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
n- Acetyl-d-glucosamine	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
cis-Aconitic acid	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
Adonitol	5	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
D-Alaninamide	0	0	0	0	5	0	0	0	0		0				5	0		5	0	0	5	5	5	0	**2**
Alanine	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
L-Alanine	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
L-Alanyl glycine	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
2- Amino ethanol	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	5	0	0	0	**0**
D-L-Arabinose	10	0	10	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**1**
Arabitol	10	0	5	10	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**1**
L-Asparagine	10	10	10	5	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
L-Aspartic acid	10	10	10	5	10	10	10	10	10		10				10	10		10	10	10	10	10	5	10	**10**
γ-Amino butyric acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	5	0	**0**

V. splendidus group 2

Charcater	SFF2-13	SFF2-20	AE1-31	SFF1-19	MFF1-13	SYS4-13	SYS4-17	SYS4-23	SYS4-30	SYS6-17	SYS6-19	**Group average**
Voges Proskauer	0	0	0	0								**0**
0/129 10 µg	0	0	0		0	0	0	0	0	0	0	**0**
0/129 150 µg	0	0	5+		0	0	0	0	0	0	0	**0**
Agar digestion (agarase)	0	0	0	0	0	0	0	0	0	0	0	**0**
Aesculin hydrolysis	0	10	5	0								**4**
Haemolysis srbc	10	10	10	10	10(B)							**10**
Acid arbutin	0	0	0	0								**0**
Acid salicin	0	0	0	0								**0**
Acid sucrose	0	10	10	10								**8**
Catalase	10	10	10	5								**8**
Utilisation of single carbon sources (BIOLOG GN):												
Acetic acid	5	5	5	5	10	10	5	10	10	10	10	**8**
n- Acetyl-d-galatosamine	0	0	0	0	0	0	0	0	0	0	0	**0**
n- Acetyl-d-glucosamine	10	10	10	10	10	10	10	10	10	10	10	**10**
cis-Aconitic acid	10	10	10	10	10	10	10	10	10	10	10	**10**
Adonitol	0	0	0	0	0	0	0	0	0	0	0	**0**
D-Alaninamide	0	0	0	0	0	0	5	5	5	0	0	**1**
Alanine	5	5	10	10	10	10	10	10	10	10	10	**9**
L-Alanine	10	10	10	10	10	10	10	10	10	10	10	**10**
L-Alanyl glycine	10	10	10	10	10	10	10	10	10	10	10	**10**
2- Amino ethanol	0	0	0	0	0	0	0	0	0	0	0	**0**
D-L-Arabinose	0	0	0	0	5	0	0	0	0	0	0	**1**
Arabitol	0	0	0	0	0	0	0	0	0	0	0	**0**
L-Asparagine	10	10	10	10	10	10	10	10	10	10	10	**10**
L-Aspartic acid	5	10	10	10	10	10	10	10	10	10	10	**10**
γ-Amino butyric acid	0	0	0	0	0	0	0	0	0	0	0	**0**

Appendix 2d (continued)

V. splendidus group 1

Utilisation of single carbon sources (BIOLOG GN):	OFA1-15	OFA1-17	OFA1-19	OFF2-14	OFF2-16	OFF2-18	SFF4-01	SFF4-03	SFF4-05	SFF4-07	SFF4-10	SFF4-14	SFF4-17	SFF4-19	AE1-26	FFW2-16	MFF1-03	MFF1-10	MFF1-14	MFF2-03	MFF2-05	MFF2-12	MA1-03	MA1-09	**Group average**
Bromo-succinic acid	5	0	5	5	10	10	10	10	10		10				5	5		10	5	5	10	10	0	5	**7**
2,3 Butanediol	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
D,L-Carnitine	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	5	0	0	0	**0**
Cellobiose	0	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
Citric acid	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
α-Cyclodextrin	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
Dextrin	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
I- Erythritol	0	0	0	5	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
Formic acid	10	5	10	0	5	10	0	0	0		0				0	0		5	0	5	5	5	0	0	**3**
D-Fructose	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	5	10	**10**
L-Fucose	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	5	5	0	0	**1**
D- Galactonic acid lactone	0	0	0	0	5	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
D-Galactose	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
D-Galacturonic acid	0	0	0	0	5	0	0	0	0		0				0	0		0	0	0	0	0	5	0	**0**
Gentiobiose	0	0	0	0	5	0	10	10	5		10				0	0		0	0	0	5	0	5	0	**3**
D-Gluconic acid	10	10	10	10	10	10	10	10	10		10				10	0		10	10	10	10	10	10	10	**10**
D-Glucosamic acid	0	0	0	0	5	0	0	0	0		0				0	10		0	0	0	5	0	5	0	**1**
α-D-Glucose	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	5	10	**10**
Glucose-1-phosphate	0	10	5	10	10	10	5	0	10		0				0	10		10	10	10	10	5	5	5	**7**
Glucose-6-phosphate	0	10	10	10	10	10	10	10	10		5				5	10		10	10	10	10	10	10	10	**9**
D-Glucuronic acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
L-Glutamic acid	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
Glycerol	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
D-L-α-Glycerol phosphate	10	10	10	10	10	10	10	10	5		10				10	0		10	10	10	10	10	10	10	**9**
Glycyl-L-aspartic acid	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
Glycyl-L-glutamic acid	0	10	10	10	10	10	10	10	10		10				10	10		1	10	10	10	10	10	10	**9**

V. splendidus group 2

Utilisation of single carbon sources (BIOLOG GN):	SFF2-13	SFF2-20	AE1-31	SFF1-19	MFF1-13	SYS4-13	SYS4-17	SYS4-23	SYS4-30	SYS6-17	SYS6-19	**Group average**
Bromo-succinic acid	5	5	5	10	5	5	10	5	10	5	10	**7**
2,3 Butanediol	0	0	0	0	0	0	0	0	0	0	0	**0**
D,L-Carnitine	0	0	0	0	0	0	0	0	0	0	0	**0**
Cellobiose	10	10	10	10	10	10	10	10	10	10	10	**10**
Citric acid	10	10	10	10	10	10	10	10	10	10	10	**10**
α-Cyclodextrin	0	0	0	0	0	10	0	0	0	0	0	**1**
Dextrin	10	10	10	10	10	10	10	10	10	10	10	**10**
I- Erythritol	0	0	0	0	0	0	0	0	0	0	0	**0**
Formic acid	0	0	0	5	10	10	10	0	10	10	10	**6**
D-Fructose	10	10	10	10	10	10	10	10	10	10	10	**10**
L-Fucose	0	0	0	0	0	0	0	0	0	0	0	**0**
D- Galactonic acid lactone	5	0	0	0	0	0	0	0	0	0	0	**1**
D-Galactose	10	10	10	10	10	10	10	10	10	10	10	**10**
D-Galacturonic acid	0	0	0	0	0	0	0	0	0	0	0	**0**
Gentiobiose	0	0	0	0	5	0	0	5	0	0	0	**1**
D-Gluconic acid	10	10	10	10	10	10	10	10	10	10	10	**10**
D-Glucosamic acid	0	0	0	0	0	0	0	0	0	0	0	**0**
α-D-Glucose	10	10	10	10	10	10	10	10	10	10	10	**10**
Glucose-1-phosphate	10	10	10	10	10	10	10	10	10	10	10	**10**
Glucose-6-phosphate	10	10	10	10	10	10	10	10	10	10	10	**10**
D-Glucuronic acid	0	0	0	0	10	0	0	0	0	0	0	**1**
L-Glutamic acid	10	10	10	10	10	10	10	10	10	10	10	**10**
Glycerol	10	10	10	10	10	10	10	10	10	10	10	**10**
D-L-α-Glycerol phosphate	10	5	5	10	10	10	10	10	10	10	10	**9**
Glycyl-L-aspartic acid	10	10	10	10	10	10	10	10	10	10	10	**10**
Glycyl-L-glutamic acid	10	10	10	10	10	10	10	10	10	10	10	**10**

Appendix 2d (continued)

Utilisation of single carbon sources (BIOLOG GN):	*V. splendidus* group 1																								*V. splendidus* group 2											
	OFA1-15	OFA1-17	OFA1-19	OFF2-14	OFF2-16	OFF2-18	SFF4-01	SFF4-03	SFF4-05	SFF4-07	SFF4-10	SFF4-14	SFF4-17	SFF4-19	AE1-26	FFW2-16	MFF1-03	MFF1-10	MFF1-14	MFF2-03	MFF2-05	MFF2-12	MA1-03	MA1-09	**Group average**	SFF2-13	SFF2-20	AE1-31	SFF1-19	MFF1-13	SYS4-13	SYS4-17	SYS4-23	SYS4-30	SYS6-17	SYS6-19	**Group average**
Glucuronamide	0	0	0	0	5	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**	0	0	0	0	5	0	0	0	0	0	0	**1**
Glycogen	5	10	5	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
L-histidine	10	0	10	5	10	10	10	10	10		5				5	5		10	10	5	10	10	5	5	**8**	0	0	10	10	5	10	10	10	10	10	5	**7**
α-Hydroxy butyric acid	0	0	0	0	0	0	0	0	0		0				0	0		5	0	0	0	0	5	0	**1**	0	0	0	0	5	0	0	0	0	0	0	**1**
β-Hydroxy butyric acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
γ-Hydroxy butyric acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
Hydroxy L-proline	0	0	0	0	10	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	**0**
p-Hydroxy phenylacetic acid	0	0	0	0	5	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
Inosine	10	10	10	10	10	10	10	10	10		10				10	0		10	10	10	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
meso-Inositol	5	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
Itaconic acid	0	0	0	0	0	0	0	0	5		0				0	0		5	0	5	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	**0**
α-Ketobutyric acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
α-Ketoglutaric acid	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
α-Ketovaleric acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
D,L-lactic acid	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
α-lactose	0	0	0	10	0	0	50	0	0		5				0	0		5	5	10	5	0	5	5	**5**	0	0	0	0	0	0	0	0	0	10	0	**1**
α-D-lactose-lactulose	0	0	0	0	0	0	10	0	0		0				0	0		0	0	0	0	0	5	0	**1**	0	0	0	0	0	0	0	0	0	0	0	**0**
L-Leucine	0	0	0	0	0	0	0	0	0		0				0	0		0	0	5	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**
Malonic acid	0	0	0	0	5	0	0	0	0		0				0	0		0	5	0	0	0	5	0	**1**	0	0	0	0	5	0	0	0	0	0	0	**1**
D-Mannitol	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**	10	10	10	10	10	10	10	10	10	10	10	**10**
D-Mannose	0	10	10	10	10	10	10	10	10		10				10	10		10	10	5	10	10	5	10	**9**	10	10	10	10	10	10	10	10	10	10	10	**10**
D-Melibiose	0	0	0	0	5	0	0	0	5		5				5	0		0	5	0	5	5	0	5	**2**	0	0	5	0	10	0	0	0	5	0	0	**2**
Methyl pyruvate	10	10	10	5	10	10	10	10	10		10				10	5		10	10	10	10	10	10	10	**10**	0	5	10	10	10	10	10	10	10	10	10	**9**
β-Methyl-D-glucoside	5	0	0	0	0	0	5	0	0		5				0	0		0	0	0	0	5	0	0	**1**	0	0	0	0	0	0	0	0	0	0	0	**0**
Mono-methyl succinate	0	0	10	0	10	10	0	0	5		10				0	0		5	5	0	0	5	0	0	**3**	0	0	0	0	0	10	10	10	10	5	10	**5**
L-Ornithine	10	0	10	5	10	5	5	5	5		0				0	0		10	10	5	10	10	5	5	**6**	0	0	0	5	5	0	0	5	5	0	0	**2**
L-Phenyl alanine	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	**0**

Appendix 2d (continued)

V. splendidus group 1

Characteristic	OFA1-15	OFA1-17	OFA1-19	OFF2-14	OFF2-16	OFF2-18	SFF4-01	SFF4-03	SFF4-05	SFF4-07	SFF4-10	SFF4-14	SFF4-17	SFF4-19	AE1-26	FFW2-16	MFF1-03	MFF1-10	MFF1-14	MFF2-03	MFF2-05	MFF2-12	MA1-03	MA1-09	**Group average**
Maltose	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
Phenyl ethylamine	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
L-Proline	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
Propionic acid	10	0	10	0	10	5	5	10	10		0				10	0		5	5	5	10	10	5	5	**6**
Psicose	5	0	5	5	5	10	10	10	10		10				10	5		5	5	0	0	5	0	0	**5**
Putrescine	0	0	0	0	0	0	0	0	0		5				0	0		0	0	0	0	0	0	0	**0**
L-Pyro glutamic acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	5	0	0	**0**
Quinic acid	0	0	0	0	0	0	0	0	5		0				0	0		0	0	0	5	5	0	0	**1**
D-Raffinose	0	0	0	0	5	0	0	0	0		0				0	0		10	0	0	5	0	5	0	**1**
L-Rhamnose	0	0	0	0	5	0	0	0	0		0				0	0		0	0	0	5	0	5	0	**1**
D-Saccharic acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
Sebacic acid	0	0	0	0	0	0	0	0	0		0				0	0		0	0	0	0	0	0	0	**0**
D-Serine	0	0	0	0	0	0	5	0	5		0				0	0		0	0	5	0	0	5	0	**1**
L-Serine	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**
D-Sorbitol	0	0	0	0	10	5	5	5	10		0				5	0		5	5	0	5	5	5	5	**4**
Succinamic acid	0	0	0	0	5	0	5	0	5		5				0	0		0	5	0	5	5	0	5	**2**
Sucrose	10	10	10	10	10	0	10	10	10		10				10	0		5	10	10	10	10	10	10	**9**
Succinic acid	0	10	10	10	10	10	10	10	10		10				10	10		10	10	5	10	10	0	10	**9**
Thymidine	10	10	10	10	10	10	10	10	10		10				10	0		10	10	10	10	10	10	10	**10**
D-Trehalose	10	10	10	10	10	10	10	10	10		10				10	10		10	5	10	10	10	5	10	**10**
Turanose	0	0	0	0	0	5	10	5	10		5				5	0		10	5	0	0	5	0	0	**3**
Tween 40	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	5	10	**10**
Tween 80	10	10	5	5	5	10	10	10	10		10				10	10		10	5	5	5	10	0	5	**8**
Uridine	10	10	10	10	10	10	10	10	10		10				10	0		10	10	10	10	10	10	10	**10**
Urocanic acid	0	0	0	0	0	0	0	0	5		0				0	0		0	0	0	0	0	0	0	**0**
Xylitol	0	0	0	0	10	0	0	0	0		0				0	0		5	0	0	0	0	0	0	**1**
L-Threonine	10	10	10	10	10	10	10	10	10		10				10	10		10	10	10	10	10	10	10	**10**

V. splendidus group 2

Characteristic	SFF2-13	SFF2-20	AE1-31	SFF1-19	MFF1-13	SYS4-13	SYS4-17	SYS4-23	SYS4-30	SYS6-17	SYS6-19	**Group average**
Maltose	10	10	10	10	10	10	10	10	10	10	10	**10**
Phenyl ethylamine	0	0	0	0	0	0	0	0	0	0	0	**0**
L-Proline	10	10	10	10	10	10	10	10	10	10	10	**10**
Propionic acid	0	0	5	0	10	5	10	5	5	10	10	**6**
Psicose	5	10	10	10	5	10	10	5	10	5	10	**8**
Putrescine	0	0	0	0	0	0	0	0	0	0	0	**0**
L-Pyro glutamic acid	0	0	0	0	5	0	0	0	0	0	0	**1**
Quinic acid	0	0	0	0	0	0	0	0	0	0	0	**0**
D-Raffinose	0	0	0	0	5	0	0	0	0	0	0	**1**
L-Rhamnose	0	0	0	0	0	0	0	0	0	0	0	**0**
D-Saccharic acid	0	0	0	0	0	0	0	0	0	0	0	**0**
Sebacic acid	0	0	0	0	0	0	0	0	0	0	0	**0**
D-Serine	0	0	0	0	0	0	0	5	5	0	0	**1**
L-Serine	10	10	10	10	10	10	10	10	10	10	10	**10**
D-Sorbitol	5	5	5	0	0	10	10	5	5	5	5	**5**
Succinamic acid	0	0	0	0	5	0	0	5	5	0	0	**1**
Sucrose	0	10	0	10	5	0	0	0	0	0	0	**2**
Succinic acid	10	10	10	10	10	10	10	10	10	10	10	**10**
Thymidine	10	10	10	10	10	10	10	10	10	10	10	**10**
D-Trehalose	10	10	10	10	10	10	10	10	10	10	10	**10**
Turanose	5	10	5	10	0	0	5	5	5	0	5	**5**
Tween 40	5	10	10	10	10	10	10	10	10	10	10	**10**
Tween 80	0	5	10	10	10	10	10	10	10	10	10	**9**
Uridine	10	10	10	10	10	10	10	10	10	10	10	**10**
Urocanic acid	0	0	0	5	0	0	0	0	5	0	0	**1**
Xylitol	0	0	0	0	0	0	0	0	0	0	0	**0**
L-Threonine	10	10	10	10	10	10	10	10	10	10	10	**10**

Appendix 2d (continued). Antibiotic sensitivity and resistance of *V. splendidus* groups 1 and 2.

V. splendidus group 1

Antibiotic sensitivity:	OFA1-15	OFA1-17	OFA1-19	OFF2-14	OFF2-16	OFF2-18	SFF4-01	SFF4-03	SFF4-05	SFF4-07	SFF4-10	SFF4-14	SFF4-17	SFF4-19	AE1-26	FFW2-16	MFF1-03	MFF1-10	MFF1-14	MFF2-03	MFF2-05	MFF2-12	MA1-03	MA1-09	**Group average**
Chloramphenicol 25 µg		10	10	10	10	10	0	3	0	0	5	10	10	10	10	10	5	10	5	10	10	10	10	10	**8**
Erythromycin 5 µg		5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Fusidic acid 10 µg		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Methicillin 10 µg		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Novobiocin 5 µg		5	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10**
Penicillin G 1 unit		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
Streptomycin 10 µg		5	10	10	0	0	0	0	0	0	0	0	0	5	5	0	5	10	3	10	0	10	10	5	**4**
Tetracycline 25 µg		0	0	10	5	10	0	0	3	0	10	10	3	5	10	10	0	0	0	0	0	0	0	0	**3**
Tetracycline 100 µg		10	0	10	5	10	0	10	5	10	10	10	5	10	10	10	0	0	0	0	0	0	0	0	**5**
Ampicillin 10 µg		5	10	5	5	0	3	3	3	3	3	3	3	0	10	10	0	0	0	0	0	0	0	0	**3**
Ampicillin 25 µg		10	10	10	10	0	3	3	3	3	3	3	3	3	10	10	0	0	0	0	0	0	0	0	**4**
Cephalothin 5 µg		5	0	10	0	3	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	3	**1**
Colistin sulphate 25 µg		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10**
Gentamycin 10 µg		10	10	10	0	0	10	5	5	10	5	10	5	5	10	5	5	10	10	10	5	10	10	10	**8**
Sulphatriad 200 µg		0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	**0**
Cotrimoxazole 25 µg		10	10	10	3	10	0	0	3	0	0	0	0	3	10	0	0	0	0	0	0	0	0	0	**3**
Nitrofurantoin 50µg		10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10**
Ticarcillin 75 µg		5	10	0	10	0	0	3	3	3	5	10	3	3	10	10	0	0	0	0	0	0	0	0	**3**
Nalidixic acid 30 µg		5	5	10	10	10	0	0	0	0	0	0	0	0	5	10	0	3	0	10	10	0	0	0	**3**
Trimethoprim 2.5 µg		10	0	5	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	**1**
Sulphamethoxazole 50µg		0	0	0	0	0	0	3	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	**0**

V. splendidus group 2

Antibiotic sensitivity:	SFF2-13	SFF2-20	AE1-31	SFF1-19	MFF1-13	SYS4-13	SYS4-17	SYS4-23	SYS4-30	SYS6-17	SYS6-19	**Group average**
Chloramphenicol 25 µg	10	10		10	10	10	10	10	10	10	10	**10**
Erythromycin 5 µg	0	0		0	0	3	0	0	0	0	0	**0**
Fusidic acid 10 µg	0	0		0	0	0	0	0	0	0	0	**0**
Methicillin 10 µg	0	0		0	0	0	0	0	0	0	0	**0**
Novobiocin 5 µg	10	10		10	10	10	10		10	10	10	**10**
Penicillin G 1 unit	0	0		0	0	0	0		0	3	0	**0**
Streptomycin 10 µg	0	0		0	0	0	0		0	0	0	**0**
Tetracycline 25 µg	10	10		10	5	10	10		10	10	10	**9**
Tetracycline 100 µg	10	10		10	0	10	10		10	10	10	**9**
Ampicillin 10 µg	10	10		0	0	10	10		10	5	0	**6**
Ampicillin 25 µg	10	10		0	0	10	10		10	10	0	**7**
Cephalothin 5 µg	0	10		0	10	0	0		0	0	0	**2**
Colistin sulphate 25 µg	10	10		10	10	10	10		10	10	10	**10**
Gentamycin 10 µg	10	5		3	5	0	0		5	10	3	**4**
Sulphatriad 200 µg	0	0		5	0	0	10		0	0	5	**2**
Cotrimoxazole 25 µg	10	10		5	0	0	10		0	0	5	**4**
Nitrofurantoin 50µg	10	10		10	10	10	10		10	10	10	**10**
Ticarcillin 75 µg	10	10		0	0	10	10		10	10	0	**7**
Nalidixic acid 30 µg	0	0		0	0	10	10		5	0	0	**3**
Trimethoprim 2.5 µg	0	0		0	0	0	0		0	0	0	**0**
Sulphamethoxazole 50µg	0	0		0	0	0	0		5	0	0	**1**

10 = > 2mm zone of clearing outside of test disc, 5 = < 2mm zone of clearing, 2.5 = disruption of growth, 0 = resistant.

Appendix 2e Biochemical, physiological and antibiotic susceptibility test results for isolates classified as *Vibrio fisheri*-like, *Photobacterium phosphoreum*, *Vibrio salmonicida*-like and '*Vibrio splendidus*' group 3. Each test was scored on a scale of between 0 –10 for a strongly positive reaction. Isolates are coded in Table 1.

	V. fischeri-like				*P. phosphoreum*										*V. salmonicida*-like							'*V. splendidus*' group 3			
Character	W2-16	W2-21	W2-07*	**Group average**	OFE1-04	SFF3-01	SFF3-03	E1-01	E1-02	E1-03	S1-01	S1-04*	S2-03	**Group average**	W1-08*	OFF1-01	OFF1-05*	OFF1-10	OFF2-17	OFF2-13	**Group average**	SYS6-10*	TG4--07*	TG4-14	**Group average**
PCR-RFLP pattern	X	X	X	**X**		X		X		X	X	X		**X**	XII	XII	XII	XII	XII	XII	**XII**	Xib	Xib		**Xib**
[a]Biolog	G	G	G	**G**	G	G		G	G	G	G	G	G	**G**	G		G		G	G	**G**	G	G	G	**G**
Gram stain	0	0	0	**0**	[c]	0	0	0	0	0	0	0	0	**0**	0		0				**0**	0	0	0	**0**
Motility	10	10	10	**10**		5	5	5	5	5	5	5	5	**5**	10							10	10	10	**10**
[b]Pigment	0	0	0	**0**		0	0	0	0	0	0	0	0	**0**	0							0	0	0	**0**
Morphology	10	10	10	**10**		10	10	10	10	10	10	10	10	**10**	10							10	10	10	**10**
Oxidase	10	10	10	**10**		5	5	5	5	5	5	5	5	**5**	10							10	10	10	**10**
TCBS	10	10	10	**10**		10	10	10	10	10	10	10	10	**10**	10		10		10		**10**	10	10	10	**10**
TCBS – sucrose positive	10	10	10	**10**		0	0	0	0	0	0	0	0	**0**	0		0		0		**0**	0			**0**
Oxidation of glucose			10			10				10	10	10	10	**10**	0							0			**0**
Fermentation of glucose			0			10				10	10	10	10	**10**	10							10			**10**
Swarming			0	**0**	0	0	0	0	0	0	0	0	0	**0**	0					0	**0**	0			**0**
Luminesence			0	**0**	0	10	10	10	10	10	10	10	10	**10**	0					0	**0**	0			**0**
Growth at 4 °C			10	**10**	10	10				10	10	10	10	**10**	10						**10**	10			**10**
Growth at 20 °C			10	**10**	10	10				10	10	10	10	**10**	10						**10**	10			**10**
Growth at 37 °C			0	**0**		5				0	0	0	0	**1**	0						**0**	0			**0**
CLED			0	**0**		0				0	0	0	0	**0**	0						**0**	0			**0**
Arginine decarboxylase			10	**10**		10		10	10	10	10	10	10	**10**	10						**10**	10			**10**
Lysine decarboxylase			10	**10**		10		10	10	10	10	10	10	**10**	10						**10**	0			**0**
Ornithine decarboxylase			0	**0**		10		0	0	5	5	5	0	**2.5**	0						**0**	0			**0**
Nitrate reduction			10	**10**		10 10	10	10	10	10	10	10	10	**10**	10						**10**	10			**10**

[a] G more than 3 positive reactions in BIOLOG GN microplate; NG not enough positive reactions for identification using BIOLOG
[b] Pigment production. Y, yellow; B, black; Br, brown.
[c] Where no data is shown tests were not done.
*Denotes isolate 16S rDNA gene partially sequenced- refer to Figure 6 for phylogenetic affiliation.

Appendix 2e (continued)

Character	*V. fischeri*-like				*P. phosphoreum*										*V. salmonicida*-like							'V. *splendidus*' group 3			
	W2-16	W2-21	W2-07	**Group average**	OFE1-04	SFF3-01	SFF3-03	E1-01	E1-02	E1-03	S1-01	S1-04	S2-03	**Group average**	W1-08	OFF1-01	OFF1-05	OFF1-10	OFF2-17	OFF2-13	**Group average**	SYS6-10	TG4-07	TG4-14	**Group average**
Indole			10	**10**		0	0	0	0	0	NG	NG	NG	**0**	10						**10**	NG			**0**
ONPG			ng	**ng**		10	0	0	0	10	0	10	0	**3**	0						**0**	NG			**0**
Voges Proskauer						0	0	10	0	0	0	0	0	**1**	0						**0**	0			**0**
0/129 10 µg	10	0	0								0	0	0	**0**	10						**10**	0			**0**
0/129 150 µg	10	0	0								10	0	5		10						**10**	0			**0**
Agar digestion (agarase)			0	**0**		0	0			0	0	0	0	**0**	0						**0**	0			**0**
Aesculin hydrolysis			0	**0**		0				0	0	0	0	**0**	0						**0**	0			**0**
Haemolysis srbc			0	**0**		0				0	0	0	0	**0**	0				0	0	**0**	10			
Acid arbutin			0	**0**		0				0	0	0	0	**0**	0						**0**	0			
Acid salicin			ng	**ng**		0				0	0	0	0	**0**	0						**0**	0			
Acid sucrose			10	**10**		0				0	0	0	0	**0**	0						**0**	0			
Catalase			10	**10**		10				10	10	10	10	**10**	10						**10**	10			
Utilisation of single carbon sources (BIOLOG GN):																									
Acetic acid	5	0	0	**1.7**	0	0		0	0	5	5	5	10	**3**	10		0		0	0	**2.5**	0	10	10	**6.7**
n- Acetyl-d-galatosamine	0	5	0	**1.7**	0	0		0	0	10	5	0	10	**3**	5		0		0	0	**1.3**	0	10	10	**6.7**
n- Acetyl-d-glucosamine	10	10	10	**10**	10	10		10	10	10	10	10	10	**10**	10		10		10	10	**10**	10	10	10	**10.0**
cis-Aconitic acid	0	0	0	**0.0**	0	0		0	0	10	0	0	0	**1**	5		0		0	0	**1.3**	5	10	10	**8.3**
Adonitol	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**1**	5		0		0	0	**1.3**	0	10	5	**5.0**
D-Alaninamide	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0**	0		0		0	0	**0.0**	0	0	0	**0.0**
Alanine	0	0	0	**0.0**	0	0		0	0	5	0	0	5	**1**	0		0		0	0	**0.0**	5	10	10	**8.3**
L-Alanine	5	0	0	**1.7**	0	0		5	10	10	10	10	10	**8**	5		5		0	0	**2.5**	10	0	0	**3.3**
L-Alanyl glycine	5	0	0	**1.7**	0	0		5	10	10	10	10	10	**8**	10		10		0	0	**5.0**	10	0	0	**3.3**
2- Amino ethanol	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**1**	0		0		0	0	**0.0**	0	0	0	**0.0**
γ-Amino butyric acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0**	0		0		0	0	**0.0**	0	0	0	**0.0**
D-L-Arabinose	0	0	0	**0.0**	0	0		0	0	10	0	0	0	**1**	0		0		0	0	**0.0**	0	0	0	**0.0**
Arabitol	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0**	0		0		0	0	**0.0**	0	10	10	**6.7**
L-Asparagine	10	10	10	**10**	0	0		0	0	5	5	10	10	**4**	10		10		0	5	**6.3**	10	5	0	**5.0**

Appendix 2e (continued)

Utilisation of single carbon sources (BIOLOG GN):	*V. fischeri*-like							*P. phosphoreum*							*V. salmonicida*-like							'*V. splendidus*' group 3			
	W2-16	W2-21	W2-07	**Group average**	OFE1-04	SFF3-01	SFF3-03	E1-01	E1-02	E1-03	S1-01	S1-04	S2-03	**Group average**	W1-08	OFF1-01	OFF1-05	OFF1-10	OFF2-17	OFF2-13	**Group average**	SYS6-10	TG4-07	TG4-14	**Group average**
L-Aspartic acid	10	10	0	**6.7**	0	0		0	0	45	5	10	10	**10**	10		10		0	0	**5.0**	5	5	0	**3.3**
Bromo-succinic acid	5	0	0	**1.7**	0	0		0	0	0	0	0	5	**1**	5		0		0	0	**1.3**	0	0	0	**0.0**
2,3 Butanediol	0	0	0	**0.0**	0	0		0	0	10	0	0	0	**1**	0		0		0	0	**0.0**	0	10	10	**6.7**
D,L-Carnitine	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0**	0		0		0	0	**0.0**	0	0	0	**0.0**
Cellobiose	10	0	0	**3.3**	0	0		0	0	10	0	0	0	**1**	10		0		10	0	**5.0**	10	5	0	**5.0**
Citric acid	0	0	0	**0.0**	0	0		0	0	10	0	0	0	**1**	5		0		0	0	**1.3**	5	0	0	**1.7**
α-Cyclodextrin	0	0	0	**0.0**	0	0		0	0	10	0	0	0	**1**	0		0		0	0	**0.0**	0	10	10	**6.7**
Dextrin	10	10	10	**10**	10	0		0	5	10	5	10	10	**6**	10		10		10	10	**10**	10	10	10	**10.0**
I- Erythritol	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0**	0		0		0	0	**0.0**	0	5	0	**1.7**
Formic acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0**	0		0		0	0	**0.0**	5	0	0	**1.7**
D-Fructose	10	10	10	**10**	10	10		10	10	10	10	10	0	**7**	10		10		10	10	**10**	10	0	0	**3.3**
L-Fucose	0	0	0	**0.0**	0	0		0	0	0	0	0	10	**1**	5		0		0	0	**1.3**	0	10	10	**6.7**
D- Galactonic acid lactone	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0**	0		0		0	0	**0.0**	0	5	0	**1.7**
D-Galactose	0	0	0	**0.0**	0	10		10	10	0	10	10	10	**7**	5		10		0	0	**3.8**	10	5	0	**5.0**
D-Galacturonic acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0**	0		0		0	0	**0.0**	0	0	0	**0.0**
Gentiobiose	10	0	0	**3.3**	0	0		0	0	0	0	0	0	**0**	10		0		10	0	**5.0**	0	5	0	**1.7**
D-Gluconic acid	10	10	10	**10**	10	0		0	0	0	0	0	0	**4**	10		10		10	0	**7.5**	10	10	10	**10.0**
D-Glucosamic acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	5	0	**1.7**
α-D-Glucose	10	10	10	**10**	10	10		10	10	5	10	10	10	**0.8**	10		10		10	10	**10**	10	0	0	**3.3**
Glucose-1-phosphate	0	0	0	**0.0**	0	0		10	10	5	10	10	10	**0.8**	0		0		0	10	**2.5**	0	5	5	**3.3**
Glucose-6-phosphate	0	0	0	**0.0**	0	10		10	10	0	10	10	10	**0.7**	0		0		0	10	**2.5**	0	0	0	**0.0**
Glucuronamide	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	5		0		0	0	**1.3**	0	0	0	**0.0**
D-Glucuronic acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	10	5	**5.0**
L-Glutamic acid	10	10	10	**10**	0	0		0	5	5	0	5	10	**0.4**	10		10		10	10	**10**	10	10	5	**8.3**
Glycerol	10	10	10	**10**	10	0		0	0	5	0	5	0	**0.1**	10		10		0	0	**5.0**	0	0	0	**0.0**
D-L-α-Glycerol phosphate	0	0	0	**0.0**	0	0		0	0	10	10	5	20	**0.6**	5		10		0	0	**3.8**	0	0	0	**0.0**
Glycyl-L-aspartic acid	10	5	0	**5.0**	5	0		0	5	5	5	10	10	**0.5**	10		10		0	0	**5.0**	10	0	0	**3.3**

Appendix 2e (continued)

Utilisation of single carbon sources (BIOLOG GN):	*V. fischeri*-like					*P. phosphoreum*									*V. salmonicida*-like							'*V. splendidus*' group 3			
	W2-16	W2-21	W2-07	**Group average**	OFE1-04	SFF3-01	SFF3-03	E1-01	E1-02	E1-03	S1-01	S1-04	S2-03	**Group average**	W1-08	OFF1-01	OFF1-05	OFF1-10	OFF2-17	OFF2-13	**Group average**	SYS6-10	TG4-07	TG4-14	Group average
Glycyl-L-glutamic acid	5	5	5	**5.0**	0	0		0	5	10	5	10	10	**0.6**	10		10		0	0	**5.0**	10	0	0	3.3
Glycogen	5	5	0	**3.3**	0	0		0	0	5	0	5	5	**0.2**	10		0		0	10	**5.0**	10	0	0	3.3
L-histidine	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	0		0		0	0	**0.0**	0	0	0	0.0
α-Hydroxy butyric acid	0	0	0	**0.0**	0	0		0	0	0	0	0	5	**0.1**	0		0		0	0	**0.0**	0	0	0	0.0
β-Hydroxy butyric acid	0	0	0	**0.0**	0	0		0	0	5	0	0	5	**0.1**	0		0		0	0	**0.0**	0	0	0	0.0
γ-Hydroxy butyric acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	0	0	0.0
Hydroxy L-proline	0	5	0	**1.7**	0	0		0	0	5	0	0	0	**0.1**	5		0		0	0	**1.3**	0	0	0	0.0
p-Hydroxy phenylacetic acid	0	0	0	**0.0**	0	0		0	0	0	0	5	0	**0.1**	5		0		0	0	**1.3**	0	0	0	0.0
Inosine	10	10	10	**10**	10	10		10	10	5	10	10	10	**0.8**	10		10		10	10	**10**	10	0	0	3.3
m-Inositol	0	0	0	**0.0**	5	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	0	0	0.0
Itaconic acid	0	5	0	**1.7**	0	0		0	0	0	0	5	5	**0.1**	0		0		0	0	**0.0**	0	0	0	0.0
α-Ketobutyric acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	10	10	6.7
α-Ketoglutaric acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	5	0	0	1.7
α-Ketovaleric acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	5	0	1.7
D,L-lactic acid	0	0	0	**0.0**	0	0		0	10	0	10	10	10	**0.6**	0		0		0	0	**0.0**	10	5	0	5.0
α-lactose	0	5	0	**1.7**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	5	0	1.7
α-D-lactose-lactulose	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	0	0	0.0
L-Leucine	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	10	10	6.7
Malonic acid	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	0		0		0	0	**0.0**	0	5	5	3.3
Maltose	10	10	10	**10**	10	10		10	10	10	10	10	10	**0.9**	10		10		10	5	**8.8**	10	0	0	3.3
D-Mannitol	0	10	10	**6.7**	0	0		0	0	0	0	0	0	**0.0**	5		0		0	0	**1.3**	10	0	0	3.3
D-Mannose	10	0	0	**3.3**	10	10		10	10	10	10	10	10	**0.9**	10		10		10	0	**7.5**	10	0	0	3.3
D-Melibiose	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	5	0	1.7
Methyl pyruvate	0	0	0	**0.0**	0	0		0	10	5	10	10	10	**0.6**	5		0		0	0	**1.3**	0	10	10	6.7
β-Methyl-D-glucoside	0	5	0	**1.7**	0	0		0	0	5	0	0	0	**0.1**	5		0		0	0	**1.3**	0	10	10	6.7
Mono-methyl succinate	0	0	0	**0.0**	0	0		0	0	0	0	0	10	**0.1**	5		0		0	0	**1.3**	0	10	5	5.0
L-Ornithine	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	0		0		0	0	**0.0**	0	5	5	3.3

Appendix 2e (continued)

Utilisation of single carbon sources (BIOLOG GN):	*V. fischeri*-like				*P. phosphoreum*										*V. salmonicida*-like							*'V. splendidus'* group 3			
	W2-16	W2-21	W2-07	**Group average**	OFE1-04	SFF3-01	SFF3-03	E1-01	E1-02	E1-03	S1-01	S1-04	S2-03	**Group average**	W1-08	OFF1-01	OFF1-05	OFF1-10	OFF2-17	OFF2-13	**Group average**	SYS6-10	TG4-07	TG4-14	**Group average**
L-Phenyl alanine	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	0		0		0	0	**0.0**	0	10	5	**5.0**
Phenyl ethylamine	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	5	0	**1.7**
L-Proline	5	0	0	**1.7**	0	0		0	0	10	0	0	5	**0.2**	5		5		0	0	**2.5**	5	10	5	**6.7**
Propionic acid	0	5	0	**1.7**	0	0		0	0	5	0	5	10	**0.3**	0		0		0	0	**0.0**	0	0	0	**0.0**
Psicose	10	5	0	**5.0**	0	0		0	5	5	5	10	10	**0.5**	10		5		0	0	**3.8**	5	0	0	**1.7**
Putrescine	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	0		0		0	0	**0.0**	0	0	0	**0.0**
L-Pyro glutamic acid	0	0	0	**0.0**	0	0		0	0	10	0	0	0	**0.1**	5		0		0	0	**1.3**	0	0	0	**0.0**
Quinic acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	0	0	**0.0**
D-Raffinose	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	5		0		0	0	**1.3**	0	10	5	**5.0**
L-Rhamnose	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	0	0	**0.0**
D-Saccharic acid	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	0		0		0	0	**0.0**	0	0	0	**0.0**
Sebacic acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	10	10	**6.7**
D-Serine	0	0	0	**0.0**	0	0		0	0	10	0	0	0	**0.1**	0		0		0	0	**0.0**	0	10	10	**6.7**
L-Serine	0	0	0	**0.0**	0	0		10	10	10	10	10	10	**0.9**	10		10		0	0	**5.0**	10	0	0	**3.3**
D-Sorbitol	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	0		0		0	0	**0.0**	0	0	0	**0.0**
Succinamic acid	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	10		0		0	0	**2.5**	0	0	0	**0.0**
Sucrose	0	10	10	**6.7**	0	0		0	0	10	0	0	5	**0.2**	0		0		0	10	**2.5**	0	10	10	**6.7**
Succinic acid	5	10	5	**6.7**	0	0		0	0	10	0	5	10	**0.4**	10		10		0	0	**5.0**	5	10	10	**8.3**
L-Threonine	5	0	0	**1.7**	0	10		0	0	5	0	0	10	**0.2**	5		0		0	0	**1.3**	5	5	5	**5.0**
Thymidine	10	10	10	**10**	10	5		5	10	0	10	10	10	**0.6**	10		10		10	0	**7.5**	5	0	0	**1.7**
D-Trehalose	10	0	0	**3.3**	10	0		0	0	10	0	0	0	**0.1**	10		10		10	10	**10**	10	0	0	**3.3**
Turanose	0	0	0	**0.0**	0	0		0	0	0	0	5	0	**0.1**	5		0		0	0	**1.3**	0	0	0	**0.0**
Tween 40	5	5	0	**3.3**	10	0		0	0	10	10	10	10	**0.6**	10		0		5	0	**3.8**	5	0	0	**1.7**
Tween 80	0	0	0	**0.0**	0	0		0	0	10	0	0	10	**0.3**	5		0		0	0	**1.3**	10	0	10	**6.7**
Uridine	10	10	10	**10**	0	5		5	10	0	10	10	10	**0.6**	10		10		5	0	**6.3**	10	10	10	**10.0**
Urocanic acid	0	0	0	**0.0**	0	0		0	0	5	0	0	0	**0.1**	0		0		0	0	**0.0**	0	0	0	**0.0**
Xylitol	0	0	0	**0.0**	0	0		0	0	0	0	0	0	**0.0**	0		0		0	0	**0.0**	0	10	10	**6.7**

Appendix 2e (continued) Antibiotic sensitivity and resistance of isolates classified as *V. fisheri-like, P. phosphoreum and V. splendus* group 3

	V. fischeri-like				*P. phosphoreum*										*V. salmonicida*-like							'V. splendidus' group 3			
Isolate	W2-16	W2-21	W2-07	Group average	OFE1-04	SFF3-01	SFF3-03	E1-01	E1-02	E1-03	S1-01	S1-04	S2-03	**Group average**	W1-08	OFF1-01	OFF1-05	OFF1-10	OFF2-17	OFF2-13	**Group average**	SYS6-10	TG4-07	TG4-14	**Group average**
Antibiotic sensitivity:																									
Chloramphenicol 25 µg	10	10	10	**10**		10	10	10	10	10	10	10	10	**10.0**	10	10	10	10	10	10	**10**	10			
Erythromycin 5 µg	0	0	0	**0.0**		0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0	0	**0.0**	0			
Fusidic acid 10 µg	0	0	0	**0.0**		0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0	10	**1.7**	0			
Methicillin 10 µg	0	0	0	**0.0**		0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0	0	**0.0**	0			
Novobiocin 5 µg	10	10	10	**10**		0	0	0	0	0	0	0	0	**0.0**	10	10	10	10	10	10	**10**	10			
Penicillin G 1 unit	0	0	0	**0.0**		0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0	0	**0.0**	0			
Streptomycin 10 µg	0	0	0	**0.0**		0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0	0	**0.0**	0			
Tetracycline 25 µg	10	10	10	**10**		5	10	10	10	10	10	10	3	**8.4**	10	10	10	10	10	10	**10**	10			
Tetracycline 100 µg	10	10	10	**10**		10	10	10	10	10	10	10	3	**9.1**	10	10	10	10	10	10	**10**	10			
Ampicillin 10 µg	5	3	0	**2.5**		0	0	0	0	0	0	0	0	**0.0**	5	0	0	0	0	0	**0.8**	5			
Ampicillin 25 µg	10	10	10	**10**		0	0	5	0	3	0	0	0	**0.9**	5	0	0	0	0	0	**0.8**	10			
Cephalothin 5 µg	10	10	10	**10**		10	10	10	10	10	0	0	0	**6.3**	10	10	10	10	3	10	**8.8**	0			
Colistin sulphate 25 µg	10	10	10	**10**		10	10	10	10	10	10	10	10	**10.0**	10	10	10	10	10	3	**8.8**	10			
Gentamycin 10 µg	5	5	10	**6.7**		10	10	10	10	10	10	0	10	**8.8**	5	0	0	0	0	0	**0.8**	10			
Sulphatriad 200 µg	0	3	0	**0.8**		0	0	10	0	0	0	0	0	**1.3**	0	0	0	0	0	10	**1.7**	0			
Cotrimoxazole 25 µg	3	3	0	**1.7**		5	10	10	5	5	0	0	0	**4.4**	10	5	5	5	5	10	**6.7**	10			
Nitrofurantoin 50µg	10	10	10	**10**		10	10	10	10	10	10	10	3	**9.1**	10	10	10	10	10	10	**10**	10			
Ticarcillin 75 µg	10	3	10	**7.5**		0	0	0	0	0	0	0	0	**0.0**	10	0	0	0	0	0	**1.7**	10			
Nalidixic acid 30 µg	10	10	10	**10**		5	10	10	10	10	10	3	3	**7.5**	0	10	10	10	10	10	**8.3**	0			
Trimethoprim 2.5 µg	5	3	3	**3.3**		0	0	0	0	0	0	0	0	**0.0**	0	3	3	0	5	0	**1.7**	0			
Sulphamethoxazole 50µg	0	0	0	**0.0**		0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0	0	**0.0**	0			

10 = >2mm zone of clearing outside of test disc, 5 = < 2mm zone of clearing, 2.5 = disruption of growth, 0 = resistant.

Appendix 2f Biochemical, physiological and antibiotic susceptibility test results for isolates classified as *Vibrio alginolyticus,* other unidentified *Vibrio* species and *Halomonas marina*-like. Each test was scored on a scale of between 0 –10 for a strongly positive reaction. Isolates are coded as in Table 1

Character	*V. alginolyticus*														Other *Vibrio* spp.										Unidentified copepod-associated organisms					*Halomonas marina*-like			
	OFA1-02	OFF2-05	OFA1-09	OFF2-20	SFF5-02	SFF5-03	AE1-24*	SFF6-02	SFF6-09	SFF6-05	SSF6-19	SFF6-18	SSF6-20	**Group average**	SFF6-03	SFF5-08	OFF2-01	SFF5-13	*V. tubiashii* SFF6-15*	SYS5-25	OFC1-21	MYS1-06	SFF2-18	MFF1-07	OFC1-01	OFC1-02	OFC1-05	OFC1-10	**Group average**	MYS2-06	MYS2-08	MYS2-09	**Group average**
[a]PCR-RFLP pattern	F	F	F	F	F	F	F	F	F	F	F	F	F	**F**	Xia	F	F	XIb	F		VIII	IX			F	F	F	F					
[b]Biolog	G	G	G	G	G	G	G	G	c					**G**		G	G	G	G	G	G	G	G	G	G	G	G	G	**G**	G	G	G	**G**
Gram stain					0	0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0			0	0						**0**				
Motility					10	10	10	10	10	10	10	10	10	**10.0**	10	10	10	10	10			10	10										
Pigment					0	0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0			0	0						**0**				
Morphology					10	10	10	10	10	10	10	10	10	**10.0**	10	10	10	10	10			10	10										
Oxidase					10	10	10	10	10	10	10	10	10	**10.0**	10	10	10	10	10			10	10										
TCBS		10			10	10	10	10	10	10	10	10	10	**10.0**	10	10	10	10	0	10	10	10	10		10	10	10	10	**10**				
TCBS - sucrose positive		10			10	10	10	10	10	10	10	10	10	**10.0**	10	10	10	10	0	0	10	10	10		0	0	0	0	**0**				
Oxidation of glucose					5	5	5	10	5	10	10	5	5	**6.7**	0	10		5	10				0										
Fermentation of glucose					10	10	5	10	10	0	10	10	10	**8.3**	10	10		10	10				10										
Swarming	10	10			10	10	10	10	10	10	10	10	10	**10.0**	0	0	10	0	5		0	0	0	0									
Luminescence					0	0	0	0	0	0	0	0	0	**0.0**	0	0	0	0	0		0	0	0	0									
Growth at 4 °C					0	0	0	5	5	0	10	1	5	**2.9**	10	0		10	5				10										
Growth at 20 °C					10	10	10	10	10	10	10	10	10	**10.0**	10	10	10	10	10				10										
Growth at 37 °C					10	10	10	10	10	10	10	10	10	**10.0**	5	10		0	10				10										

[a] F Failed to PCR amplify

[b] G more than 3 positive reactions in BIOLOG GN microplate; NG not enough positive reactions for identification using BIOLOG

[c] Where no data is shown tests were not done.

Appendix 2f (continued)

Character	*V. alginolyticus*														Unidentified *Vibrio* spp.										Unidentified copepod-associated organisms					*Halomonas marina*-like			
	OFA1-02	OFF2-05	OFA1-09	OFF2-20	SFF5-02	SFF5-03	AE1-24	SFF6-02	SFF6-09	SFF6-05	SSF6-19	SFF6-18	SSF6-20	**Group average**	SFF6-03	SFF5-08	OFF2-01	SFF5-13	*V. tubiashii* SFF6-15	SYS5-25	OFC1-21	MYS1-06	SFF2-18	MFF1-07	OFC1-01	OFC1-02	OFC1-05	OFC1-10	**Group average**	MYS2-06	MYS2-08	MYS2-09	**Group average**
CLED					0	0		0	0	0	0	0	0	**0.0**	0	10		0					0										
Arginine decarboxylase					NG	NG	NG	NG	NG	NG	NG	NG	NG	**0.0**	10	NG		10	10				10										
Lysine decarboxylase					NG	NG	NG	NG	NG	NG	NG	NG	NG	**0.0**	5	NG		0	10				10										
Ornithine decarboxylase					NG	NG	NG	NG	NG	NG	NG	NG	NG	**0.0**	0	NG		0	0				0										
Nitrate reduction					10	10	10	10	10	10	10	10	10	**10.0**	10	10		10	10				10										
Indole					0	10	0	0	0	0	0	0	0	**1.1**	0	0		5	0				5										
ONPG					0	0	0	0	0	1	10	10	0	**2.3**	10	0		10	0				0										
Voges Proskauer					10	10	10	10	10	10	10	10	10	**10.0**	0	10		0	0				0										
0/129 10 µg					8	10	0	0	0	0	0	5		**2.8**	0			5	0	0		0	0	0									
0/129 150 µg					10	10	10	3	3	3	3	3		**5.3**	5-			10	10	5-		0	0	5+									
Agar digestion (agarase)					0		0	0	0	0	0	0		**0.0**	0	0	0	0	0				0										
Aesculin hydrolysis					0		0	0	0	0	0	0		**0.0**	0	0		0	0				0										
Haemolysis srbc		10			0	0	0	0	0	0	0	0		**0.0**	0	0	10	5	0				0		10	10	10	10	**10**				
Acid arbutin					0	0	0	0	0	0	0	0		**0.0**	0	0		0	0				0										
Acid salicin					0	0	0	0	0	0	0	10		**1.3**	0	0		0	0				0										
Acid sucrose					10	10	10	10	10	10	10	10		**10.0**	10	10		10	10				10										
Catalase					5	5	5	5	5	5	5	10		**5.6**	10	5		10	5				10										
Utilisation of single carbon sources (BIOLOG GN):																																	
Acetic acid	10	10	10	10	5	5	10	10						**8.8**		10	10	10	0	5	10	5	10	10	0	0	0	0	**0**	10	10	10	**10**
n- Acetyl-D-galactosamine	0	0	0	0	0	0	0	0						**0.0**		0	0	0	10	0	0	10	5	0	0	0	0	0	**0**	0	0	0	**0**
n- Acetyl-D-glucosamine	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	10	10	10	10	10	10	10	10	**10**	0	0	10	**3**
cis-Aconitic acid	10	10	10	10	10	10	5	10						**9.4**		10	10	10	0	0	10	10	10	10	0	0	0	0	**0**	10	10	10	**10**
Adonitol	0	0	0	0	0	0	0	0						**0.0**		5	5	0	0	0	0	0	5	5	0	0	0	0	**0**	0	0	0	**0**
D-Alaninamide	0	0	0	0	0	0	0	0						**0.0**		5	5	5	0	0	0	0	5	5	0	0	0	0	**0**	5	5	5	**5**

Appendix 2f (continued)

Utilisation of single carbon sources (BIOLOG GN):

	V. alginolyticus													
	OFA1-02	OFF2-05	OFA1-09	OFF2-20	SFF5-02	SFF5-03	AE1-24	SFF6-02	SFF6-09	SFF6-05	SSF6-19	SFF6-18	SSF6-20	Group average
Alanine	10	10	10	10	10	10	10	10						**10.0**
L-Alanine	10	10	10	10	10	10	10	10						**10.0**
L-Alanyl glycine	10	10	10	10	10	10	10	10						**10.0**
2- Amino ethanol	0	0	0	5	0	0	0	0						**0.6**
γ-Amino butyric acid	0	0	0	0	0	0	5	10						**1.9**
D-L-Arabinose	0	0	0	0	0	0	0	0						**0.0**
Arabitol	0	0	0	0	0	0	0	0						**0.0**
L-Asparagine	10	10	10	10	10	10	10	10						**10.0**
L-Aspartic acid	10	10	10	10	0	0	5	0						**5.6**
Bromo-succinic acid	10	5	5	10	10	10	10	10						**8.8**
2,3 Butanediol	0	0	0	0	0	0	0	0						**0.0**
D,L-Carnitine	0	0	0	0	0	0	0	10						**1.3**
Cellobiose	5	5	10	10	10	10	0	5						**6.9**
Citric acid	10	10	10	10	10	10	10	10						**10.0**
α-Cyclodextrin	10	10	10	10	10	10	10	10						**10.0**
Dextrin	10	10	10	10	10	10	10	10						**10.0**
meso- Erythritol	0	0	0	0	0	0	0	0						**0.0**
Formic acid	10	10	10	0	10	10	10	10						**8.8**
D-Fructose	10	10	10	10	10	10	10	10						**10.0**
L-Fucose	0	0	5	5	0	0	0	0						**1.3**
D- Galactonic acid lactone	0	0	0	0	0	0	0	0						**0.0**
D-Galactose	10	10	10	10	10	10	0	0						**7.5**
D-Galacturonic acid	0	0	0	0	0	0	0	0						**0.0**
Gentiobiose	0	0	0	0	0	0	0	0						**0.0**
D-Gluconic acid	10	10	10	10	10	10	10	10						**10.0**

	Unidentified *Vibrio* spp.										Unidentified copepod-associated organisms					*Halomonas marina*-like			
	SFF6-03	SFF5-08	OFF2-01	SFF5-13	*V. tubiashii* SFF6-15	SYS5-25	OFC1-21	MYS1-06	SFF2-18	MFF1-07	OFC1-01	OFC1-02	OFC1-05	OFC1-10	Group average	MYS2-06	MYS2-08	MYS2-09	Group average
Alanine		0	10	10	10	10	0	10	10	10	10	10	10	10	**10**	10	10	10	**10**
L-Alanine		10	10	10	10	10	5	10	10	10	10	10	10	10	**10**	10	10	10	**10**
L-Alanyl glycine		10	10	10	10	10	5	10	10	10	10	10	10	10	**10**	10	10	10	**10**
2- Amino ethanol		0	5	0	0	0	0	0	5	0	0	0	0	0	**0**	10	10	10	**10**
γ-Amino butyric acid		0	0	0	0	0	0	0	10	5	0	0	0	0	**0**	10	10	10	**10**
D-L-Arabinose		0	0	5	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**
Arabitol		0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	10	10	10	**10**
L-Asparagine		10	10	10	10	0	5	10	10	10	10	10	10	10	**10**	10	10	10	**10**
L-Aspartic acid		10	10	10	5	0	5	10	10	10	10	10	10	10	**10**	10	10	10	**10**
Bromo-succinic acid		10	5	10	5	5	0	10	10	5	0	0	0	0	**0**	10	10	10	**10**
2,3 Butanediol		0	0	0	0	0	0	0	5	0	0	0	0	0	**0**	0	0	10	**3**
D,L-Carnitine		0	0	0	0	0	0	0	5	0	0	0	0	0	**0**	10	10	10	**10**
Cellobiose		10	0	10	0	0	10	0	10	5	0	0	0	0	**0**	5	0	0	**2**
Citric acid		10	10	10	0	5	10	10	10	10	0	0	0	0	**0**	10	10	10	**10**
α-Cyclodextrin		10	10	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**
Dextrin		10	10	10	10	10	10	10	10	10	10	10	10	10	**10**	10	10	10	**10**
meso- Erythritol		0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**
Formic acid		10	10	0	0	0	0	5	0	0	0	0	0	0	**0**	10	10	10	**10**
D-Fructose		10	10	10	10	10	10	10	10	10	10	10	10	10	**10**	10	10	10	**10**
L-Fucose		5	0	0	0	0	0	0	0	5	0	0	0	0	**0**	0	0	0	**0**
D- Galactonic acid lactone		0	5	0	0	0	0	0	5	0	0	0	0	0	**0**	10	10	10	**10**
D-Galactose		0	10	10	0	0	10	10	10	10	0	0	0	0	**0**	10	10	10	**10**
D-Galacturonic acid		0	5	0	0	0	0	0	5	5	0	0	0	0	**0**	0	0	0	**0**
Gentiobiose		0	0	0	0	0	10	0	10	0	0	0	0	0	**0**	0	0	0	**0**
D-Gluconic acid		10	10	10	10	0	10	10	10	10	0	0	0	5	**1.25**	10	10	10	**10**

Appendix 2f (continued)

Utilisation of single carbon sources (BIOLOG GN):	*V. alginolyticus*														Unidentified *Vibrio* spp.														Unidentified copepod-associated organisms					*Halomonas marina*-like			
	OFA1-02	OFF2-05	OFA1-09	OFF2-20	SFF5-02	SFF5-03	AE1-24	SFF6-02	SFF6-09	SFF6-05	SSF6-19	SFF6-18	SSF6-20	Group average	SFF6-03	SFF5-08	OFF2-01	SFF5-13	*V. tubiashii* SFF6-15	SYS5-25	OFC1-21	MYS1-06	SFF2-18	MFF1-07	OFC1-01	OFC1-02	OFC1-05	OFC1-10	Group average	MYS2-06	MYS2-08	MYS2-09	Group average
D-Glucosamic acid	0	0	0	0	0	0	0	0						**0.0**		0	5	0	0	0	0	0	5	5	0	0	0	0	0	0	0	10	**3**
α-D-Glucose	10	10	10	10	10	10	10	10						**10.0**		0	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	**10**
Glucose-1-phosphate	10	10	10	10	10	10	10	10						**10.0**		10	10	0	0	0	0	10	5	10	10	0	10	0	5	0	0	0	**0**
Glucose-6-phosphate	10	10	10	10	10	10	10	10						**10.0**		10	10	10	0	0	0	10	10	10	10	10	10	0	7.5	5	10	10	**8**
Glucuronamide	0	0	0	0	0	0	0	0						**0.0**		0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	**0**
D-Glucuronic acid	0	0	0	0	0	0	0	0						**0.0**		0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	**0**
L-Glutamic acid	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	**10**
Glycerol	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	5	10	10	10	5	10	0	0	3.75	10	10	10	**10**
D-L-α-Glycerol phosphate	10	10	10	10	10	10	0	10						**8.8**		10	10	10	5	0	0	0	10	10	10	10	10	5	8.75	10	10	10	**10**
Glycyl-L-aspartic acid	10	10	10	10	0	0	5	0						**5.6**		10	10	10	5	0	5	10	10	10	10	10	10	10	10	10	5	5	**7**
Glycyl-L-glutamic acid	10	10	10	10	10	10	5	10						**9.4**		10	10	10	0	5	0	5	10	10	10	10	10	10	10	10	10	10	**10**
Glycogen	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	0	10	10	10	10	10	10	10	10	10	5	10	5	**7**
L-histidine	10	10	10	10	10	10	10	10						**10.0**		10	10	10	0	5	0	5	10	5	10	10	5	5	7.5	0	0	5	**2**
α-Hydroxy butyric acid	0	0	0	0	0	0	0	5						**0.6**		0	0	5	0	0	0	0	0	0	0	5	0	0	1.25	10	5	10	**8**
β-Hydroxy butyric acid	0	0	0	0	0	0	0	0						**0.0**		0	0	0	10	0	0	0	0	0	0	0	0	0	0	10	10	10	**10**
γ-Hydroxy butyric acid	0	0	0	0	0	0	0	5						**0.6**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	10	10	**10**
Hydroxy L-proline	10	10	10	10	10	10	10	10						**10.0**		10	10	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	**0**
p-Hydroxy phenylacetic acid	0	0	0	0	0	0	0	0						**0.0**		0	0	0	0	0	0	0	0	0	0	5	0	0	1	5	5	5	**5**
Inosine	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	0	10	10	10	10	10	10	10	10	10	10	10	**10**
meso-Inositol	0	0	0	0	0	0	0	0						**0.0**		10	10	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	**0**
Itaconic acid	0	0	0	0	0	0	0	0						**0.0**		0	0	0	5	0	0	0	0	0	0	5	0	0	1	0	0	0	**0**
α-Ketobutyric acid	0	0	0	0	0	0	0	0						**0.0**		0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	5	5	**7**
α-Ketoglutaric acid	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	0	5	0	1	10	0	5	0	0	1	10	10	10	**10**
α-Ketovaleric acid	0	0	0	0	0	0	0	0						**0.0**		0	0	0	0	0	0	0	0	0	0	5	0	0	1	0	0	0	**0**
D,L-lactic acid	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	**10**

Appendix 2f (continued)

Utilisation of single carbon sources (BIOLOG GN):	*V. alginolyticus*														Unidentified *Vibrio* spp.										Unidentified copepod-associated organisms					*Halomonas marina*-like			
	OFA1-02	OFF2-05	OFA1-09	OFF2-20	SFF5-02	SFF5-03	AE1-24	SFF6-02	SFF6-09	SFF6-05	SSF6-19	SFF6-18	SSF6-20	Group average	SFF6-03	SFF5-08	OFF2-01	SFF5-13	*V. tubiashii* SFF6-15	SYS5-25	OFC1-21	MYS1-06	SFF2-18	MFF1-07	OFC1-01	OFC1-02	OFC1-05	OFC1-10	Group average	MYS2-06	MYS2-08	MYS2-09	Group average
α-lactose	0	0	0	0	0	0	0	0						0.0		10	10	0	0	0	10	0	0	5	0	0	0	0	0	0	0	10	3
α-D-lactose-lactulose	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	0	0	0	10	0	0	2.5	5	0	0	2
L-Leucine	5	5	5	5	5	5	5	0						4.4		0	10	0	0	0	0	0	5	0	5	10	5	10	7.5	10	10	10	10
Malonic acid	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	5	5	0	0	5	0	1.25	10	10	10	10
Maltose	10	10	10	10	10	10	10	10						10.0		0	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
D-Mannitol	10	10	10	10	10	10	10	10						10.0		0	10	10	10	10	10	10	10	10	0	5	0	0	1	10	10	10	10
D-Mannose	5	10	5	0	0	0	0	0						2.5		0	10	10	10	0	10	10	0	10	0	0	0	0	0	10	10	10	10
D-Melibiose	0	0	0	0	0	0	0	5						0.6		5	0	5	0	0	10	0	10	5	0	0	0	0	0	5	5	5	5
Methyl pyruvate	10	10	10	10	10	10	10	10						10.0		0	10	10	10	0	5	10	10	5	0	5	0	0	1	10	10	10	10
β-Methyl-D-glucoside	0	0	0	0	0	0	0	5						0.6		10	0	0	0	0	0	0	10	5	10	10	10	10	10	5	5	5	5
Mono-methyl succinate	10	10	10	10	5	10	0	5						7.5		0	10	0	0	0	0	0	0	0	0	0	0	0	0	10	10	10	10
L-Ornithine	5	5	5	5	0	0	0	0						2.5		5	5	5	5	0	0	5	5	5	10	10	10	5	8.75	10	10	10	10
L-Phenyl alanine	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	5	0	0	0	0	0	0	5	5	5	5
Phenyl ethylamine	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	5	0	0	0	0	0	0	10	0	0	3
L-Proline	10	10	10	10	10	10	10	10						10.0		10	10	10	5	10	5	10	10	10	10	10	10	10	10	10	10	10	10
Propionic acid	10	10	10	10	10	10	10	10						10.0		0	0	10	0	0	0	10	10	10	0	0	0	0	0	10	10	10	10
Psicose	5	0	0	5	5	5	5	5						3.8		5	5	10	5	0	5	5	10	0	0	0	0	0	0	10	5	10	8
Putrescine	0	0	0	0	10	10	10	10						5.0		10	0	0	0	0	0	0	5	5	0	0	0	0	0	10	10	10	10
L-Pyro glutamic acid	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	10	5	0	0	0	0	0	10	10	10	10
Quinic acid	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	5	5	0	0	5	0	1	0	0	0	0
D-Raffinose	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	5	5	0	0	0	0	0	0	0	0	0
L-Rhamnose	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
D-Saccharic acid	0	0	0	0	0	0	0	0						0.0		0	0	5	0	0	0	0	0	5	0	0	5	0	1	0	0	0	0
Sebacic acid	0	0	0	0	0	0	0	0						0.0		0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Serine	0	0	0	0	0	0	0	0						0.0		0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	5	10	5

Appendix 2f (continued)

Utilisation of single carbon sources (BIOLOG GN):	*V. alginolyticus*													Group average	Unidentified *Vibrio* spp.										Unidentified copepod-associated organisms				**Group average**	*Halomonas marina*-like			**Group average**
	OFA1-02	OFF2-05	OFA1-09	OFF2-20	SFF5-02	SFF5-03	AE1-24	SFF6-02	SFF6-09	SFF6-05	SSF6-19	SFF6-18	SSF6-20		SFF6-03	SFF5-08	OFF2-01	SFF5-13	V. tubiashii SFF6-15	SYS5-25	OFC1-21	MYS1-06	SFF2-18	MFF1-07	OFC1-01	OFC1-02	OFC1-05	OFC1-10		MYS2-06	MYS2-08	MYS2-09	
L-Serine	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	5	10	10	10	10	10	10	10	**10**	10	10	10	**10**
D-Sorbitol	0	0	0	0	0	0	0	0						**0.0**		0	5	0	0	0	0	0	5	5	0	0	0	0	**0**	5	5	5	**5**
Succinamic acid	0	0	0	0	0	0	5	10						**1.9**		10	0	0	0	0	0	0	5	0	0	0	0	0	**0**	10	10	10	**10**
Sucrose	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	0	10	10	10	10	10	0	0	0	**0**	10	10	10	**10**
Succinic acid	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	5	10	10	10	10	10	10	5	**8.75**	10	10	10	**10**
L-Threonine	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	0	10	10	10	10	10	10	10	**10**	10	10	10	**10**
Thymidine	10	10	10	10	10	10	10	10						**10.0**		10	10	10	0	10	5	10	10	10	5	10	5	0	**5**	5	0	5	**3**
D-Trehalose	10	10	10	10	10	10	10	10						**10.0**		0	10	10	10	0	10	10	10	10	0	10	10	10	**7.5**	10	10	10	**10**
Turanose	0	0	0	0	0	5	5	5						**1.9**		0	0	5	0	0	5	10	10	0	0	0	0	0	**0**	10	10	10	**10**
Tween 40	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	10	10	10	5	10	10	10	10	**10**	10	10	10	**10**
Tween 80	5	10	5	5	10	10	10	10						**8.1**		10	5	10	10	10	10	5	10	5	0	5	0	0	**1.25**	10	10	10	**10**
Uridine	10	10	10	10	10	10	10	10						**10.0**		10	10	10	10	10	5	10	10	10	10	10	10	5	**8.75**	10	10	10	**10**
Urocanic acid	0	0	0	0	0	0	0	0						**0.0**		0	5	0	0	0	0	0	10	0	0	5	0	0	**1.25**	0	0	10	**3**
Xylitol	0	0	0	0	0	0	0	0						**0.0**		0	0	5	0	0	0	0	0	5	0	0	0	0	**0**	0	0	0	**0**

Appendix 2f (continued) Antibiotic sensitivity and resistance of organisms classified as *Vibrio alginolyticus*, other unidentified *Vibrio* species and *Halomonas marina*-like.

	V. alginolyticus														Unidentified *Vibrio* spp.										Unidentified copepod-associated organisms					*Halomonas marina*-like			
	OFA1-02	OFF2-05	OFA1-09	OFF2-20	SFF5-02	SFF5-03	AE1-24	SFF6-02	SFF6-09	SFF6-05	SSF6-19	SFF6-18	SSF6-20	**Group average**	SFF6-03	SFF5-08	OFF2-01	SFF5-13	*V. tubiashii* SFF6-15	SYS5-25	OFC1-21	MYS1-06	SFF2-18	MFF1-07	OFC1-01	OFC1-02	OFC1-05	OFC1-10	**Group average**	MYS2-06	MYS2-08	MYS2-09	**Group average**
Antibiotic sensitivity:																																	
Chloramphenicol 25 µg	10	10	10	10	5	10	10	10	10	10	10	10		**9.6**	10	10		10	10	10		10	10	10						0	0	10	
Erythromycin 5 µg	0	0	0	0	0	5	0	0	0	0	0	0		**0.4**	0	5		0	0	0		0	0	0						3	0	0	
Fusidic acid 10 µg	0	0	0	0	0	3	0	0	0	0	0	0		**0.2**	0	0		0	0	0		0	0	0						0	0	0	
Methicillin 10 µg	0	0	0	0	0	0	0	0	0	0	0	0		**0.0**	0	0		0	0	0		0	0	0						0	0	0	
Novobiocin 5 µg	10	5	10	10	10	10	10	10	10	10	10	10		**9.6**	10	10		10	10	10		10	10	10						0	0	10	
Penicillin G 1 unit	0	0	0	0	0	0	0	0	0	0	0	0		**0.0**	0	0		0	0	0		0	0	0						0	0	0	
Streptomycin 10 µg	0	0	0	0	5	10	10	3	10	3	5	5		**4.2**	3	10		5	5	0		0	0	5						10	10	10	
Tetracycline 25 µg	10	5	3	5	5	3	10	10	10	10	10	10		**7.5**	10	0		10	10	10		10	10	0						10	10	0	
Tetracycline 100 µg	10	5	3	5	10	5	10	10	10	10	10	10		**8.1**	10	0		10	10	10		10	10	0						10	10	0	
Ampicillin 10 µg	0	0	0	0	3	3	0	10	0	0	0	0		**1.3**	0	0		10	5	0		0	10	0						0	0	0	
Ampicillin 25 µg	0	0	0	0	3	3	0	10	0	0	0	0		**1.3**	10	3		10	10	0		0	10	0						0	0	0	
Cephalothin 5 µg	0	0	0	0	10	0	0	0	0	0	0	0		**0.8**	10	0		10	0	10		5	0	0						0	0	0	
Colistin sulphate 25 µg	10	10	10	10	10	10	10	10	10	10	10	10		**10.0**	10	10		10	10	10		10	10	10						10	0	10	
Gentamycin 10 µg	5	0	5	5	10	10	10	10	10	10	10	10		**7.9**	10	10		10	10	10		10	10	10						10	10	10	
Sulphatriad 200 µg	0	3	0	0	0	0	0	0	3	0	0	0		**0.4**	0	0		0	3	10		0	0	0						0	0	0	
Cotrimoxazole 25 µg	3	3	5	0	3	0	10	3	5	10	10	3		**4.4**	3	0		10	10	10		0	10	0						0	0	0	
Nitrofurantoin 50µg	10	10	10	10	10	10	10	10	10	10	10	10		**10.0**	10	10		10	10	10		10	10	10						0	0	10	
Ticarcillin 75 µg	0	0	0	0	0	0	0	10	0	0	0	0		**0.8**	10	0		10	10	0		10	10	0						0	0	0	
Nalidixic acid 30 µg	10	10	10	10	0	0	3	0	3	0	10	10		**5.4**	0	0		0	10	10		0	0	0						0	0	0	
Trimethoprim 2.5 µg	0	0	0	0	0	0	3	0	0	0	0	0		**0.2**	0	0		10	0	5		0	0	0						0	0	0	
Sulphamethoxazole 50µg	0	0	0	0	0	0	0	0	0	0	0	0		**0.0**	0	0		0	0	0		0	0	0						0	0	0	

10 = >2mm zone of clearing outside of test disc, 5 = < 2mm zone of clearing, 2.5 = disruption of growth, 0 = resistant.

Appendix 3

16s rDNA sequences

NAME *Flexibacter ovolyticus*- like

LENGTH 578 base

AFFILIATION cytophaga-flavobacter-bacteroides phylum

ORIGIN Halibut yolk-sac incubator water, SFIA Ardtoe

```
  1 GGAACGCTAG CGGCAGGCTT TAACACATGC AAGTCGAGGG GTAACAGGTA GCTTGCTATG
 61 CTGACGACCG GCGAACGGGT GCGTAACGCG TATAGAATCT GCCTTGTACA GGAGGATAGC
121 CTTTAGAAAT GAAGATTAAT ACTCCATAAT GTTAATGATT GGCATCAGTT ATTAATTAAA
181 CATTTATGGG TACAAGATGA CTATGCGTCC TATTAGCTAG ATGGTAAGGT AACGGCTTAC
241 CATGGCAACG ATAGGTAGGG GGTCTGAGAG GATTATCCCC CACACTGGTA CTGAGACACG
301 GACCAGACTC CTACGGGAGG CAGCAGTGAG GAATATTGGT CAATGGAGGC AACTCTGAAC
361 CAGCTATGCC GCGTGCAGGA CGACTGCCCT ATGGGTTGTA AACTGCTTTT ATACAGGAAG
421 AAACCGATCT ACGTGTAGAT CCTTGACGGT ACTGTAAGAA TAAGGACCGG CTAACTCCGT
481 GCCAGCAGCC GCGGTAATAC GGAGGGTCCA AGCGTTATCC GGAATCATTG GGTTTAAAGG
541 GTCCGCAGGC GGTCAATTAA GTCAGAGGTG AAATCCCA
```

NAME SYS5-03

LENGTH 571 nucleotides

AFFILIATION γ-proteobacteria, *Pseudoalteromonas* group

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAWGTCG AGCGGTAACA XAAAGTAGCT TTGCTACTTT GCTGACGAGC
 61 GGCGGACGGG TGAGTAATGC TTGGGAATAT GCCTTTTGGT GGGGGACAAC AGTTGGAAAC
121 GACTGCTAAT ACCGCATAAT GTCTACGGAC CAAAGTGGGG GACCTTCGGG CCTCACGCCA
181 AAAGATTAGC CCAAGTGGGA TTAGCTAGTT GGTAAGGTAA TGGCTTACCA AGGCAACGAT
241 CCCTAGCTGG TTTGAGAGGA TGATCAGCCA CACTGGGACT GAGACACGGC CCAGACTCCT
301 ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGCAA GCCTGATGCA GCCATGCCGC
361 GTGTGTGAAG AAGGCCTTCG GGTTGTAAAG CACTTTCAGT AAGGAGGAAA GGGTAGTCTT
421 TAATAGAGGC TATCTGTGAC GTTACTTACA GAAGAAGCAC CGGCTAACTC CGTGCCAGCA
481 GCCGCGGTAA TACGGAGGGT GCGAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGTACGCA
541 GGCGGTTTGT TAAGCGAGAT GTGAAAGCCC C
```

NAME TG15-07

LENGTH 571 nucleotides

AFFILIATION γ-proteobacteria, *Pseudoalteromonas* group

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGTAACA GAAAGTAGCT TGCTACTTTT GCTGACGAGC
 61 GGCGGACGGG TGAGTAATGC TTGGGAATAT GCCTTTTGGT GGGGGACAAC AGTTGGAAAC
121 GACTGCTAAT ACCGCATAAT GTCTACGGAC CAAAGTGGGG GACCTTCGGG CCTCACGCCA
181 AAAGATTAGC CCAAGTGGGA TTAGCTAGTT GGTAAGGTAA TGGCTTACCA AGGCAACGAT
241 CCCTAGCTGG TTTGAGAGGA TGATCAGCCA CACTGGGACT GAGACACGGC CCAGACTCCT
301 ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGCAA GCCTGATGCA GCCATGCCGC
361 GTGTGTGAAG AAGGCCTTCG GGTTGTAAAG CACTTTCAGT AAGGAGGAAA GGGTAGTCTT
421 TAATAGAGGC TATCTGTGAC GTTACTTACA GAAGAAGCAC CGGCTAACTC CGTGCCAGCA
481 GCCGCGGTAA TACGGAGGGT GCGAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGTACGCA
541 GGCGGTTTGT TAAGCGAGAT GTGAAAGCCC C
```

NAME TG10-11

LENGTH 566 nucleotides

AFFILIATION γ-proteobacteria, related to *Pseudoalteromonas*

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGTAACA GAGAGGTGCT TTGCACCTTT GCTGACGAGC
 61 GGCGGACGGG TGAGTAATGC TTGGGAACAT GCCTTAAGGT GGGGGACAAC AGTTGGAAAC
121 GACTTGCTAA TACCGCATAA TGTCTACGGA CCAAAGGGGG CTTCGGCTCT CGCCTTTAGA
181 TTGGCCCAAG TGGGATTAGC TAGTTGGTGA GGTAATGGCT CACCAAGGCG ACGATCCCTA
241 GCTGGTTTGA GAGGATGATC AGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG
301 AGGCAGCAGT GGGGAATATT GCACAATGGG CGCAAGCCTG ATGCAGCCAT GCCGCGTGTG
361 TGAAGAAGGC CTTCGGGTTG TAAAGCACTT TCAGTCAGGA GGAAAGGTTA GTAGTTAATA
421 CCTGCTAGCT GTGACGTTAC TGACAGAAGA AGCACCGGCT AACTCCGTGC CAGCAGCCGC
481 GGTAATACGG AGGGTGCGAG CGTTAATCGG AATTACTGGG CGTAAAGCGT ACGCAGGCGG
541 TTTGTTAAGC GAGATGTGAA AGCCCT
```

NAME MA1-13

LENGTH 562 nucleotides

AFFILIATION γ-proteobacteria, related to *Pseudoalteromonas*

ORIGIN Mannin Seafarms enriched *Artemia*

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGTAACA TTTCTAGCTT GCTAGAAGAT GACGAGCGGC
 61 GGACGGGTGA GTAATGCTTG GGAACATGCC TTGAGGTGGG GGACAACAGT TGGAAACGAC
121 TGCTAATACC GCATAATGTC TACGGACCAA AGGGGGCTTC GGCTCTCGCC TTTAGATTGG
181 CCCAAGTGGG ATTAGCTAGT TGGTGAGGTA ATGGCTCACC AAGGCGACGA TCCCTAGCTG
241 GTTTGAGAGG ATGATCAGCC ACACTGGGAC TGAGACACGG CCCAGACTCC TACGGGAGGC
301 AGCAGTGGGG AATATTGCAC AATGGGCGCA AGCCTGATGC AGCCATGCCG CGTGTGTGAA
361 GAAGGCCTTC GGGTTGTAAA GCACTTTCAG TCAGGAGGAA AGGTTAGTAG TTAATACCTG
421 CTAGCTGTGA CGTTACTGAC AGAAGAAGCA CCGGCTAACT CCGTGCCAGC AGCCGCGGTA
481 ATACGGAGGG TGCGAGCGTT AATCGGAATT ACTGGGCGTA AAGCGTACGC AGGCGGTTTG
541 TTAAGCGAGA TGTGAAAGCC CC
```

NAME SFF1-01

LENGTH 565 nucleotides

AFFILIATION γ-proteobacteria, related to *Pseudoalteromonas*

ORIGIN Halibut first-feeding larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGTAACA GAGAGTAGCT TGCTACTTTT GCTGACGAGC
 61 GGCGGACGGG TGAGTAATGC TTGGGAACAT GCCTTGAGGT GGGGGACAAC AGTTGGAAAC
121 GACTGCTAAT ACCGCATAAT GTCTACGGAC CAAAGGGGGC TTCGGCTCTC GCCTTTAGAT
181 TGGCCCAAGT GGGATTAGCT AGTTGGTGAG GTAATGGCTC ACCAAGGCGA CGATCCCTAG
241 CTGGTTTGAG AGGATGATCA GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA
301 GGCAGCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTGT
361 GAAGAAGGCC TTCGGGTTGT AAAGCACTTT CAGTCAGGAG GAAAGGTTAG TAGTTAATAC
421 CTGCTAGCTG TGACGTTACT GACAGAAGAA GCACCGGCTA ACTCCGTGCC AGCAGCCGCG
481 GTAATACGGA GGGTGCGAGC GTTAATCGGA ATTACTGGGC GTAAAGCGTA CGCAGGCGGT
541 TTGTTAAGCG AGATGTGAAA GCCCC
```

NAME OFC1-21

LENGTH 571 nucleotides

AFFILIATION γ-proteobacteria,

ORIGIN Copepods, Otter Ferry Seafarms

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGTAACA TTTCTAGCTT GCTAGAAGAT GACGAGCGGC
 61 GGGACGGGTG AGTAATGCTT GGGAATATGC CTTTACGTGG GGGACAACAG TTGGAAACGA
121 CTGCTAATAC CGCATAATGT CTTCGGACCA AAGGAGGGGA CGCTTCGGCA CCTTTCGCGT
181 ATTGATTAGC CCAAGTGGGA TTAGCTAGTT GGTAAGGTAA TGGCTTACCA AGGCGACGAT
241 CCCTAGCTGG TTTGAGAGGA TGATCAGCCA CACTGGGACT GAGACACGGC CCAGACTCCT
301 ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGAAA GCCTGATGCA GCCATGCCGC
361 GTGTGTGAAG AAGGCCTTCG GGTTGTAAAG CACTTTCAGT TGTGAGGAAG AGTTAAGGGT
421 TAATACCCCT TATCTTTGAC GTTAGCAACA GAAGAAGGAC CGGCTAACTC CGTGCCAGCA
481 GCCGCGGTAA TACGGAGGGT CCGAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGTACGCA
541 GGCGGCTTTT TAAGCCAGAT GTGAAATCCC C
```

NAME S1-04

LENGTH 570 nucleotides

AFFILIATION γ-proteobacteria, *Photobacterium phosphoreum*

ORIGIN Adult halibut gut, SFIA Ardtoe

```
  1 CCTAACACAT GCAAGTCGAG CGGTAACAGA AAGAAAGCTT GCTTTCTTTG CTGACGAGCG
 61 GCGGACGGGT GAGTAATGCC TGGGAATATA CCCTGATGTG GGGGATAACT ATTGGAAACG
121 ATAGCTAATA CCGCATAATC TCTTCGGAGC AAAGAGGGGG ACCTTCGGGC CTCTCGCGTC
181 AGGATTAGCC CAGGTGGGAT TAGCTAGTTG GTGGGGTAAT GGCTCACCAA GGCGACGATC
241 CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC CAGACTCCTA
301 CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGGGAAAC CCTGATGCAG CCATGCCGCG
361 TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG CGTTGGAGTT
421 AATAGCTTCA GCGCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC GTGCCAGCAG
481 CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TCGGAATTAC TGGGCGTAAA GCGCATGCAG
541 GCGGTCTGTT AAGCAAGATG TGAAAGCCCG
```

NAME MYS1-06

LENGTH 568 nucleotides

AFFILIATION γ-proteobacteria, *Photobacterium profundum* subgroup

ORIGIN Halibut yolk-sac larvae, Mannin Seafarms

```
  1 CCTAACACAT GCAAGTCGAG CGGTAACAGG AATTAGCTTG CTAATTCGCT GACGAGCGGC
 61 GGACGGGTGA GTAATGCCTG GGAATATGCC TTAGTGTGGG GGATAACTAT TGGAAACGAT
121 AGCTAATACC GCATAACGTC TTCGGACCAA AGAGGGGGAC CTTCGGGCCT CTCGCGCTAA
181 GATTAGCCCA GGTGGGATTA GCTAGTTGGT GGGGTAAAGG CCCACCAAGG CAACGATCCC
241 TAGCTGGTCT GAGAGGATGA TCAGCCACAC TGGAACTGAG ACACGGTCCA GACTCCTACG
301 GGAGGCAGCA GTGGGGAATA TTGCACAATG GGGGAAACCC TGATGCAGCC ATGCCGCGTG
361 TATGAAGAAG GCCTTCGGGT TGTAAAGTAC TTTCAGTCGT GAGGAAGGCW TTGTAGTTAA
421 TAGCTGCXXX GCTTGACGTT AGCGACAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC
481 GCGGTAATAC GGAGGGTGCG AGCGTTAATC GGAATTACTG GGCGTAAAGC GCATGCAGGC
541 GGTCTGTTAA GCAAGATGTG AAAGCCCG
```

NAME W1-08

LENGTH 569 nucleotides

AFFILIATION γ-proteobacteria, *V. salmonicida* group

ORIGIN Halibut larva freshly transferred to inert diet. SFIA Ardtoe

```
  1 cctaAACACA GCAAGTCGAG CGGTAACAGG AATTAGCTTG CTAATTCGCT GACGAGCGGC
 61 GGACGGGTGA GKTAATGCCT GGGAATATGC CTTAATGTGG GGGATAACTA TTGGAAACGA
121 TAGCTAATAC CGCATAATGT CTTCGGACCA AAGAGGGGGA TCTTCGGACC TCTCGCGCTA
181 AGATTAGCCC AGGTGAGATT AGCTAGTTGG TGAGGTAAGA GCTCACCAAG GCGACGATCT
241 CTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGAACTGA GACACGGTCC AGACTCCTAC
301 GGGAGGCAGC AGTGGGGAAT ATTGCACAAT GGGCGAAAGC CTGATGCAGC CATGCCGCGT
361 GTATGAAGAA GGCCTTCGGG TTGTAAAGTA CTTTCAGTCG TGAGGAAGGG TGTGTAGTTA
421 ATAGCTGCAT ATCTTGACGT TAGCGACAGA AGAAGCACCG GCTAACTYCG TGCCAGCAGC
481 CGCGGTAATA CGGAGGGTGC GAGCGTTAAT CGGAATTACT GGGCGTAAAG CGCATGCAGG
541 TGGTTCATTA AATCAAATGT GAAAGCCCG
```

NAME OFF1-05

LENGTH 570 nucleotides

AFFILIATION γ-proteobacteria, V. *salmonicida* subgroup

ORIGIN First feeding halibut larvae, Otter Ferry Seafarms

```
  1 GCCTTAACAC ATGCAAGTCG AGCGGTACCA GAAATTAGCT TGCTAATTTG CTGACGAGCG
 61 GCGGACGGGT GAGTAATGCC TGGGAATATG CCTTGATGTG GGGGATAACT ATTGGAAACG
121 ATAGCTAATA CCGCATAATG TCTTCGGACC AAAGAGGGGG ATCTTCGGAC CTCTCGCGTC
181 AAGATTAGCC CAGGTGAGAT TAGCTAGTTG GTGGGGTAAG AGCTCACCAA GGCGACGATC
241 TCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC CAGACTCCTA
301 CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG CCATGCCGCG
361 TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTC STGAGGAAGG GTGTGTAGTT
421 AATAGCTGCA CATCTTGACG TTAGCGACAG AAGAAGCACC GGCTAACTCC GTGCCAGCAG
481 CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TYGGAATTAC TGGGCGTAAA GCGCATGCAG
541 GTGGTTCATT AAGTCAGATG TGAAAGCCCG
```

NAME AE1-24

LENGTH 585 nucleotides

AFFILIATION γ-proteobacteria, V. *harveyi* subgroup

ORIGIN Enriched *Artemia*, SFIA Ardtoe

```
  1 GGCXTAACAC ATGCAAGTCG AGCGGTAACG AGGTTATCTG AACCTTCGGG GAACGATAAC
 61 GGCCGTCGAG CGGCGGACGG GTGAGTAATG CCTAGGAAAT TGCCCTGATG TGGGGGATTA
121 ACAGTTGAAA CGATGGCTAA TACCGCATGA TGCCTACGGG CCAAAGAGGG GGACCTTCGG
181 GCCTCTCGCG TCAGGATATG CCTAGGTGGG ATTAGCTAGT TGGTGAGGTA AGGGCTCACC
241 AAGGCGACGA TCCCTAGCTG GTCTGAGAGG ATGATCAGCC ACACTGGAAC TGAGACACGG
301 TCCAGACTCC TACGGGAGGC AGCAGTGGGG AATATTGCAC AATGGGCGCA AGCCTGATGC
361 AGCCATGCCG CGTGTGTGAA GAAGGCCTTC GGGTTGTAAA GCACTTTCAG TCAGTGAGGA
421 AGGCGGCAGC GTTAATAGCC TGCTATCGTT TGACGTTAGC GACAGAAGAA GCACCGGCTA
481 ACTCCGTGCC AGCAGCCGCG GTAATACGGA GGGTGCGAGC GTTAATCGGA ATTACTGGGC
541 GTAAAGCGCA TGCAGGTGGT TTGTTAAGTC AGATGTGAAA GCCCG
```

NAME SFF6-15

LENGTH 584 nucleotides

AFFILIATION γ-proteobacteria, V*ibrio harveyi* subgroup

ORIGIN First feeding halibut larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGAAACG ACTTAACTGA ACCTTCGGGG AACGTTAAGG
 61 GCGTCRAGCG GCGGACGGGT GAGTAATGCC TGGGAAATTG CCCTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATAATA GCTTCGGCTC AAAGAGGGGG ACCTTCGGGC
181 CTCTCGCGTC AGGATATGCC CAGGTGGGAT TAGCTAGTTG GTGAGGTAAG GGCTCACCAA
241 GGCAACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGCAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGCA AGTGAGGAAG
421 GTTCATGCGT TAATAGCGTA TGGATTTGAC CGTTAGCTGC AGAAGAAGCA CCGGCTAACT
481 CCGTGCCAGC AGCCCGCGGT AATACGGAGG GTGCGAGCGT TAATCGGAAT TACTGGGCGT
541 AAAGCGCATG CAGGTGGTTT GTTAAGTCAG ATGTGAAAAG CCCG
```

NAME SFF5-03

LENGTH 582 nucleotides

AFFILIATION γ-proteobacteria, V. *harveyi* subgroup

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 GCCTTAACAC ATGCAAGTCG AGCGGAAACG AGTTATCTTG AACCTTCGGG GAACGATAAC
 61 GGCGTCGAGC GGCGGACGGG TGAGTAATGC CTAGGAAATT GCCCTGATGT GGGTGGATAA
121 CCATTSKAAA CGATGGCTAA TACCGCATGA TGCCTACGGG CCAAAGAGGG GGACCTTCGG
181 GCCTCTCGCG TCAGGATATG CCTAGGTGGG ATTAGCTAGT TGGTGAGGTA AGGGCTCACC
241 AAGGCGACGA TCCCTAGCTG GTCTGAGAGG ATGATCAGCC ACACTGGAAC TGAGACACGG
301 TCCAGACTCC TACGGGAGGC AGCAGTGGGG AATATTGCAC AATGGGCGCA AGCCTGATGC
361 AGCCATGCCG CGTGTGTGAA GAAGGCCTTC GGGTTGTAAA GCACTTTCAG TCGTGAGGAA
421 GGTGGSKTCG TTAATAGCGK XTTTGTTTGA CGTTAGCGAC AGAAGAAGCA CCGGCTAACT
481 CCGTGCCAGC AGCCGCGGTA ATACGGAGGG TGSTAGCGTT AATCKGAATT ACTGGGCGTA
541 AAGCGCATGC AGGTGGTTTG TTAAGTCAGA TGTGAAAGCC CG
```

NAME AE1-26

LENGTH 580 nucleotides

AFFILIATION γ-proteobacteria, V*ibrio splendidus* subgroup

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GCGGACGGGT GAGTAATGCC TAGGAAATTG CCTTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATGATG CCTACGGGCC AAAGAGGGGG ACCTTCGGGC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GTGTGTAGTT AATAGCTGCG CATCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC
481 GTGCCAGCAG CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TCGGAATTAC TGGGCGTAAA
541 GCGCATGCAG GTGGTTCATT AAGTCAGATG TGAAAGCCCG
```

NAME SFF4-03

LENGTH 580 nucleotides

AFFILIATION γ-proteobacteria, V. *splendidus* subgroup

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GCGGACGGGT GAGTAATGCC TAGGAAATTG CCTTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATGATG CCTACGGGCC AAAGAGGGGG ACCTTCGGGC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GTGTGTAGTT AATAGCTGCG CATCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC
481 GTGCCAGCAG CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TCGGAATTAC TGGGCGTAAA
541 GCGCATGCAG GTGGTTCATT AAGTCAGATG TGAAAGCCCG
```

NAME SFF2-18

LENGTH 579 nucleotides

AFFILIATION γ-proteobacteria, V. *splendidus* subgroup

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 GGCXTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GCGgACGGGT GAGTAATGCC TAGGAAATTG CCTTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATGATG CCTACGGGCC AAAGAGGGGG ACCTTCGGGC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GTGTGTAGTT AATARCTGCG CATCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC
481 GTGCCAGCAG CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TCGXAATTAC TGGGCGTAAA
541 GCGCATGCAG GTGKTCATTA AGTCASATGT GAAAGCCCG
```

NAME SFF4-19

LENGTH 581 nucleotides

AFFILIATION γ-proteobacteria, V. *splendidus* subgroup

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 GCCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GCGGACGGGT GAGTAATGCC TAGGAAATTG CCTTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATGATG CCTACGGGCC AAAGAGGGGG ACCTTCGGGC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AAGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GTGTGTAGTT AATAGCTGCG CATCTTGACG TTAGCAACAG AAGAAGCACC CGGCTAACTC
481 CGTGCCAGCA GCCGCGGTAA TACGGAGGGT GCGAGCGTTA ATCGGAATTA CTGGGCGTAA
541 AGCGCATGCA GGTGGTTCAT TAAGTCAGAT GTGAAAGCCC G
```

NAME MFF1-11

LENGTH 581 nucleotides

AFFILIATION γ-proteobacteria, V*ibrio splendidus* subgroup

ORIGIN First-feeding halibut larvae, Mannin Seafarms.

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GCGGACGGGT GAGTAATGCC TAGGAAATTG CCTTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATGATG CCTACGGGCC AAAGAGGGGG ACCTTCGGGC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GTGTGXAGTT AATAGCTGCG CATCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC
481 GTGCCAGCAG CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TCGGAATTAC TGGGCGTAAA
541 GCCGCATGCA GGTGGTTCAT TAAGTCAGAT GTGAAAGCCC G
```

NAME MA1-03

LENGTH 583 nucleotides

AFFILIATION γ-proteobacteria, V*ibrio splendidus* subgroup

ORIGIN Enriched *Artemia*, Mannin Seafarms.

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTTAACA ATCCTTCGGG TGCGTTAATG
 61 GGCGTCGAGC GGCGGACGGG TGAGTAATGC CTAGGAAATT GCCTTGATGT GGGGGATAAC
121 CATTGGAAAC GATGGCTAAT ACCGCATGAT GCCTACGGGC CAAAGAGGGG GACCTTCGGG
181 CCTCTCGCGT CAAGATATGC CTAGGTGGGA TTAGCTAGTT GGTGAGGTAA TGGCTCACCA
241 AGGCGACGAT CCCTAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGAACT GAGACACGGT
301 CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGAAA GCCTGATGCA
361 GCCATGCCGC GTGTATGAAG AAGGCCTTCG GGTTGTAAAG TACTTTCAGT TGTGAGGAAG
421 GGTGTGTAGT TAATAGCTGC GCATCTTGAC GTTAGCAACA GAAGAAGCAC CCGGCTAACT
481 CCGTGCCAGC AGCCCGCGGT AATACGGAGG GTGCGAGCGT TAATCGGAAT TACTGGGCGT
541 AAAGCGCATG CAGGTGGTTC ATTAAGTCAG ATGTGAAAGC CCG
```

NAME MFF2-03

LENGTH 582 nucleotides

AFFILIATION γ-proteobacteria, V. *splendidus* subgroup

ORIGIN First-feeding halibut larvae, Mannin Seafarms.

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GCGGACGGGT GAGTAATGCC TAGGAAATTG CCTTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATGATG CCTACGGGCC AAAGAGGGGG ACCTTCGGGC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AXACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GTGTGTAGTT AATAGCTGCG CATCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC
481 GTGCCAGCAG CCGCGGTAAT ACGGATGGGT GCGAGCGTTA ATCGGAATTA CCTGGGCGTA
541 AAGCGCATGC AGGTGGTTCA TTAAGTCAGA TGTGAAAGCC CG
```

NAME MFF2-03

LENGTH 580 nucleotides

AFFILIATION γ-proteobacteria, V. *splendidus* subgroup

ORIGIN First-feeding halibut larvae, Mannin Seafarms.

```
  1 GGCTTAACAC ATGCAAGTCG AGCGGGAAAC GACACTAACA ATCCTTCGGG TGCGTTAATG
 61 GGCGTCGAGC GGCGGACGGG TGAGTAATGC CTAGGAAATT GCCTTGATGT GGGGGATAAC
121 CATTGGAAAC GATGGCTAAT ACCGCATAAT GCCTACGGGC CAAAGAGGGG GATCTTCGGC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GGGTXACGTT AATAGCGXTA TCTCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC
481 GTGCCAGCAG CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TCGGAATTAC TGGGCGTAAA
541 GCGCATGCAG GTGGTTCATT AAGTCAGATG TGAAAGCCCG
```

NAME MA1-09

LENGTH 581 nucleotides

AFFILIATION γ-proteobacteria, V. *splendidus* subgroup

ORIGIN Enriched *Artemia*, Mannin Seafarms

```
  1 TGCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GCGGACGGGT GAGTAATGCC TAGGAAATTG CCTTRATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTTAAT ACCGCATGAT GCCTACGGGC CAAAGAGGGG GACCTTCGGG
181 CCTCTCGCGT CAAGATATGC CTAGGTGGGA TTAGCTAGTT GGTGAGGTAA TGGCTCACCA
241 AGGCGACGAT CCCTAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGAACT GAGACACGGT
301 CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGAAA GCCTGATGCA
361 GCCATGCCGC GTGTATGAAG AAGGCCTTCG GGTTGTAAAG TACTTTCAGT TGTGAGGAAG
421 GGTGTGTAGT TAATAGCTGC GCATCTTGAC GTTAGCAACA GAAGAAGCAC CGGCTAACTC
481 CGTGCCAGCA GCCGCGGTAA TACGGAGGGT GCGAGCGTTA ATCGGAATTA CTGGGCGTAA
541 AGCGCATGCA TTCRRTWCAT TAAGTCAGAT GTGAAAGCCC G
```

NAME OFC1-22

LENGTH 584 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Copepods, Otterferry

```
  1 GgCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TTCTTCGGAT GCTTTAATGG
 61 GCGTCGAGCG GCGGACGGGT GAGTAATGCC TAGGAAATTG CCTTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATAATG CCTACGGGCC AAAGAGGGGG ATCTTCGGAC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GTGTGTAGTT AATAGCTGCG CATCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC
481 GTGCCAGCAG CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TCGGAATTAC TGGGCGTAAG
541 CGCATGCAGG TGGTTCATTA AGTCAGATGT GATGTGAAAG CCCG
```

NAME OFC1-17

LENGTH 580 nucleotides

AFFILIATION γ-proteobacteria, *V. splendidus* subgroup

ORIGIN Copepods, Otterferry

```
  1 gGcCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GCGGACGGGT GAGTAATGCC TAGGAAATTG CCTTGATGTG GGGGATAACC
121 ATTGGAAACG ATGGCTAATA CCGCATAATG CCTACGGGCC AAAGAGGGGG ATCTTCGGGC
181 CTCTCGCGTC AAGATATGCC TAGGTGGGAT TAGCTAGTTG GTGAGGTAAT GGCTCACCAA
241 GGCGACGATC CCTAGCTGGT CTGAGAGGAT GATCAGCCAC ACTGGAACTG AGACACGGTC
301 CAGACTCCTA CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGAAAG CCTGATGCAG
361 CCATGCCGCG TGTATGAAGA AGGCCTTCGG GTTGTAAAGT ACTTTCAGTT GTGAGGAAGG
421 GGGTGACGTT AATAGCGTTA TCTCTTGACG TTAGCAACAG AAGAAGCACC GGCTAACTCC
481 GTGCCAGCAG CCGCGGTAAT ACGGAGGGTG CGAGCGTTAA TCGSMMTTAC TGGGCGTAAA
541 GCGCATGCAG GTGRTTCAWT AAGTCAGATG TGAAAGCCCG
```

NAME SFF4-14

LENGTH 582 nucleotides

AFFILIATION γ-proteobacteria, V. *splendidus* subgroup

ORIGIN First-feeding halibut larvae, SFIA,

```
  1 GGcCTATAAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTCGAGCG GACGGACGGG TGAGTAATGC CTAGGAAATT gCXTTGATGT GGGGGATAAC
121 CATTGGAAAC GATGGCTAAT ACCGCATGAT GCCTACGGGC CAAAGAGGGG GACCTTCGGG
181 CCTCTCGCGT CAAGATATGC CTAGGTGGGA TTAGCTAGTT GGTGAGGTAA TGGCTCACCA
241 AGGCGACGAT CCCTAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGAACT GAGACACGGT
301 CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGAAA GCCTGATGCA
361 GCCATGCCGC GTGTATGAAG AAGGCCTTCG GGTTGTAAAG TACTTTCAGT TGTGAGGAAG
421 GGTGTGTAGT TAATAGCTGC XCATCTTGAC GTTAGCAACA GAAGAAGCAC CGGCTAACTC
481 CGTGCCAGCA GCCGCGGTAA TACTGGAGGG TGCGAGCGTT AATCGGAATT ACTGGGCGTA
541 AAGCGCATGC AGGTGGTTCA TTAAGTCAGA TGTGAAAGCC CG
```

NAME MFF2-11

LENGTH 583 nucleotides

AFFILIATION γ-proteobacteria, V. *splendidus* subgroup

ORIGIN First-feeding halibut larvae, Mannin

```
  1 ggCCTAACAC ATGCAAGTCG AGCGGAAACG ACACTAACAA TCCTTCGGGT GCGTTAATGG
 61 GCGTTCGAGC GGCGGACGGG TGAGTAATGC CTAGAAAATT GCCTTRATGT GGGGGATAAC
121 CATTGGAAAC GATGGCTAAT ACCGCATGAT GCCTACGGGC CAAAGAGGGG GACCTTCGGG
181 CCTCTCGCGT CAAGATATGC CTAGGTGGGA TTAGCTAGTT GGTGAGGTAA TGGCTCACCA
241 AGGCGACGAT CCCTAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGAACT GAGACACGGT
301 CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGAAA GCCTGATGCA
361 GCCATGCCGC GTGTATGAAG AAGGCCTTCG GGTTGTAAAG TACTTTCAGT TGTGAGGAAG
421 GGTGTGTAGT TAATAGCATG YGCATCTTGA CGTTAGCAAC AGAAGAAGCA CCGGCTAACT
481 CCGTGCCAGC AGCCGCGGTA ATACGGAGGG TGCGAGCGTT AATCGGAATT ACTGGGCGTT
541 AAGSGCATGC AGGTGGTTCA TTAAGTCAGA TGTGAAAAXC CCC
```

NAME TG4-07

LENGTH 582 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio* genus

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGAAAAC GACAACATTG ATTCTTCGGA TGATTTGTTG
 61 GGCGTCGAGC GGCGGACGGG TGAGTAATGC CTAGGAAGTT GCCCGGTAGA GGGGGATAAC
121 CATTGGAAAC GATGGCTAAT ACCGCATAAT CTCTATGGAG CAAAGCAGGG GACCTTCGGG
181 CCTTGTGCTA CCGGATACGC CTAGGTGGGA TTAGCTAGTT GGTGAGGTAA TGGCTCACCA
241 AGGCGACGAT CCCTAGCTGG TCTGAGAGGA TGATCAGCCA CACTGGAACT GAGACACGGT
301 CCAGACTCCT ACGGGAGGCA GCAGTGGGGA ATATTGCACA ATGGGCGAAA GCCTGATGCA
361 GCCATGCCGC GTGTATGAAG AAGGCCTTCG GGTTGTAAAG TACTTTCAGT TGTGAGGAAG
421 GGTGTGTAGT TAATAACTGC ACATCTTGAC GTTAGCAACA GAAGAAGCAC CGGCTAACTC
481 CGTGCCAGCA GCCGCGGTAA TACGGAGGGT GCGAGCGTTA ATCGGAATTA CTGGGCCGTA
541 AAGCGCATGC AGGTGGTTCA TTAAGTCAGA TGTGAAAGCC CG
```

NAME MYS2-06

LENGTH 566 nucleotides

AFFILIATION *V. splendidus* subgroup

ORIGIN Halibut yolk-sac larvae, Mannin Seafarms

```
  1 GGCTTAACAC ATGCAAGTCG AGCGGAAACG ATTCTAGCTT GCTAGAAGGC GTCGAGCGGC
 61 GGACGGGTGA GTAATGCATG GGAATCTGCC CGATAGTGGG GGACAACCTG GGGAAACTCA
121 GGCTAATACC GCATACGTCC TACGGGAGAA AGCAGGGGAT CTTCGGACCT TGCGCTATCG
181 GATGAGCCCA TGTCGGATTA GCTAGTTGGT GAGGTAATGG CTCACCAAGG CGACGATCCG
241 TAGCTGGTCT GAGAGGATGA TCAGCCACAC TGGGACTGAG ACACGGCCCA GACTCCTACG
301 GGAGGCAGCA GTGGGGAATA TTGGACAATG GGCGCAAGCC TGATCCAGCC ATGCCGCGTG
361 TGTGAAGAAG GCCTTCGGGT TGTAAAGCAC TTTCAGCGAG GAAGAAAGCT TCTGGATTAA
421 TACTCTGGAG GAAGGACATC ACTCGCAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC
481 GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG GGCGTAAAGC GCGCGTAGTG
541 RMTWAGTMAC CAGGTGTGAA AGCCCC
```

NAME SYS3-02

LENGTH 564 nucleotides

AFFILIATION γ-proteobacteria, *Pseudoalteromonas* group

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 GGCCTAACAC ATGCAAGTCG AGCGGTAACA GAAAGTAGCT TGCTACTTTG CTGACGAGCG
 61 GCGGACGGGT GAGTAATGCT TGGGAACATG CCTTGAGGTG GGGGACAACA GTTGGAAACG
121 ACTGCTAATA CCGCATAATG TCTACGGACC AAAGGGGGCT TCGGCTCTCG CCTTTAGATT
181 GGCCCAAGTG GGATTAGCTA GTTGGTGAGG TAATGGCTCA CCAAGGCGAC GATCCCTAGC
241 TGGTTTGAGA GGATGATCAG CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG
301 GCAGCAGTGG GGAATATTGC ACAATGGGCG CAAGCCTGAT GCAGCCATGC CGCGTGTGTG
361 AAGAAGGCCT TCGGGTTGTA AAGCACTTTC AGTCAGGAGG AAAGGTTAGT AGTTAATACC
421 TGCTAGCTGT GACGTTACTG ACAGAAGAAG CACCGGCTAA CTCCGTGCCA GCAGCCGCGG
481 TAATACGGAG GGTGCGAGCG TTAATCGGAA TTACTGGGCG TAAAGCGTAC GCAGGCGGTT
541 TGTTAAGCGA GATGTGAAAG CCCC
```

NAME TG16-05

LENGTH 550 nucleotides

AFFILIATION γ-proteobacteria, *Pseudoalteromonas* group

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 TACTAGCGGX GCXACGGXTG AGAAACAGCG CTTTXATCTX CCTAXTTATA GGGGGACAAC
 61 ATGTGGGAAX CGCATGCTXX TACCGXAGAC XCCCTAAGGG GGAAAGGAGG GGACTCTTCG
121 GAGCCTTCCG CTATTAGATX AAGCCTGCGC GAGATTAGCT TAGXTGGTAG GGTAAAGGCC
181 TACCAAGGCG ACXATXTCTA ACTGGCTGAG AGGATGACCA GTCACACTGG GACTGAGACA
241 CGGGCCAXAC TTXCTACGGG AGGCAXCAAT GGGGAATATT TGGACAATGG GCGCAAGCCT
301 GATCCAGCCA TGCCCGCGTG TGTAAGAAGG CCTTAGGGTT GTAAAGCACT TTCAGGGGTG
361 AGGAAGGGTG XTAGCTTAAT ACGTTATCAT TTTGACCTTX AGCCCCAGAA TAAGCACCGG
421 CTAACTXTTT GCCAXCAGXX CGCAGGTAAT ACAAGAGGGT GGCAAGCCGT TAATCGGAAT
481 TACTGGGGCG TAAAGCGCGC XTATCGTXCG CXXGTTTAAG TCGGGATGTX AAAATCCCXG
541 GGXCTXAACA
```

NAME TG4-11

LENGTH 651 nucleotides

AFFILIATION γ-proteobacteria, *Pseudoalteromonas* group

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 TTTXGATTCA TGGCTCAGAT TGAACGCTGG CGGCAGCTAc ACATGCAAGT CGAGCGGTAA
 61 CAGAGAGTAG CTTGCTACTT TGCTGACGAG CGGCGGACGG GTXAGTAATG CTTGGGAACA
121 TGCCTTGAGG TGGGGGACAA CAGTTGGAAA CGACTGCTAA TACCGCATAA TGTCTACGGA
181 CCAAAGGGGG CTTCGGCTCT CGCCTTTAGA TTGGCCCAAG TGGGATTAAG CTAGTTGGTG
241 AGGTAATGGC TCACCAAGGC GACGATCCCT AGCTGGtTTT GAGAGGATGA TCAGCCACAC
301 TGGGACTGAG ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGCACAATG
361 GGCGCAAGCC TGATGCAGCC ATGCCGCGTG TGTGAAGAAG GCCTTCGGGT TGTAAAGCAC
421 TTTCAGTCAG GAGGAAAGGT TAGTAGTTAA TACCTGCTAG CcTGTGACGT TACTGACAGA
481 AGAAGCACCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA CGGAGGGTGC GAGCGTTAAT
541 CGGAATTACT GGGCGTAAAG CGTACGCAGG CGGTTTGTTA AGCGAGATGT GAAAGCCCTG
601 GGCTCAACCT GGGAACTGCA TTTCGAACTG GCAAACTAGA GgTGTGATAG A
```

NAME TG7-01

LENGTH 630 nucleotides

AFFILIATION γ-proteobacteria, *Pseudoalteromonas* group

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 ATCCAGGGCT AXAATTAACC GCGGCGCCAG GCTTTACXCA TGCATGKCGA RCGGTRRCWG
 61 GGRARCTTGC TCTGSTtGAC GAGCGGCGAC GGGTgAGTAA CGCGTAKGAA TCTGCYTAGT
121 AGAGGGGGAC AACATGTGGA AACGCATGCT AATACCGCAT ACGCCCTAAG GGGGAAAGGA
181 GGGGaCTCTT CGGAGCCTTC CGCTATWAGA TGAGCCTGCG TGAGATWAGC TAGTTGGTAG
241 GGTAAAGGCC TACCAGGCGA CGATCTCTAA CTGGTCTGAG AGGATGACCA GTCACACTGG
301 GACTGAGACA CGGCCCAGAC TCTACGGAGG CAGCAGTGGG GAATATTGGA CAATGGGCGC
361 AAGCCTGATC CAGCCATGCC GCGTGTGTGA AGAARGCCTT AGGGTTGTAA AGCACTTTCA
421 GGGGTGAGGA AGGGTAATGG ATTAATACTT CATTACTTTG ACGTTAGCCC CAGAAGAAGC
481 ACCGGCTAAC TCTGTGCCAG CAGCCGCGGT AATACAGAGG GTGCAAGCGT TAATCGGAAT
541 TACTGGGCGT AAAGCGCGCG TAGGTGGTTT GTTAAGTCGG ATGTGAAATC CCAGGGCTCA
601 ACCTGGAATG GCACCCGATA CTGGCTAGCT
```

NAME TG8-01

LENGTH 630 nucleotides

AFFILIATION γ-proteobacteria, *Pseudoalteromonas* group

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 GGGCTTAXGA TTTAAACCGC GGGGGGCCAG GCTTAACACC XTGCAWGTCK AGCGGTAAAX
 61 GAGAGGgTgC TTGCXCCTTT gCTGACXAGC GGCGGACGGG TGAGTAATGC TTGGGAACAT
121 GCCTTGAGGT GGGGGACAAC AGTGGAAACG ACTGCTAATA CCGCATAATG TCTACGACCA
181 AAGGGGGCTT CGGCTCTCGC CTTTAGATTG GCCCAAGTGG GATWAGCTAG TTGGTGAGGT
241 AATGGCTCAC CAAGGCGACG ATCCCTAGcT TGGTTTGAGA GGATGATCAG CCACACTGGG
301 ACTGAGACAC GGCCCAGACT CTACGGAGGC AGCAGTGGGG AATATTGCAC AATGGGCGCA
361 AGCCTGATGC AgCCATGCCG CGTGTGTGAA GAAGGXCTTC GGGTTGTAAA GCACTTTCAG
421 TCAGGAGGAA AGGTTAGTAG TTAATACCTG CTAGCTGTGA CGTTACTGAC AGAAGAAGCA
481 CCGGCTAACT CCGTGCCAGC AGCCGCGGTA ATACGGAGGG TGCGAGCGTT AATCGGAATT
541 ACTGGGCGTA AAGCGTACGC AGGCGGTTTG TTAAGCGAGA TGTGAAAGCC CTGGGCTCAA
601 CCTGGGAACT GCATTTCGAA CTGGCAAACT
```

NAME SYS6-10

LENGTH 681 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio* genus

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 TTTGATCATG GCTCAXATTG AACGCTGGCG GCAGGCCTAA CACATGCAAG TCGAGCGXAA
 61 AACGACAACA TTGACTCTTC GGATGATTTX TTGGGCGTCX AGCGGCGXAC GGGTGAGTAA
121 TGCCTAGGAA GTTGCCCGGT AGAGGGGGAT AACCATTGGA AACGATGGCT AATACCGCAT
181 AATCTCTATG GAGCAAAGCA GGGGACCTTC GGGCCTTGTG CTACCGGATA CGCCTAGGTG
241 GGATTAGCTA GTTGGTGAGG TAATGGCTCA CCAAGGCAAC GATCCCTAGC TGGTCTGAGA
301 GGATGATCAG CCACACTGGA ACTGAGACAC GGTCCAGACT CCTACGGGAG GCAGCAGTGG
361 GGAATATTGC ACAATGGGCG AAAGCCTGAT GCAGCCATGC CGCGTGTATG AAGAAGGCCT
421 TCGGGTTGTA AAGTACTTTC AGTTGTGAGG AAGGGTGTGT AGTTAATAAC TGCGCATCTT
481 GACGTTAGCA ACAGAAGAAG CACCGGCTAA CTCCGTGCCA GCAGCCGCGG TAATACGGAG
```

```
541 GGTGCGAGCG TTAATCGGAA TTACTGGGCG TAAAGCGCAT GCAGGTGGTT CATTAAGTCA
601 GATGTGAAAG CCCGGGGCTC AACCTCGGAA CTGCATTTGA AACTGGTAAA TAGAGTACTG
661 GCAAATGGAA ATGAATTCAA A
```

NAME TG3-15

LENGTH 650 nucleotides

AFFILIATION γ-proteobacteria, *Pseudoalteromonas* group

ORIGIN Halibut yolk-sac larvae, SFIA Ardtoe

```
  1 TATCCAXGGC TAAAATTTGA ACCGCXGGGG GGCAGGCTTA CCXCTGCAAG TCGAXCGGTA
 61 AXAAAGAGTA GCTTGTTCXT TTGCTCXCGA GGGCGGAXGG TGAGTAATGC TTTGGGAATA
121 TGCCTTTTGG TGGGGGACAA CAGTTGGAAA CGACTTGCTA ATACCGCATG ATGTXTACGG
181 ACCAAAGTGG GGGACCTTCG GGCCTCACGC CAAAAGATTA GCCCAAGTGG GATXAGCTAG
241 TTGGTAAGGT AATGGCTTAC CAAGGCAACG ATCCCTAGCT GGTTTGAGAG GATGATCAGC
301 CACACTGGGA TTGAGACACG GCCCAGACTC CTACGGGAGG CAGCAGTGGG GAATATTGCA
361 CAATGGGCGC AAGCCTGATG CAGCCATGCC GCGTGTGTGA AGAAGGCTTC GGGTTGTAAA
421 GCACTTTCAG TCAGGAGGAA AGGGTGTGTT TTAATAGAGC ATATCTGTGA CGTTACTGAC
481 AGAAGAAGCA CCGGCTAACT CCGTGCCAGC AGCCGCGGTA ATACGGAGGG TGCGAGCGTT
541 AATCGGAATT ACTGGGCGTA AAGCGTACGC AGGCGGTTTG TTAAGCGAGA TGTGAAAGCC
601 CCGGGCTCAA CCTGGGAAXT GCATTTCGAA CTGGCAAACT AGAGTGTXAG
```

NAME W1-07

LENGTH 567 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio fischeri* group

ORIGIN Halibut larvae freshly transferred to an inert diet, SFIA Ardtoe

```
  1 CCTACACATG CAAGTCGAGC GGTAACAGAA ATTAGCTTGC TAATTTGCTG ACGAGCGGCG
 61 GACGGGTGAG TAATGCCTGG GAATATGCCT TAKTGTGGGG GATAACTATT GGAAACGATA
121 GCTAATACCG CATAATGTCT TCGGACCAAA GAGGGGGACC TTCGGGCCTC TCGCGCTAAG
181 ATTAGCCCAG GTGAGATTAG CTAGTTGGTG AGGTAAGAGC TCACCAAGGC GACGATCTCT
241 AGCTGGTCTG AGAGGATGAT CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG
301 GAGGCAGCAG TGGGGAATAT TGCACAATGG GCGAAAGCCT GATGCAGCCA TGCCGCGTGT
361 ATGAAGAAGG CCTTCGGGTT GTAAAGTACT TTCAGTAGGG AGGAAGGCGT TGTCGTTAAT
421 AGCGGCTTCG TTTGACGTTA CCTACAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG
481 CGGTAATACG GAGGGTGCGA GCGTTAATCG GAATTACTGG GCGTAAAGCG CATGCAGGTG
541 GTTCATTAAG TCAGATGTGA AAGCCCG
```

NAME SFF5-16

LENGTH 560 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio salmonicida* group

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 CTTAACCCTT GCAGTCGAGC GGTACAGGAA TWAGCTTGCT ATTCGCTGAC AGCGGCGGAC
 61 GGKTGAGTAA TGCCTGGGAA TATGCCTTAG TGTGGGGGAT AACTATKGGA AMCGATAGCT
121 AATACCGCAT AAGGTCTTCG GACCAAAGAG GGGATCTTCG GACCTCTCGC GYTAAGATTA
181 GCCCAGGTGA GATWAGCTAG TTGTGAGGTA AGAGCTCACC AAGGCGACGA TCTCTAGCTG
241 GTCTGAGAGG ATGATCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC TACGGGAGGC
301 AGCAGTGGGG AATATTGCAC AATGGGCGAA AGCCTGATGC AGCCATGCCG CGTGTATGAA
361 GAAGGCTTCG GGTTGTAAAG TACTTTCAGT CGTGAGGAAG GGTGTGTAGT TAATAGCTGC
```

```
421 ATATCTTGAC GTAGCGACAG AAGAAGCACC GGCTAACTCC GTGCCAGCAG CCGCGGTAAT
481 ACGGAGGGTG CGAGCGTTAA TCGGAATTAC TGGGCGTAAA GCGCATGCAG GTGGTTCATT
541 AAGTCAGATG TGAAAGCCCG
```

NAME MFF1-05

LENGTH 568 nucleotides

AFFILIATION γ-proteobacteria, *Vibrio salmonicida* group

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 CCTAACCCAT GCAWGTCGAG CGGGTRACAA GAAGTAGCTT GCTACTTTGC TGACKAGCGG
 61 CGGACGGGTG AGTAATGCCT GGGAATATGC CTTGATGTGG GGGATAACTA TGGAAACGAT
121 AGCTAATACC GCATAATGTC TTCGGACCAA AGAGGGGGAT CTTCGGACCT CTCGCGTCAA
181 GATTAGCCCA GGTGAGATWA GCTAGTTGGT GGGGTAAGAG CTCACCAAGG CGACGATCTC
241 TAGCTGGTCT GAGAGGATGA TCAGCCACAC TGGAACTGAG ACACGGTCCA GACTCCTACG
301 GGAGGCAGCA GTGGGGAATA TTGCACAATG GGCGAAAGCC TGATGCAGCC ATGCCGCGTG
361 TATGAAGAAG GCCTTCGGGT TGTAAAGTAC TTTCAGTCGT GAGGAAGGGT GTGCAGTTAA
421 TAGCTGCACA TCTTGACGTT AGCGACAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC
481 GCGGTAATAC GGAGGGTGCG AGCGTTAATC GGAATTACTG GGCGTAAAGC GCATGCAGGT
541 GGTTCATTAA GTCAGATGTG AAAGCCCG
```

NAME MYS1-06

LENGTH 568 nucleotides

AFFILIATION γ-proteobacteria, *Photobacterium profundum* subgroup

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 CCTAACACAT GCAAGTCGAG CGGTAACAGG AATTAGCTTG CTAATTCGCT GACGAGCGGC
 61 GGACGGGTGA GTAATGCCTG GGAATATGCC TTAGTGTGGG GGATAACTAT TGGAAACGAT
121 AGCTAATACC GCATAACGTC TTCGGACCAA AGAGGGGGAC CTTCGGGCCT CTCGCGCTAA
181 GATTAGCCCA GGTGGGATTA GCTAGTTGGT GGGGTAAAGG CCCACCAAGG CAACGATCCC
241 TAGCTGGTCT GAGAGGATGA TCAGCCACAC TGGAACTGAG ACACGGTCCA GACTCCTACG
301 GGAGGCAGCA GTGGGGAATA TTGCACAATG GGGGAAACCC TGATGCAGCC ATGCCGCGTG
361 TATGAAGAAG GCCTTCGGGT TGTAAAGTAC TTTCAGTCGT GAGGAAGGCW TTGTAGTTAA
421 TAGCTGCXXX GCTTGACGTT AGCGACAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC
481 GCGGTAATAC GGAGGGTGCG AGCGTTAATC GGAATTACTG GGCGTAAAGC GCATGCAGGC
541 GGTCTGTTAA GCAAGATGTG AAAGCCCG
```

NAME W2-07

LENGTH 580 nucleotides

AFFILIATION γ-proteobacteria, *Photobacterium profundum* subgroup

ORIGIN First-feeding halibut larvae, SFIA Ardtoe

```
  1 TGACGAGCgG CGGACGGGTG AGTAATGCCT GGGAATATGC CTTAKTGTGG GGGATAACTA
 61 TTGGAAACGA TaGCTAATAC CGCATAATGT CTTCGGACCA AAGAGGGGGA CCTTCGGGCC
121 TCTCGCGCTA AGATTAGCCC AGGTGAGATT AGCTAGTTGG TGAGGTAAGA GCTCACCAAG
181 GCGACGATCT CTAGCTGGTC TGAGAGGATG ATCAGCCACA CTGGAACTGA GACACGGTCC
241 AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGCACAAT GGGCGAAAGC CTGATGCAGC
301 CATGCCGCGT GTATGAAGAA GGCCTTCGGG TTGTAAAGTA CTTTCAGTAG GGAGGAAGGC
361 GTTGTCGTTA ATAGCGGCTT CGTTTGACGT TACCTACAGA AGAAGCACCG GCTAACTCCG
421 TGCCAGCAGC CGCGGTAATA CGGAGGGTGC GAGCGTTAAT CGGAATTACT GGGCGTAAAG
481 CGCATGCAGG TGGTTCATTA AGTCAGATGT GAAAGCCCGG GGCTCAACCT CGGAACCGCA
541 TTTGAAACTG GTAACTAGAG TGCTGTAGAG GGGGGAAGGT
```

NAME OFE1-10

LENGTH 628 nucleotides

AFFILIATION γ-proteobacteria, *Collwelia group*

ORIGIN Halibut eggs, Otter Ferry Seafarms

```
  1 GGCTCCATTA CCTXCTTAGA GTTTGATCAT GGCTCAGGGT GGCTGGAXCA CCTCCTTAGA
 61 GTTTGATCAT GGCTCAGGGT GGCTGGXXXA CCTCCTTAgT AGCTYGGGMA TRtGCTTATS
121 GTGGGTGGAC AACCTCTGTA AAcGAXCGcT AatxCcgcAT MaGGTTtACG GACCAWAGGG
181 KGScATCTTC GGCCTCTCGC CATTTGATTA GCCCAAGTGA GATTAGCTAG TTGGTGAGGT
241 AATGGCTCAC CAAGGCGACG ATCTCTAGCT GGTTTGAGAG GATGATCAGC CACACTGGGA
301 YTGAGACACG GCCCAGACTC YTACGGGAGG CASCAGTGGG GAATATTGCA CAATGGGCGA
361 AAGCCTGATG CACCATGCCG CGTGTGTGAA GAARGCCTTC RGGTTGTAAA GCACTTTCAG
421 TTGTGAGGAA AGGGGAGTAa GTTAATAGCT GCWTXCTGTG ACGxTTACAA CAGAAGAAGC
481 CCGGCTAACT TCGTGCCAGC AGCCGCGGTA ATACSAXGGG TGCAAGCGTT AATCSGAATT
541 ACTGGGCGTA AAXCGTTCGT AXGCcGGTCT ATTaAAGCAA GATGTGAAAG CCCAGGGCTC
601 AACCTTGGgA ACTGCATTTT GAACTGGG
```

Appendix 4 a Summary data for individual 2L flasks in Infection Experiment 5 (Section 4.3.4.5)

Treatment	Sterile immediately prior to inoculation.	Numbers of bacteria present in flask water at end of experiment.	Mean CFU ml^{-1} for each treatment	Number surviving to 200 day-degrees / 25	Number Deformed.	Mean surviva (±SEM) for eac treatment.
CONTROL	[1]YES	None detected		19	7	84
CONTROL	YES	None detected		19	8	(±9.8)
CONTROL	YES	None detected		25	7	
SYS6-10	YES	$>10^6$ CFU ml^{-1}	6.06×10^6	7	1	41
SYS6-10	YES	$>10^6$ CFU ml^{-1}	$(\pm 9.8 \times 10^5)$	16	4	(±10.7)
SYS6-10	YES	$>10^6$ CFU ml^{-1}		6	3	
SYS6-10		$>10^6$ CFU ml^{-1}		12	4	
TG11-16	YES	Not done	2.54×10^6	6	3	52
TG11-16	YES	$>10^6$ CFU ml^{-1}		16	4	(±12.2)
TG11-16	YES	$>10^6$ CFU ml^{-1}		17	4	
TG15-07	YES	$>10^6$ CFU ml^{-1}	5.74×10^6	9	1	41.3
TG15-07	YES	$>10^6$ CFU ml^{-1}	(1.92×10^5)	0	0	(±14.5)
TG15-07	YES	$>10^6$ CFU ml^{-1}		11	2	
TG4-07	YES	$>10^6$ CFU ml^{-1}	1.58 x 106	9	4	57.6
TG4-07	YES	$>10^6$ CFU ml^{-1}	(± 3.98 x 105)	9	1	(± 11.6)
TG4-07	YES	$>10^6$ CFU ml^{-1}		15	7	
TG4-07	YES	$>10^6$ CFU ml^{-1}		16	4	
TG4-07	YES	$>10^6$ CFU ml^{-1}		23	4	
TG7-01	YES	$>10^6$ CFU ml^{-1}	2.88×10^6	9	2	64
TG7-01	YES	$>10^6$ CFU ml^{-1}	$(\pm 1.48 \times 10^6)$	13	7	(± 19.1)
TG7-01	YES	$>10^6$ CFU ml^{-1}		14	5	
TG7-01	YES	$>10^6$ CFU ml^{-1}		28	7	

Appendix 4 b Summary data for individual 2L flasks in Experiment 6 (Section 4.3.4.6). Due to high flask contamination rate average CFU ml^{-1} per treatment not calculated.

Treatment	Sterile or apparently pure culture in flask at end of experiment.	Types and numbers of bacteria present in flask water at end of experiment.	Number of surviving larvae	Number of deformed larvae	Mean % survival (±SEM) for each treatment
CONTROL	NO	Cream > 10^6 ml^{-1}	14	2	51.47(± 7.8)
CONTROL	NO	Pale cream & yellow pigmented > 10^6 ml^{-1}	12	1	
CONTROL	NO	Yellow pigmented	6	3	
CONTROL	NO	> 10^6 ml^{-1} cream & yellow pigmented	17	4	
CONTROL	NO	> 10^6 ml^{-1} cream	18	0	
CONTROL	NO	Pale cream dark cream & yellow pigmented > 10^6 ml^{-1}	7	0	
CONTROL	NO	Dark cream >10^6 ml^{-1}	16	3	

Appendix 4b Summary data for individual 2L flasks in Experiment 6 (Section 4.3.4.6). Due to high flask contamination rate average CFU ml^{-1} per treatment not calculated.

Treatment	Sterile or apparently pure culture in flask at end of experiment.	Types and numbers of bacteria present in flask water at end of experiment.	Number of surviving larvae	Number of deformed larvae	mean % survival (±SEM) for each treatment
E1-03	NO	Pale cream & yellow pigmented > 10^6 ml^{-1}	13	0	43 (± 11.8)
E1-03	NO	> 10^6 ml^{-1} E1-03	2	2	
E1-03	NO	cream & yellow pigmented > 10^6 ml^{-1}; none luminous	13	2	
E1-03	YES	> 10^6 ml^{-1} E1-03	15	4	
OFE1-19	NO	Two cream morphotypes & yellow pigmented > 10^6 ml^{-1}	14	0	47 (±5.74)
OFE1-19	NO	Cream > 10^6 ml^{-1}	8	5	
OFE1-19	YES	Pale cream & yellow pigmented > 10^6 ml^{-1}	11	1	
OFE1-19	YES	Two yellow pigmented morphotypes > 10^6 ml^{-1}	14	3	

Appendix 4b Summary data for individual 2L flasks in Experiment 6 (Section 4.3.4.6). Due to high flask contamination rate average CFU ml^{-1} per treatment not calculated.

Treatment	Sterile or apparently pure culture in flask at end of experiment.	Types and numbers of bacteria present in flask water at end of experiment.	Number of surviving larvae	Number of deformed larvae	mean % survival (±SEM) for each treatment
OFE1-19	NO	Two cream morphotypes & yellow pigmented > 10^6 ml^{-1}	14	0	47 (±5.74)
OFE1-19	NO	Cream > 10^6 ml^{-1}	8	5	
OFE1-19	YES	Pale cream & yellow pigmented > 10^6 ml^{-1}	11	1	
OFE1-19	YES	Two yellow pigmented morphotypes > 10^6 ml^{-1}	14	3	
OFF1-05	NO	Pale cream & yellow pigmented > 10^6 ml^{-1}	18	4	47 (± 13.3)
OFF1-05	YES	>10^6 ml^{-1} OFF1-05	6	1	
OFF1-05	YES	Pale cream and OFF1-05 >10^6 ml^{-1}	6	6	
OFF1-05	NO	>10^6 ml^{-1} OFF1-05 (67)	17	2	

Appendix 4b Summary data for individual 2L flasks in Experiment 6 (Section 4.3.4.6). Due to high flask contamination rate average CFU ml^{-1} per treatment not calculated.

Treatment	Sterile or apparently pure culture in flask at end of experiment.	Types and numbers of bacteria present in flask water at end of experiment.	Number of surviving larvae	Number of deformed larvae	Mean survival % (±SEM) for each treatment
TG15-08	NO	> 10^6 ml^{-1} TG15-08 & cream contaminant	13	6	52 (± 1.63)
TG15-08	YES	> 10^6 ml^{-1} TG15-08	12	4	
TG15-08	YES	> 10^6 ml^{-1} TG15-08	14	1	
TG15-08	NO	> 10^6 ml^{-1} TG15-08	13	3	
TG2-11	NO	Pale cream & yellow pigmented > 10^6 ml^{-1}	4	1	50 (± 12.1)
TG2-11	YES	Pale cream & yellow pigmented > 10^6 ml^{-1}	15	3	
TG2-11	YES	Pale cream & dark cream > 10^6 ml^{-1}	18	3	
TG2-11	YES	Yellow pigmented > 10^6 ml^{-1}	13	1	

Appendix 4b Summary data for individual 2L flasks in Experiment 6 (Section 4.3.4.6). Due to high flask contamination rate average CFU ml^{-1} per treatment not calculated.

Treatment	Sterile or apparently pure culture in flask at end of experiment.	Types and numbers of bacteria present in flask water at end of experiment.	Number of surviving larvae	Number of deformed larvae	Mean survival % (±SEM) for each treatment
TG2-12	NO	$> 10^6$ ml^{-1} cream & yellow pigmented	3	0	34 (± 18)
TG2-12	NO	$> 10^6$ ml^{-1} cream & yellow pigmented	21	0	
TG2-12	YES	Cream $> 10^6$ ml^{-1}	9	9	
TG2-12	NO	Cream and dark cream $> 10^6$ ml^{-1}	1	0	
TG4-11	NO	$> 10^6$ ml^{-1} cream & yellow pigmented	5	0	27 (±9.3)
TG4-11	NO	$> 10^6$ ml^{-1} cream & yellow pigmented	7	5	
TG4-11	YES	Cream $> 10^6$ ml^{-1}	13	2	
TG4-11	YES	Pale cream $> 10^6$ ml^{-1}	2	3	

Appendix 4b Summary data for individual 2L flasks in Experiment 6 (Section 4.3.4.6). Due to high flask contamination rate average CFU ml^{-1} per treatment not calculated.

Treatment	Sterile or apparently pure culture in flask at end of experiment	Types and numbers of bacteria present in flask water at end of experiment.	Number surviving to 200 day-degrees / 25	Number of deformed larvae	mean % survival (±SEM) for each treatment
V. anguillarum 91079	NO	$>10^6$ ml^{-1} TCBS culturable contaminant	0	0	0
V. anguillarum 91079	YES	$>10^6$ ml^{-1} cream & yellow pigmented	0	0	
V. anguillarum 91079	YES	$> 10^6$ ml^{-1} 91079 & cream contaminant	0	0	
V. anguillarum 91079	NO	$> 10^6$ ml^{-1} 91079 & cream contaminant	0	0	

Appendix 4c Summary data for individual 2L flasks in Experiment 7 (Section 4.3.4.6).

Treatment	Sterile immediately prior to inoculation.	Numbers of bacteria present in flask water at end of experiment.	Mean CFU ml^{-1} for each treatment	Number surviving to 200 day-degrees / 25	Number Deformed.	Mean surviva (±SEM) for eac treatment.
CONTROL	[a]YES	None detected		10	1	49.0
CONTROL	YES	None detected		22	0	(±4)
CONTROL	YES	> 1000 CFU ml^{-1}		16	3	
CONTROL	YES	None detected	< 1	0	0	
CONTROL	YES	None detected		12	12	
CONTROL	YES	None detected		17	3	
CONTROL	YES	None detected		21	6	
CONTROL	YES	None detected		0	0	
AE1-26	YES	None detected	4.76×10^6	15	0	45.0
AE1-26	YES	>10^7 CFU ml^{-1} TCBS sucrose positive	(± 3.19×10^6)	9	4	(±5.4)
AE1-26	YES	None detected		8	3	
AE1-26	YES	> 10^7 CFU ml^{-1} TCBs sucrose positive		13	3	
TG10-01	YES	<100 CFU ml^{-1}	9.12×10^5	16	6	60.0
TG10-01	YES	None detected	(1.05×10^6)	20	4	(±10.15)
TG10-01	YES	None detected		18	7	
TG10-01	YES	>107 CFU ml^{-1}		6	3	
TG15-19	YES	>10^7 CFU ml^{-1}	2.48×10^7	9	1	20.0
TG15-19	YES	>10^7 CFU ml^{-1}	(7.9×10^6)	0	0	(±9.52)
TG15-19	YES	>10^7 CFU ml^{-1}		11	2	
TG15-19	YES	>10^7 CFU ml^{-1}		0	0	

[a] All control flasks were sterile at the termination of the experiment (as assessed by plating onto MA and inoculating MB)

Appendix 4c Summary data for individual 2L flasks in Experiment 7 (Section 4.3.4.6).

Treatment	Sterile just prior to inoculation	Numbers of bacteria present in flask water at end of experiment.	Mean CFU ml^{-1} for each treatment	Number Surviving to 200 day-degrees / 25	Number deformed	Mean survival (±SEM) for each treatment.
TG16-05	YES	$>10^6$ CFU ml^{-1}	2.01×10^6	12	4	49
TG16-05	YES	$>10^7$ CFU ml^{-1}	(1.26×10^6)	14	3	(±2.79)
TG16-05	YES	$>10^6$ CFU ml^{-1}		13	8	
TG16-05	YES	$>10^7$ CFU ml^{-1}		10	1	
TG2-14	YES	$>10^6$ CFU ml^{-1}	6.16×10^6	14	1	55
TG2-14	YES	$>10^6$ CFU ml^{-1}	(6.76×10^5)	15	2	(±8.15)
TG2-14	NO	$>10^7$ CFU ml^{-1} (contaminant)		7	0	
TG2-14	YES	$>10^6$ CFU ml^{-1}		19	3	
TG2-18	YES	$>10^7$ CFU ml^{-1}	1.16×10^7	12	1	45
TG2-18	YES	$>10^7$ CFU ml^{-1}	(3.6 x 106)	8	3	(±10.61)
TG2-18	YES	$>10^7$ CFU ml^{-1}		5	4	
TG2-18	NO	$>10^7$ CFU ml^{-1}		20	6	
TG4-02	YES	$>10^7$ CFU ml^{-1}	1.4×10^7	11	2	[a]83
TG4-02	NO	$>10^7$ CFU ml^{-1}	(6.23×10^6)	19	3	(±15.7)
TG4-02	YES	$>10^7$ CFU ml^{-1}		19	5	
TG4-02	YES	$>10^7$ CFU ml^{-1}		34	2	
TG8-01	YES	$>10^7$ CFU ml^{-1}	1.68×10^7	14	3	47
TG8-01	YES	ND	(1.04×10^7)	10	3	(±15.6)
TG8-01	NO	$>10^7$ CFU ml^{-1}		23	4	
TG8-01	YES	$>10^7$ CFU ml^{-1}		0	0	

[a]High % survival includes survivals in flask that has obviously been over-stocked.

Appendix 4c Summary data for individual 2L flasks in Experiment 7 (Section 4.3.4.6).

Treatment	Sterile (20/03/00)	Numbers of bacteria present in flask water at end of experiment.	Mean CFU ml^{-1} for each treatment	Number Surviving to 200 day-degrees / 25	Number deformed	Mean % survival (±SEM)
V. viscosus 236	YES	[a] None detected		10	0	33.33333
V. viscosus 236	YES	None detected	[b]<10	15	3	(±12.47)
V. viscosus 236	NO	> 10^7 CFU ml^{-1} (contaminant)		0	0	
V. viscosus 236	YES	None detected		16	7	

[a]Only one of the flasks which had been inoculated with *V. viscosus* contained MA culturable bacteria at the end of the experimental period and this was clearly a contaminant. *V. viscosus* is not readily cultured on MA so it may have been present in all the flasks.

[b] Calcualted mean excluded contaminated flask.

Appendix 5

List of suppliers

BIOLOG	Biolog, Inc., 3938 Trust Way, Hayward, CA 94545 UK agent: Don Whitley Scientific, 14 Otley Road, Shipley, W. Yorks. BD17 7ST
BIORAD	Biorad House, Marylands Avenue, Hemel Hampstead, Herts.
Difco	Becton Dickinson Microbiology Systems, Becton Dickinson and Company, Sparks, MD 21152, USA
Mast Diagnostics Limited	Mast International Limited, Mast House, Derby Road, Bootle, Merseyside L20 1EA
Oswel DNA Service	Lab 5005, Medical & Biological Services Building, University of Southampton, Boldrewood, Bassett Crescent East, Southampton SO16 7PX
PNACL	Protein and Nucleic acid laboratory, University of Leicester
Quiagen	Unit 1, Tillingbourne Court, Dorking Business Park, Dorking, Surrey.
R. S. Hygiene Ltd	PO Box 8, Mildenhall, Suffolk IP28 7HE
Sigma-Aldrich Company Ltd.	Fancy Road, Poole, Dorset, BH12 4HQ

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