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# Effect of Gastrointestinal Microbiota on Growth in Mangrove Killifish (Kryptolebias marmoratus) and Atlantic Cod (Gadus morhua)

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## Abstract

All animals live in symbiosis with complex microbial communities. The gastrointestinal system in vertebrates is a natural environment for microbes, and this leads to a complex and numerous microbiota. The gastrointestinal (GI) microbiota has several functions of importance to the host, and the development of molecular biological methods for investigation of microbial communities has lead to a new understanding of this environment.

The hypothesis of this thesis was that growth rate in larval fish is partly explained by the composition of the GI microbiota. This was tested by comparing the GI microbiota of slow and fast growing Atlantic cod (*Gadus morhua*) and mangrove killifish (*Kryptolebias marmoratus*) of the same age. The GI microbiota was characterized by PCR/DGGE (denaturing gradient gel electrophoresis) and sequencing of bands from the DGGE gels.

There was a significant difference between the GI microbiota in fast and slow growing individuals from cod and the mangrove killifish strain DAN. The mangrove killifish strain PAN-RS also showed differences, but these findings were only marginally significant. This can partially be explained by the low number of samples analyzed. The GI microbiota of the PAN-RS juveniles had similarities with the microbial composition of both the feed and water, and showed that the GI microbiota is affected by both.

In an experimental test it was attempted to examine if exposure to the culturable microbiota from either slow or fast growing individuals could reproduce size differences. However, the cultured bacteria from fast and slow growing mangrove killifish PAN-RS larvae were not significantly different in composition. Thus it was not expected to find any size difference between the fish larvae supplied with the different cultured bacteria. This was confirmed analytically, but the fish larvae supplied with the bacteria had a larger variation in size than the control group.

The results in this thesis indicate a difference in the composition of the GI microbiota between fast and slow growing fish in the early stages of development. Further studies are required to verify if this is a causal relationship where differences in the GI microbiota of individuals results in differences in somatic growth.

## Abbreviations

3D – 3 dimensional

AASW – autoclaved artificial sea water

ANOSIM – analysis of similarity

APS – ammonium persulfate

bp – base pair

DAH – days after hatching

DGGE – denaturing gradient gel electrophoresis

DNA – deoxyribonucleic acid

FISH – fluorescent *in situ* hybridization

GI – gastrointestinal

H’ – Shannon diversity index (Shannon index)

J’ – Pielou’s evenness index (Evenness)

k – band richness

MS 222 – tricaine methane sulphonate

PCR – polymerase chain reaction

ppt – parts per thousand

RDP – ribosomal database project

rRNA – ribosomal ribonucleic acid

SL – standard length

TAE – tris base, acetic acid and EDTA

TEMED – tetramethylethylenediamine

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# 1. Introduction

The microbiota is the bacterial composition of an ecosystem, such as a host organism. Microbiota and its composition have lately started getting more attention. This increase in attention is due to the development of methods to examine the non-cultivable microorganisms which earlier was difficult and sometimes impossible to investigate. This development can be exemplified by “The Human Microbiome Project” started in 2008, which has a goal of identifying and characterizing the microorganisms found in association with humans (Turnbaugh *et al*., 2007).

In light of these recent advances studies have been done comparing the human gut flora with the weight of individuals. It was found that, in humans and mice, the gut microbiota is a factor contributing to the pathophysiology of obesity, and it has been proposed that the microbiome of overweight individuals has an increased capacity to harvest energy from the nutrients (Turnbaugh *et al*., 2006).

Recent studies on the microbiota of Atlantic cod (*Gadus morhua*) have demonstrated that the individual variation in the composition within one rearing tank is comparable to that found between individuals from tanks with very different holding regimes. It was also observed that genetically similar individuals (*e.g*. siblings) bred under the same conditions grow at a different rate (Fjellheim *et al*., 2006).

The hypothesis of this thesis was that growth rate in larval fish is partly explained by the composition of the GI microbiota. This was tested by comparing the GI microbiota of slow and fast growing Atlantic cod (*Gadus morhua*) and mangrove killifish (*Kryptolebias marmoratus*). Cod is an important fish for Norwegian fisheries, and with potential for use in aquaculture. Mangrove killifish is the only known vertebrate which is capable of self-fertilization, and after breeding one can get genetically very similar individuals (Harrington, 1961). The GI microbiota was characterized by PCR/DGGE (denaturing gradient gel electrophoresis) and sequencing of bands from the DGGE gels.

## 1.1 The gastrointestinal microbiota

All animals are living in symbiosis with complex microbial communities. The intestinal system in vertebrates is a natural environment for microbes, and this leads to a complex and numerous microbiota. This GI microbiota has several functions of importance to the host. The development of molecular biological methods for investigation of microbial communities has lead to a new understanding of this environment, as earlier studies of the GI microbiota used culture-based methods, which only shows a small fraction of the microbes present (Madigan and Martinko, 2006; Meier *et al*., 2008).

Studies with gnotobiotically grown animals (animals with a known microbiota, *e.g.* without) have shown that the GI microbiota is beneficial for different reasons. The GI microbiota participates in digestion and synthesizes essential growth factors and nutrients. The bacteria also play an important role in the protection against pathogens. They are a protective barrier, a key factor in development and regulation of the immune system and important for development of the epithelial cell layer. Most of the knowledge about the GI microbiota is known from studies of mammals, especially rodents and humans, and if not otherwise mentioned the examples below are from studies in mammals (Vadstein *et al*., 2004; Guarner and Malagelada, 2003).

The metabolic functions of the GI microbiota are diverse. A major function is the fermentation of non-digestible dietary residue and of endogenous mucus produced by the epithelia. The diversity in the microbial community contributes to the decomposition of residues by various enzymes and biochemical pathways. This results in the recovery of otherwise lost metabolic energy which can be absorbed by the host, and the supply of nutritive products like essential growth factors and nutrients (Macfarlane and Macfarlane, 1997).

Epithelial cell growth and differentiation in the intestinal system is affected by the presence of GI microbiota. Studies with gnotobiotical rodents (Bry *et al*., 1996) and zebrafish (*Danio rerio*) (Bates *et al*., 2006) have shown that the microbiota is necessary in the maturation of the gastrointestinal tract.

The main interface between the immune system and the external environment is the intestinal mucosa. The mucosal interface has a continuous dialogue between the host and the bacteria attached to it. These complex and integrated interactions between the epithelium, immune components in the mucosa and microbes are responsible for the development and maturation of the gut-associated immune system of the host (Guarner and Malagelada, 2003).

The mucosal barrier also functions as a protective barrier against colonization by exogenous microbes. This resistance does not only apply to exogenous pathogens, but also to opportunistic bacteria which are present in the gut but have restricted growth. The indigenous microbiota can prevent attachment and subsequent entry of pathogenic invasive bacteria into the epithelial cells (Bernet *et al*., 1994; Hooper *et al*., 1999). Figure 1.1 shows a draft of the mucosal barrier in the gastrointestinal system, with microvilli, mucus layer and epithelial cells.

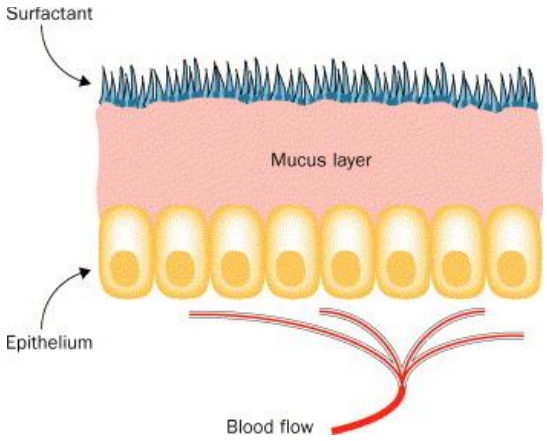


**Figure 1.1:** An overview of the mucosal barrier, which separates the internal environment from the luminal. The barrier is dependent on the integrity of the mucosa and the dynamic defensive factors (mucosal blood flow, epithelial secretions and immunocompetent cells). The mucus layer is formed by various mucosal secretions, and the resident bacteria of this layer are important for resistance against exogenous microbes (Guarner and Malagelada, 2003).

Recent studies in mammals have shown that the GI microbiota is a contributing factor in the pathophysiology of obesity (Turnbaugh *et al*., 2006). Turnbaugh *et al*. suggested that obese mammals have a microbiome with increased capacity to harvest energy from the diet. They also showed that this trait is transmissible. This was done by transferring an “obese microbiota” from obese individuals to bacteria-free normal weight individuals, which after the transfer had a significant increase in body fat compared with a control group.

In mammals obesity and increased energy harvest is associated with phylum-level changes in the microbiota, reduced bacterial diversity and altered representation of bacterial genes and metabolic pathways (Ley *et al*., 2006; Turnbaugh *et al*., 2006; Turnbaugh *et al*., 2009). These findings have not been investigated in fish with molecular methods, only with traditional techniques (Sun *et al*., 2009). Sun *et al*. found that the composition of the GI microbiota in fast and slow growing grouper (*Epinephelus coioides*) showed some differences. There were lower number and less species of pathogenic *Vibrio* in the gut of fast growing grouper, and *Bacillus* and *Psychrobacter* species with antagonistic effect against pathogenic *Vibrio* were only isolated from fast growing grouper.

## 1.2 Fish-microbe interactions

Fish live in a far more hostile microbial environment than organisms living on land. This is due to the higher microbial concentrations, which in marine and fresh water environment is known to be approximately around $10^7$ bacteria $mL^{-1}$ and for viruses one or two orders of magnitude higher. Only a small fraction of these organisms are known to be harmful to the fish, and the vast majority of host-microbe interactions are non-pathogenic (Vadstein *et al*., 2004; Kaiser *et al*., 2005; Brönmark and Hansson, 2005).

When comparing fish to mammals another difference is the total bacterial load in the gastrointestinal tract. This is lower in fish than in warm-blooded animals and have generally believed to be simpler (Nayak, 2010), but this has been questioned (Ringø et al., 2006; Bakke-McKellan et al., 2007). The digestive systems in fish and mammals share an extensive homology. Even though it has been shown distinctive differences between the bacterial composition in fish and mammals, it seems as though several host responses are shared. This has been shown in zebrafish, where conserved host responses include fortification of the innate immune defenses, enhancement of nutrient digestion, regulation of intestinal glycan expression and stimulation of epithelial cell renewal (Bates *et al*., 2006; Rawls *et al*., 2004; Rawls *et al*., 2006). This point to an evolutionary conserved role of the microbiota in vertebrate development, and that the roles of the GI microbiota on host biology are similar between zebrafish and mammals, despite the difference in composition (Kanther and Rawls, 2010).

The interactions between fish and microbes can be divided in three different categories: mutualism, commensalism and parasitism. These, and the relationship between them are schematically presented in Figure 1.2. Mutualism is a beneficial situation where two organisms live together for mutual benefit, while parasitism is a situation where one symbiont lives at the expense of (or harms) the host. Commensalism has one symbiont which benefits, while the other is neither helped nor harmed. Pathogenic bacteria can be roughly divided in obligate and opportunistic. Obligate pathogen is dependent on infecting and causing disease in the host, while opportunistic pathogen is a microorganism that does not ordinarily cause disease in an uncompromised host. The boundaries between the categories can be blurred, and should be regarded as more of a continuum rather than strict categories (Seifert and DiRita, 2006; Vadstein *et al*., 2004; Madigan and Martinko, 2006).

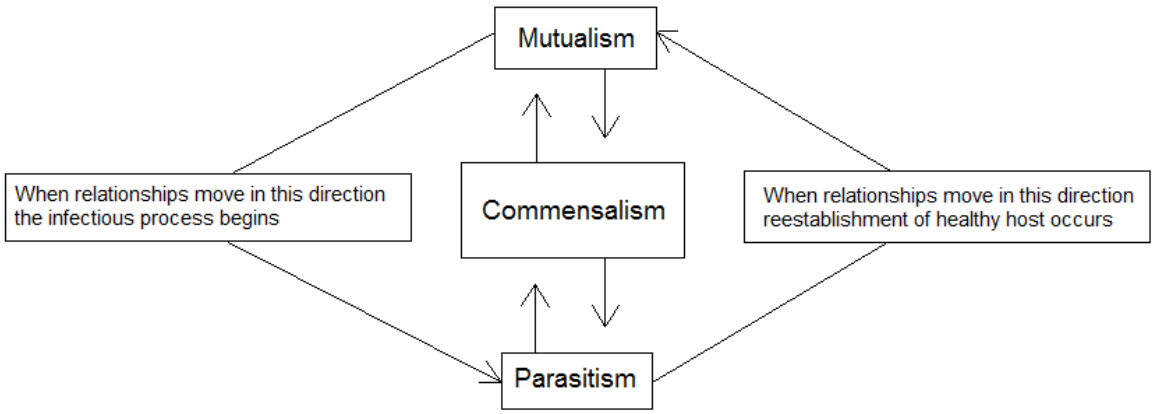


**Figure 1.2:** Symbiotic relationships in host-microbe interactions. These relationships are highly dynamic, and shifts may occur due to shifts at community level. Redrawn from Prescott *et al*. (1999).

The microbial colonization, composition and diversity in the GI tract of fish are established through a complex process and believed to be a reflection of the microbial composition of the rearing water, diet and their environment. The bacteria of the GI tract can be autochthonous (adherent) or allochthonous (transient), where the adherent group is bacteria which are able to attach to the intestinal mucosa and succeed in colonizing the mucosa on the epithelial surface. Transient bacteria lack the ability to attach to the intestinal mucosa or are outcompeted by other bacteria (Nayak, 2010).

The initial colonization process takes place during larval and juvenile stages, and is a complex process dependent on several factors, like fish type, nutrients and surrounding conditions. In an adult fish bacteria which enter the fish must pass the stomach. The stomach is an important barrier against pathogens, with its anti-bacterial substances and low pH. However, fish in the larval stage lack a well developed stomach and have a more accessible intestine. Before active feeding commences the larvae possesses a low bacterial load. It is believed that this is a generally non-fermentative microbiota, and that it changes after the onset of feeding. The total bacterial load increases with feeding and the microbiota changes and becomes dominated by fermentative bacteria (Bergh *et al*., 1994; Vadstein *et al*., 2004; Nayak, 2010).

Thus the fish is at its most vulnerable in the larval stage, and several factors play a role in development to get viable or non-viable larvae. The three significant factors are the microbial environment, physiochemical environment and larvae's innate properties. As shown in Figure 1.3, different conditions influence these factors (Vadstein *et al*., 2004).

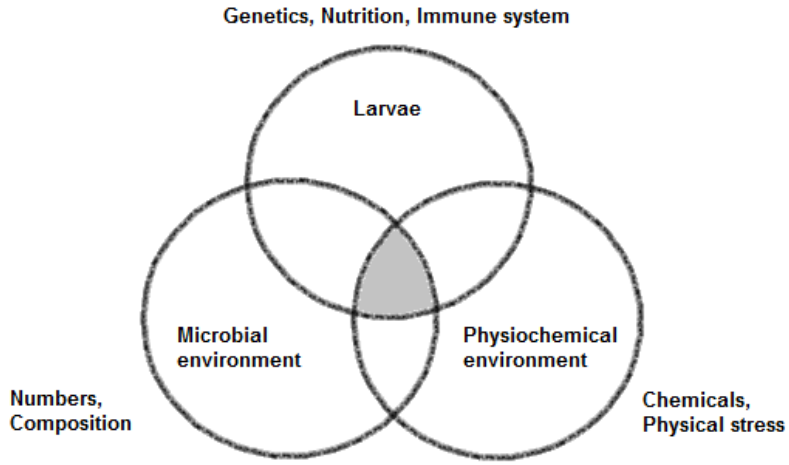


**Figure 1.3:** The larvae itself, the microbial and the physiochemical environment are the three factors of significance for the probability of viable larvae. The figure also shows different conditions which influence these factors. Redrawn from Vadstein *et al*. (2004).

The composition of the GI microbiota can be influenced by many exogenous and endogenous factors, *e.g*. developmental stage of the fish, gut structure, surrounding environment, rearing conditions, stress and chemicals (pollutants, antibiotics). But generally *Carnobacterium*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Pseudomonas* and *Vibrio* constitute the predominant intestinal microbiota of a variety of marine fish species (Perez *et al*., 2010). Fresh water fish tend to have a different composition, dominated by *Actinobacter*, *Aeromonas*, *Flavobacterium*, *Lactococcus* and *Pseudomonas* representatives of the family *Enterobacteriaceae* and obligate anaerobic bacteria of the genera *Bacteroides*, *Clostridium*, and *Fusobacterium* (Perez *et al*., 2010).

## 1.3 Host-microbe interactions in aquaculture

Aquaculture is the biggest protein producer in Norway, and the second biggest export article as of 2009 (SSB, 2009). The industry is in strong growth, but certain problems are impairing the development. One of these is the problem concerning reduced growth and survival in intensive juvenile production of marine species (Bengtson, 2003).

The production of juveniles is a major bottleneck in the aquaculture of most marine species. This production is struggling with poor reproducibility of survival, growth and quality. One of the factors which seem to be lacking is the microbial control. Strategies for microbial control, like the use of probiotics and closed recirculation systems with non-opportunistic K-selected bacteria have been proposed and show promising results (Vadstein *et al*., 2004; Salvesen *et al*., 1999; Gatesoupe, 1999). Better microbial control is a premise for a stable and

reproducible production of marine larvae in intensive systems, but still there is a need for a better understanding of host-microbe interactions in aquatic organisms.

## 1.4 Species studied and used

### 1.4.1 Mangrove killifish (*Kryptolebias marmoratus*)

The mangrove killifish (*Kryptolebias marmoratus*, formerly known as *Rivulus marmoratus*) is a small estuarine teleost fish, which has its natural habitat in mangrove areas in the tropical and subtropical parts of the western Atlantic and the Caribbean basin. It is widely distributed from Brazil to Florida. This species was first reared by R. W. Harrington, Jr., which held the species in cultivation for 15 years (1961-1975) and discovered the species special reproductive system. Harrington observed that the fish can reproduce by internal self-fertilization, and thus can produce clonal lineages with genetically similar individuals (Harrington, 1961). This property, together with the high sensitivity to toxic materials and ease of culture of this species, has led to its use as a model organism (Koenig and Chasar, 1984).

The mangrove killifish reach a total length of more than 45 mm in the wild, and can be considered a juvenile after it has reached 9.8 mm in standard length (Grageda *et al*., 2005). Egg ratio in healthy adults is approximately around 1 egg day$^{-1}$ (Grageda *et al*., 2005). Three phenotypes are observed in laboratory-reared individuals: Hermaphrodites, primary males and secondary males. The hermaphrodites have marbled brownish color pattern with caudal ocellus. Primary males produce only sperm and have orange body coloration usually without caudal ocellus. Secondary males are hermaphrodites which have lost the female function. Different ratios between the phenotypes have been reported, but in the laboratory strains DAN and PAN-RS hermaphrodites are the dominating phenotype (Grageda *et al*., 2005). These strains have recently been reported to be uncontaminated and unmixed (Tatarenkov *et al.*, 2010.) Figure 1.4 shows the fish's appearance in different parts of the development, where the bars equal 1 mm (Grageda *et al*., 2004).

The standard protocol for rearing of mangrove killifish was developed by Koenig and Chasar (1984), for carcinogenicity testing. One of the qualities which make the species suitable as a model organism is the size and development of the eggs. The eggs have a size of 1.5-2.0 mm in diameter with a transparent chorion, and have a hibernation period before hatching. It is practical to induce hatching by mechanical means, so called manual dechorionation. The species has been used in other areas of research, like toxicity screening, carcino- and tumorigenesis, macrophage induction and mutation repair (Turner, 1998; Koenig and Chasar, 1984).

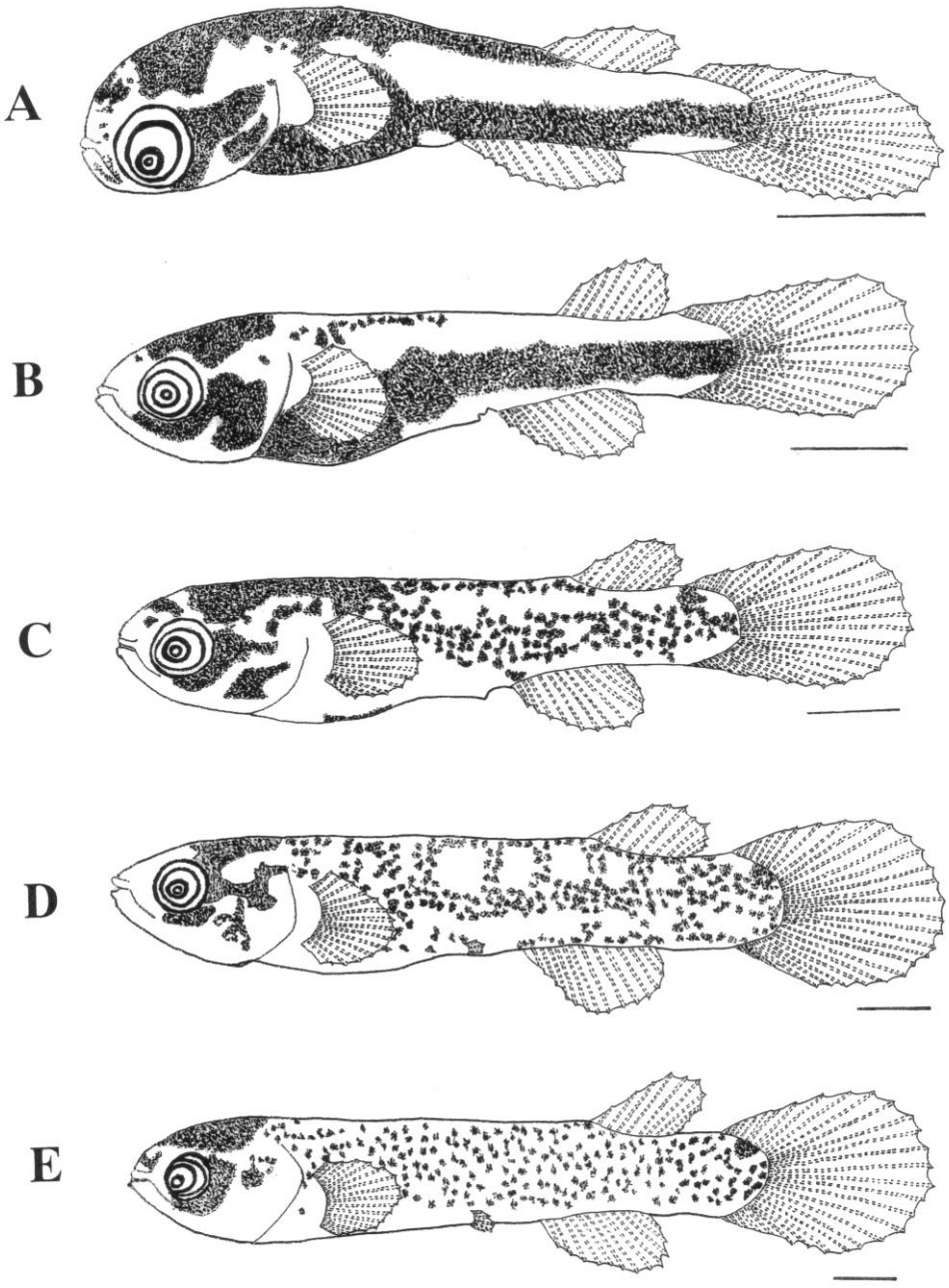


**Figure 1.4:** Morphological development of *Kryptolebias marmoratus*. **A**: a newly hatched larva 4.4 mm standard length (SL). **B**: 5 days after hatching (DAH) larva, 5.7 mm SL. **C**: 10 DAH larva, 6.7 med mer SL. **D**: 20 DAH juvenile, 9.8 mm SL. **E**: 30 DAH juvenile, 10.9 mm SL. Bars equal 1 mm (Grageda *et al*., 2004).

### 1.4.2 Atlantic cod (*Gadus morhua*)

Atlantic cod is a marine teleost fish in the Gadidae family. It is found in the North Atlantic, and thus all along the Norwegian coast. The fish is the most important commercial species for Norwegian fisheries, and is of great importance to the industry and commerce along the coast. The price of cod has led to the development of cod rearing as a part of the growing Norwegian aquaculture. It is expected that the industry will grow to become a large and important contributing factor in the future aquaculture, but the development has not been as rapid as expected. Several problems have been indentified, and one of these is the challenges in fry production described above (Muus, 1981; Nordli, 2009).

Cod, like many other marine fishes, lay small, pelagic eggs which are fertilized outside the female. These eggs are not well developed and the mother lays many eggs at a time. Many fresh water fish and some marine species lay bigger, demersal eggs at the bottom or attached to a surface (Muus, 1981). A clear example is mangrove killifish which, as mentioned above, lay fertilized, big eggs, which often is covered in a sticky adhesive for attachment to a surface or other eggs. These have a high survival rate, while cod, both in nature and in aquaculture, have a high mortality rate in the 2-3 weeks after hatching. In aquaculture this morality rate can be subscribed to microbial diseases in the rearing system, and that the specific immune system of cod is not developed until 2-3 months after hatching (Schrøder *et al*., 1998). Figure 1.5 shows a cod 35-40 days after hatching.


**Figure 1.5:** Cod larvae, 35-40 days after hatching (SINTEF, 2009).

### 1.4.3 *Artemia franciscana*

The brine shrimp *A. franciscana* is commonly used as feed for fish larvae. This is mostly due to the organisms practical cysts, which are very tough and easily hatched by placing them in a saline solution. *A. franciscana* is an aquatic crustacean, and a continuous, non–selective, filter feeding organism. In aquaculture it is become normal to enrich *A. franciscana* cultures with lipid emulsions before feeding to fish larvae to obtain the best possible nutritional composition (Dhont and Van Stappen, 2003). It has also been considered a possible vector

for other substances like antimicrobial agents (Mohney *et al*., 1990; Dixon *et al*., 1995), vaccines (Campbell *et al*., 1993) and probiotics (Makridis *et al*., 2000). Figure 1.6 shows an *A. franciscana* nauplius.


**Figure 1.6:** *A. franciscana* instar II nauplius, *i.e.* the second developmental stage (molt) after hatching (SINTEF, 2009).

## 1.5 Approaches for investigation of microbial biodiversity

Molecular methods have dramatically changed the possibilities to investigate the microbial composition of an ecosystem. Many different methods have been employed, and some will be discussed below.

The culture independent methods in molecular analyses of bacterial communities can be roughly divided in staining and polymerase chain reaction (PCR) based technologies. Staining methods are employed when it is necessary to quantify or observe microorganisms in natural samples. An example of this is fluorescent *in situ* hybridization (FISH), where specific molecular probes hybridize and stain nucleic acids of the target organisms. The PCR based technologies survey the biodiversity without observing the cells. The major techniques in this aspect are denaturing gradient gel electrophoresis (DGGE), molecular cloning and DNA sequencing (Madigan and Martinko, 2006). The new development of high-throughput sequencing, and so called “barcoded amplicon” 454 pyrosequencing has given the possibility to describe the microbial composition with PCR and direct sequencing of the different amplicons in the sample (Droge and Hill, 2008).

In the PCR based methods it is necessary to use a molecular marker to identify a particular sequence of DNA. When using molecular biological methods for studying microbial diversity its common to use sequence data of the 16S ribosomal RNA (16S rRNA) gene for identification of the bacterial species. It is the most commonly used molecular marker in

microbial ecology. 16S rRNA is a part of the small subunit in prokaryotic ribosomes, and it is highly conserved between different species of *Bacteria* and *Archea*. In addition the gene contains hypervariable regions which can provide sequences used for species specific identification of *Bacteria* or *Archea*. The gene has disadvantages, like the fact that it is observed to have intragenomic heterogeneity. Due to this other genes have been proposed, *e.g.* rpoB (Case *et al*., 2007). But through the last decades there has been a staggering growth in the amount of 16S rRNA gene sequences collected, and this information, which is stored in accessible databases, is easily available for comparison with obtained data (Weisburg *et al*., 1991).

DGGE is a culture independent molecular fingerprinting method. It was developed by Fisher and Lerman (1983), and introduced in molecular ecology by Muyzer *et al*. (1993). DGGE can thus be regarded as “old” as goes for molecular methods. Even though the method has some drawbacks, it effectively and with low costs produces a good overview of the microbial composition in a sample. Also it have the advantage that it is possible to analyze several samples at a time on the same gel, while other methods are more time consuming (molecular cloning) or more expensive (454 pyrosequencing). The method is based on separation of PCR products, which have the same size but different DNA sequences.

Electrophoresis is based on DNA’s property as a negatively charged molecule, which will migrate towards a positively charged electrode in an electric current. DGGE utilize this property, as well as DNA’s denaturing characteristics. In the presence of denaturant or at high temperatures DNA will denature, *i.e.* separation of the double helix. DGGE employs a linear gradient of a DNA denaturant (a mixture of urea and formamide) in a polyamide gel. The DNA fragment moves through the gel until it reaches a domain with sufficient denaturant and the fragment “melts” or denatures. To obtain a complete stop of the migration of the denatured DNA fragments it is important to have PCR products which only become partially melted during the denaturation. Therefore a so called GC-clamp is attached to one of the primers and becomes a part of the PCR fragments used in the DGGE. This GC clamp has higher bond strength than the rest of the DNA fragment, and keeps it from separating into two single-stranded strands. The various DNA fragments are separated by differences in the melting properties, which to a large degree is controlled by differences in base sequence. The bands which can be observed in a DGGE gel are different forms of a part of the 16s rRNA gene, which vary in their sequences (Madigan and Martinko, 2006; Muyzer and Smalla, 1998).

The different bands in the DGGE gel can be excised, reamplified by PCR and sequenced. The sequence information can be compared with known sequences to determine the species

in the bacterial community. An outline of the PCR-DGGE procedure for use in biodiversity analysis of microbial communities is shown in Figure 1.7.

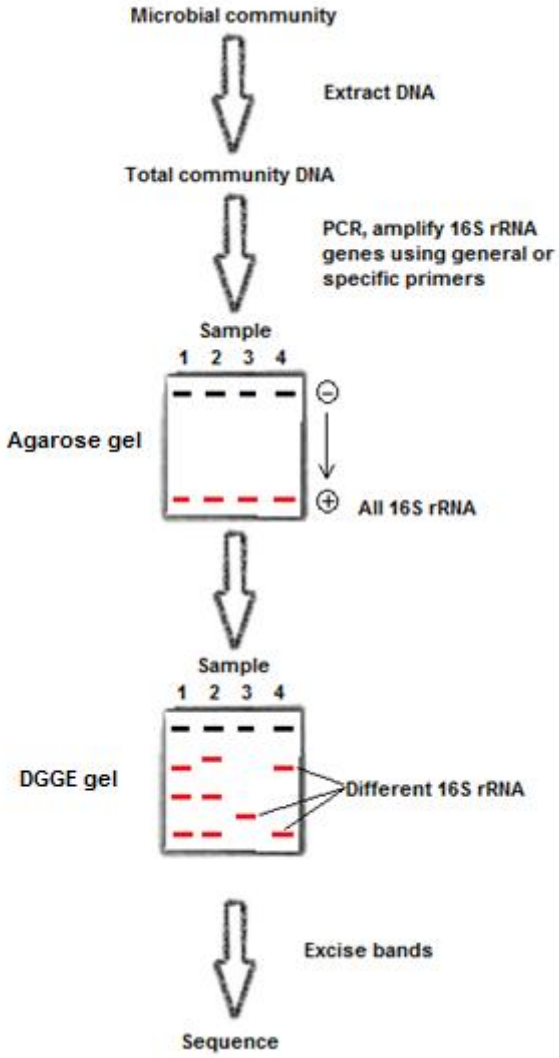


**Figure 1.7:** Steps in the PCR-DGGE procedure for biodiversity analysis of a microbial community. 16S rRNA fragments from a total community DNA are amplified by PCR, and the fragments are observed to have the same length by agarose gel electrophoresis. The different fragments are then separated by DGGE and bands from the DGGE gel can be excised and sequences determined. Redrawn from Madigan and Martinko (2006).

## 1.6 Hypothesis, goal and objectives

The hypothesis of this thesis was defined to be:

> *"The growth rate in larval fish is partly explained by the composition of the gastrointestinal microbiota."*

The main goal of this thesis was to study the GI microbiota of two species of fish at the larval/juvenile stage with regards to the individual growth of the fish. The hypothesis was thus tested by comparing the GI microbiota of slow and fast growing Atlantic cod and mangrove killifish. The objectives of this thesis were divided in three:

1. Investigate the GI microbiota in fast and slow growing fish larvae/juveniles in two strains of mangrove killifish and Atlantic cod using the molecular fingerprinting method DGGE and sequencing.

2. Culture the GI microbiota from fast and slow growing fish juveniles of one strain of mangrove killifish and investigate the culturable GI microbiota with the molecular fingerprinting method DGGE and sequencing.

3. Investigate the consequences of administration of the cultured bacteria to mangrove killifish larvae through the biological vector *A. franciscana*.

# 2. Materials and methods

## 2.1 Experimental design

To test the hypothesis two studies were done, a descriptive study and an Experimental test. Two species of fish were studied: Cod and mangrove killifish. Cod was just used in the descriptive study, while two different clonal lineages of mangrove killifish where used in the descriptive study and one clonal lineage in the Experimental test.

In the descriptive study the aim was to check for differences in the composition of GI microbiota between the largest and smallest fish bred under the same conditions. The aim of the Experimental test was to investigate how fish treated with bacteria from the gut flora of either the larger fish or the smaller fish would respond with respect to composition of the GI microbiota and growth of fish, and if there would be differences between the two groups. Figure 2.1 shows a flow chart depicting the two types of experiments and the relationship between them. Figure 2.1 apply for the work done with the mangrove killifish. The descriptive study is principally the same for the cod. An important exception between the two is that the cod larvae were reared together with other cod larvae in big tanks, whilst the mangrove killifish larvae were held individually. Thus the cod larvae were exposed to competition and interactions with respect to the GI microbiota due to defecation, while the mangrove killifish were not. Another important exception was that the mangrove killifish reached the juvenile stage, while the cod where not fully metamorphosed at the time of sampling. Furthermore there was a difference in the salinity; the cod were reared in sea water, while the mangrove killifish were reared in brackish water (17 ppt).

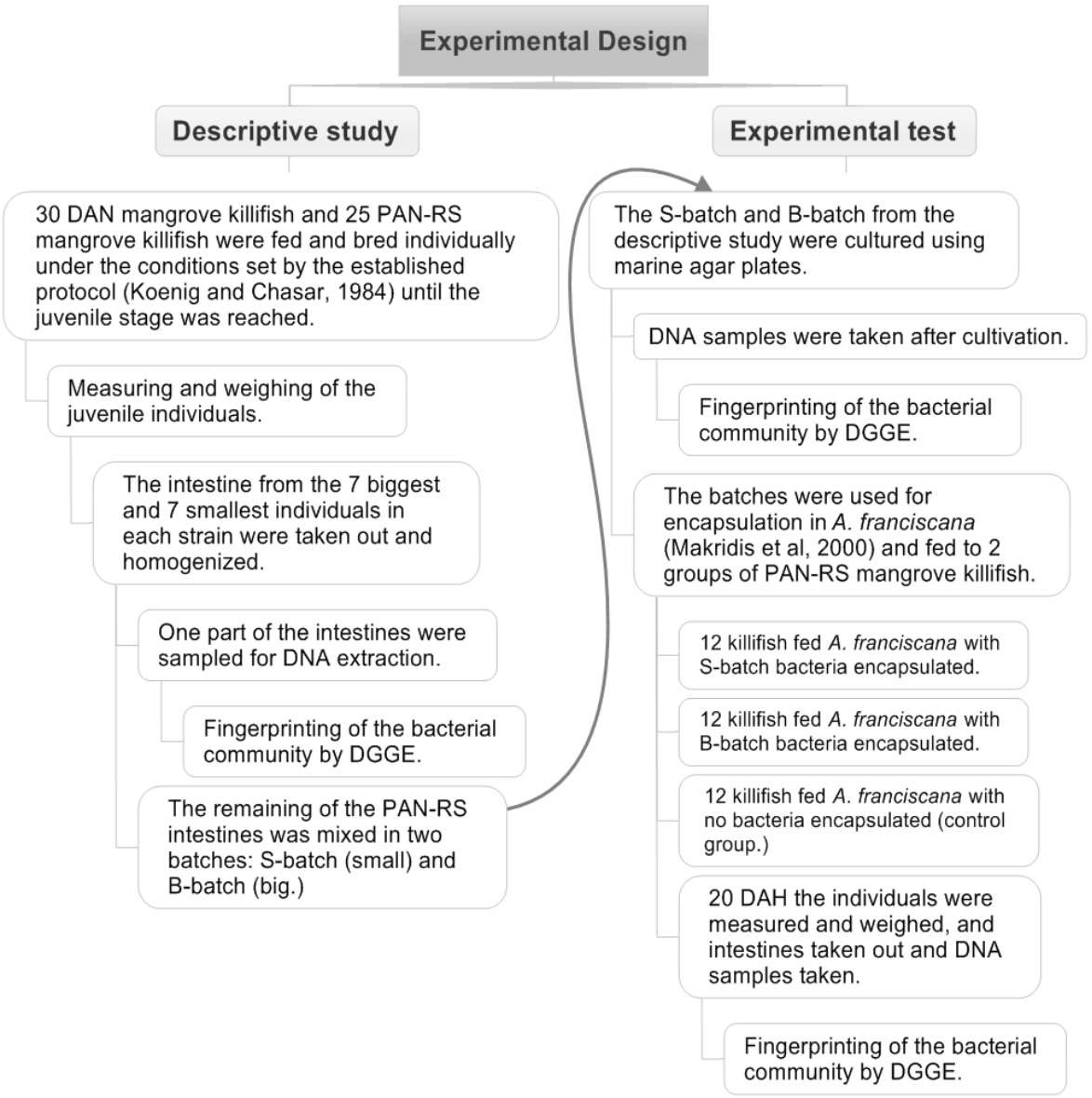


**Figure 2.1**: The experimental design in the two experiments and the relationship between them. Details consider the killifish experiment, but the cod experiment was in principle the same as in the descriptive study.
DGGE: denaturing gradient gel electrophoresis, DAH: days after hatching.

## 2.2 Biological materials and rearing conditions

### 2.2.1 Cod larvae experiment

The cod larvae were sampled at SINTEF Fisheries and Aquaculture in Trondheim at 43 days after hatching (DAH). The experimental description is written down to a minimum, because only the sampling was done by the candidate and the work was done by SINTEF Fisheries and Aquaculture. The feeding schedule is shown in Table 2.1.

**Table 2.1**: Feeding schedule for the sampled cod larvae as a function of days after hatching (DAH).

| DAH | Feed | Producer of feed |
|---|---|---|
| 1-20 | Green water (*Nannochloropsis* sp. paste) | Reed Mariculture |
| 2-22 | Rotifers (*Brachionus plicatilits* 'Nevada') with a cultivation diet of rotifer diet and *Pavlova* paste | SINTEF Fisheries and Aquaculture (Rotifers), Reed Mariculture (*Pavlova* paste) |
| 18-43 | *A. franciscana* enriched with MaroIE | SINTEF Fisheries and Aquaculture |

The supplied sea water was first sand filtered, then filtered down to 1 µm and matured in reservoir before it was supplied to the tanks. The temperature was 7 $^0$C the first 5 DAH, and gradually increased up to 11.5 $^0$C until 12 DAH. The temperature remained unaltered the rest of the period. 16 small and 14 large larvae were picked from identically treated rearing tanks. These were put to death by use of tricaine methane sulphonate (MS 222). The total length and weight was determined. The specimens were stored at -80 ºC for later microbial analyses.

### 2.2.2 Mangrove killifish experiments

Two clonal strains of mangrove killifish was used in the experiments: DAN and PAN-RS. They were obtained from W. P. Davis of the US Environmental Protection Agency, Gulf Breeze, Florida, USA. The PAN-RS strain is descendants of a single hermaphrodite, originally collected at Bocas del Toro in the Republic of Panama in 1994. The DAN strain is descendants of a fish collected at South Pelican Beach, Dangriga, Belize. The strains have been reared for over 10 generations in the Aquaculture Biology laboratory, Faculty of Fisheries at Nagasaki University, Japan (Grageda *et al*., 2005).

All the specimens used in the experiments were manually dechorionated following the protocol described by Koenig and Chasar (1984). The chorion was removed by the use of fine forceps, and the larva released.

The larvae and juveniles used in the descriptive study were reared individually in plastic cups (100 mL), containing 60 mL 17 ppt artificial seawater (Marine Art High**,** Senju, Seiyaku Co., Ltd., Osaka). See Figure 2.2. Ozonized tap water was used for the production of the seawater. The fish were held at 25 ± 1 $^{0}$C with a photoperiod of 14 hours light and 10 hours darkness. The fish in the Experimental test were kept the same way, except that autoclaved artificial seawater (AASW) was used.


**Figure 2.2**: Mangrove killifish held in 100 mL plastic cups. *A. franciscana* can be seen in the rearing water.

For the descriptive study the water was exchanged every 10$^{th}$ day, and at the same time the fish was measured and feces samples were taken. The fish were fed newly hatched *Artemia franciscana* nauplii every 2-3 days and they were held under non limited food conditions (*ad libitum*). When all specimens had reached juvenile stage (at day 40 for DAN and day 35 for PAN-RS) the fish were measured and put to death by an overdose MS 222. The intestines were sampled for later microbial analyses. The intestines were taken out by the use of fine forceps, syringe needle and fine needle (insect pin) and sampled in micro tubes. For the Experimental test the water was changed once, at day 7. The fish were fed newly hatched *A. franciscana* nauplii, with bacteria encapsulated, at day 0, 4, 7, 12 and 16. Bacteria were also added to the rearing water at a concentration of $10^{6}$ bacteria cells/mL. At 20 DAH the fish were put to death by an overdose MS 222. Lengths and weights were measured, and intestines sampled for later microbial analyses as described for the descriptive study.

### 2.2.3 Preparation and inoculation of intestinal bacteria

Autoclaved seawater (200 µL) was added to the intestines, and they were homogenized by the use of sterile plastic rod and syringe. One half of the intestines were stored at -80 $^{0}$C, and used for microbial analyses.

Half of the intestine samples from the 5 biggest and 5 smallest individuals in the descriptive study were used for inoculation of bacteria for use in the Experimental test. Before freezing 100 µL of the samples were taken out and mixed in two separate batches: Big (B) and small (S.) Autoclaved seawater (500 µL) was added and each batch was inoculated on 5 MA plates (Marine Agar 2216, Difco). In the Experimental test 5 bacterial treatments of the fish were done, and 5 plates of each batch were thus inoculated so one from each batch could be used in one treatment. The plates were incubated at 25 $^0$C, and the incubation time were 2 days for the B batch and 1 day for the S batch. The different incubation periods were due to the different growth rate of the bacteria. The plates were kept at 4 $^0$C until they were used for the treatment of the fish in the Experimental test. For the treatments in the Experimental test one plate from each of the two batches was used. Autoclaved seawater (2 mL) were added to the plates, the bacteria scraped off and sampled in micro tubes, before they were used in the encapsulation of *A. franciscana* and added directly to the fish water.

### 2.2.4 Preparation of *Artemia franciscana* as feed

One- to two-day-old *A. franciscana* were used for the feeding, and hypochlorite-mediated decapsulation of the cysts (Great Salt Lake, Aquafauna Biomarine Inc., CA, USA) were done before they were put up for hatching. Samples of *A. franciscana* in the descriptive study were taken for microbiological analyses. These samples were taken at 13, 17, 21 and 27 DAH for the DAN strain and at 1, 5, 11, 20 and 27 DAH for the PAN-RS strain.

In the Experimental test it was important to keep *A. franciscana* as bacteria free as possible before encapsulation of bacteria. Therefore care was taken when *A. franciscana* was decapsulated, hatched and bacteria encapsulated. For each treatment 1 g dry *A. franciscana* cysts were decapsulated. The cysts were hydrated in tap water (100 mL) for 1 hour under low stirring. Sodium hypochlorite (NaClO, 7 %, 5.2 mL) and sodium hydroxide (NaOH, 4 %, 2.4 mL) was added, and the reaction was stopped by adding sodium thiosulphate pentahydrate ($Na_2S_2O_3 \bullet 5H_2O$, 5 %, 8 mL) when the cysts turned orange after 8 -15 minutes. The decapsulated cysts were divided in three, and put up for hatching in autoclaved bottles containing AASW (200 mL). *A. franciscana* cultures were aerated with sterile filtered air (Millex, 0.2 µm, 50 mm Vent Filter Unit), see Figure 2.3.

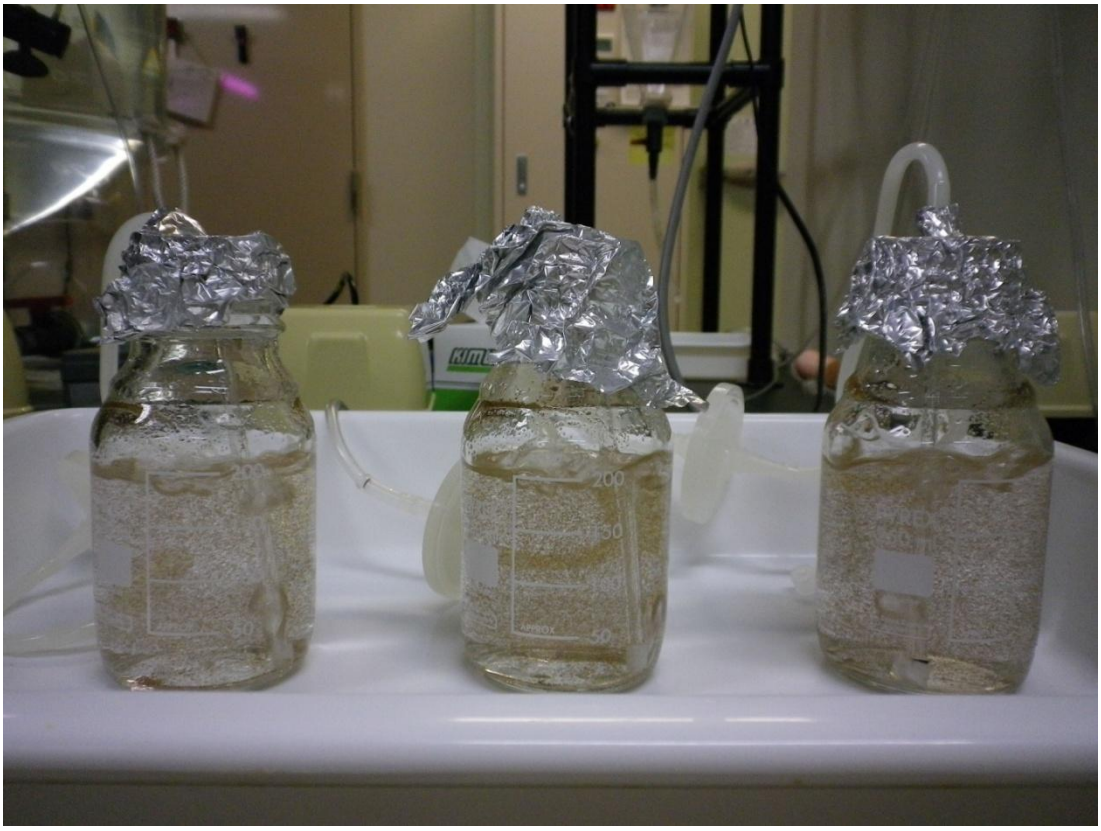

**Figure 2.3**: Set up for sterile hatching of *A. franciscana*. The three bottles contain identical amounts of newly decapsulated cysts, which were later encapsulated with bacteria.

*A. franciscana* hatched after one day, and on the 2nd day the bacteria were encapsulated. The encapsulation process was done as follows. A sterile mesh (150 µm) was used to gather *A. franciscana* and they were rinsed with autoclaved MilliQ water. The bacteria were added to 100 mL AASW and the *A. franciscana* was transferred to the solution. *A. franciscana* grazed on the bacteria for 1 hour, was rinsed with autoclaved MilliQ water and fed to the fish. Bacteria were added to the fish cups at a concentration of $10^6$ bacteria cells/mL, and to the *A.franciscana* encapsulation solution at a concentration of $10^8$ bacteria cells/mL (Makridis *et al.*, 2000). The treatments were done at 0, 4, 7, 12 and 16 DAH, and samples of the *A. franciscana* were taken from all treatments for microbial analyses.

## 2.3 Analytical methods

### 2.3.1 Measurements of length and weight of the fish

The total lengths of the cod larvae were measured by the use of a microscope. For determination of the wet weight the cod were rinsed with fresh water, dried of with KimWipes® (Kimberley-Clark Corp., Neena, USA) and weighed in distilled water.

The total lengths of the mangrove killifish were measured by the use of digital microscope (CVH 6300, Keyence Corp., Osaka, Japan). The total length of the mangrove killifish were

transferred to standard length (SL) by the use of the equation y = -0.21 + 0.84x for the DAN strain, and y=-0.19+0.84x for the PAN-RS strain, were y equals the standard length and x the total length (Grageda *et al*., 2005). The fish was dried of with kim wipes, and the wet weight was determined.

### 2.3.2 Measurement of bacterial concentration

The bacterial concentrations in the Experimental test were determined by optical density (OD) measurements with spectrophotometer (GeneSpec 3, Naka Instruments Co. Ltd.) at 600 nm. The OD measurements were transferred to bacterial concentration using a rule of thumb that each 0.1 OD unit is roughly equivalent to $10^8$ cells/mL (Lech K. and Brent R., 1988). See Appendix 1 for OD measurements and calculations.

### 2.3.3 DNA extraction

For the DNA extraction from the samples the QIAGEN DNeasy blood and tissue kit was used, with a modified protocol for marine bacteria (Hess-Erga *et al*., 2010). See Appendix 2 for protocol. The DNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and the DNA concentrations were used for calculation of the amount of template added in each PCR reaction (see below).

### 2.3.4 PCR-DGGE

The DNA extracts with DNA concentration above 20 ng/µL were diluted to get this concentration. The thermal cycler VWR™ UnoCycler was used for the PCR reactions.

From the DNA extracts a region of the 16S rRNA gene was amplified. A nested PCR protocol with two rounds of amplification was used, to avoid co-amplification of eukaryote DNA (Bakke *et al*., 2011). Two different pairs of primers were used in the two rounds, an external (EUB8F and 984YR) and internal primer pair (338F GC and 518R). The external primer pair used is prokaryote specific. See Appendix 3 for primer sequences.

The PCR reactions were run with 2 mM $MgCl_2$, 0.2 mM of each dNTP (Qiagen), 0.3 µM of each primer, Taq DNA polymerase (VWR) and reaction buffer (Qiagen). Different number of PCR cycles was necessary for different types of samples. For the external PCR 20 cycles were used and 20-30 were cycles used in the internal (95 $^0$C 30s, annealing 30 s, and 72 $^0$C 60s). An annealing temperature of 50 $^0$C was used in the external PCR and 53 $^0$C in the internal PCR. Template (1-2 µL) was added in the external PCR, and PCR product (1 µL) were transferred to the internal PCR. See Appendix 3 for an overview of PCR regimes used for the different samples.

Agarose gel was used to check quality of PCR products and contaminations. Agarose gel (1 %) with GelRed™ was casted, and 1x TAE (Appendix 4) was added. PCR product (5 µL) and

loading buffer (1 µL) was loaded to the wells. The gel was run at 140 V for approximately 1 hour, before it was photographed in UV light (G:BOX, Syngene).

For the DGGE the INGENY phorU system (Ingeny, Netherlands) was used. The gels were made with acrylamide (8 %) solution and had a 35-50 % or a 35-55 % denaturing gradient. The gels were run for 17 hours at 60 $^{0}$C.

The glass plates were cleaned and polished with 96 % ethanol. The plates were assembled with the spacer in the gel cassette. The DGGE solutions were made by mixing of 0 % and 80 % denaturing acrylamide standard solution. A mixture of formamide and urea served as denaturing agent, see Appendix 4. The required volume from each of the two solutions was pipetted to two falcon tubes (total volume 24 mL) and mixed. The 80 % solution was sterile filtrated. Tetramethylethylenediamine (TEMED, 16 µL) was added to the tubes. Ammonium persulphate (APS, 10 %, 87 µL) was added immediately before casting of the gel. A gradient maker with two chambers connected to a pump and a syringe needle, was used to cast the gradient in the INGENY phorU system. The equipment was rinsed with MilliQ water before use. The solutions were added to the chambers, and the gradient produced. When the chambers were empty the solution for the stacking gel was added to the chamber. This was made of 0 % denaturing acryl amide standard solution (8 mL), TEMED (10 µL) and APS (40 µL). The gel was allowed to polymerize for a minimum of 2 hours.

TAE (0.5x) was added to the buffer tank and heated to 60 °C. The polymerized gel was carefully placed in the buffer tank taking care to avoid bubbles below the gel. All the wells were washed with buffer to avoid particles. Samples (2.5-15 µL) and loading dye (2-4 µL) was mixed and loaded to the wells. The gel was run at 100 V for 10 minutes without circulation of buffer, and for 16 hours and 50 minutes with circulation. The gel was stained with SYBR gold solution (Invitrogen) for 1 hour and photographed in UV light (G:BOX, Syngene). See Appendix 4 for recipe for SYBR gold staining solution.

### 2.3.5 Preparation of samples for sequencing, and analysis of sequences

Dominating bands were stamped out by the use of a micropipette, added to microtubes with filtrated MilliQ water (20 µL) and allowed to elute for 24 hours at 4 ºC. The dissected bands were reamplified, purified and sent to Eurofins MWG Operon for sequencing. Reamplification was done by PCR with a specific sequencing primer, 338F M13 rev (-29). See Appendix 3 for sequence. QIAquick PCR Purification Kit (Qiagen) was used for purification of the sample.

The sequences were analyzed using Classifier tool and SeqMatch search at the Ribosomal Database Project (RDP) (Wang *et al*., 2007; Cole *et al*., 2006; Cole *et al*., 2009).

### 2.3.6 Statistical analysis

The DGGE gels were analyzed by the program GEL2k (Norland, 2004). The program produces densitometric curves, which is used to identify bands and make quantifications based on the peak areas in the curves. The peak areas for each band were normalized by dividing on the total peak area for all bands in the lane, giving fractional peak areas.

The normalized fractional peak areas were used to calculate band richness (k), Shannon diversity index (H') and Pielou's evenness index (J'). H' and J' are both diversity indices. J' quantifies how equal the community is numerically. H' takes both the number of species, *i.e.* number of bands, and the evenness of the species into account, and indicates the diversity of the sample (Peet, 1975).

H' was calculated according to Equation (2.1), where p is the normalized fractional peak area and S the species richness (in this case represented by band richness, k). H' will be at its largest when the bands have equal peak areas, and this is the instance $H'_{max}$, which is defined in Equation (2.2) (Peet, 1975).

$$H' = \sum_{i=1}^{s} p_i \ln p_i \qquad (2.1)$$

$$H'_{\max} = \ln S \qquad (2.2)$$

J' was calculated according to equation (2.3).

$$J' = \frac{H'}{H'_{\max}} = \frac{H'}{\ln S} \qquad (2.3)$$

Non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity was used for visualization of the similarities/dissimilarities between the different bacterial compositions of the samples. The analysis is based on the fractional peak areas obtained from the DGGE gel. NMDS is an exploratory ordination method, and produces a coordinate system where the different objects are placed. The similarity/dissimilarity between the samples is reflected by the distances between them in the coordinate system, where points laying close to each other reflect a similarity and vice versa. The method is, as mentioned, based on ordination, and produces a stress value, which tells how well the order from the ordination has been reproduced in the plot. Generally stress values over 0.3 indicate that the configuration is no better than arbitrary, and the results should ideally not be interpreted unless stress values are < 0.2 (Quinn *et al*., 2002; Clark, 1999; Bray and Curtis, 1957).

For hypothesis testing analysis of similarity (ANOSIM) with Bray-Curtis dissimilarity were used. This is a method which tests whether there is a significant difference between two or more groups. It is based on ordination in the same way as NMDS, but is hypothesis driven not exploratory. The procedure produces two end values, an R value and a p value. The R-value lies between -1 and 1, where values >0 implies that objects are more dissimilar between groups than within groups. An R value of 0 therefore implies that there is no difference between the groups. The p value should <0.05 to give the test significance. The normalized fractional peak areas were square root transformed, and 10 000 permutations used. (Clark, 1993; Warwick and Clark, 1995; Bray and Curtis, 1957).

Student's *t*-test (unpaired two-sample) and analysis of variance (ANOVA) were used for investigation of significance. *t*-test is used if nothing else is mentioned, and normal distribution is assumed.

All of the multivariate analysis were performed with the PAST software package (Hammer, 2005), and *t*-tests and ANOVA were performed using the Analysis ToolPak in Microsoft Excel.

# 3. Results

## 3.1 Descriptive study: Comparison of microbiota associated with large and small cod larvae

### 3.1.1 Length and weight

In total 30 cod larvae were sampled, and from these the 23 best shaped individuals were chosen. The 11 smallest individuals are referred to as S1 to S11, and the 12 largest are referred to as B1 to B12. These 23 larvae spanned a total range in length and weight of 1.1 – 1.7 cm and 0.003 - 0.053 g (Figure 3.1). The total length and weight of the analyzed cod larvae are shown in Table 3.1. The average length (Table 3.1) of the small larvae (S) is significantly different ($p < 0.05$) from the large ones (B) and the same were the case for the weight ($p < 0.05$).

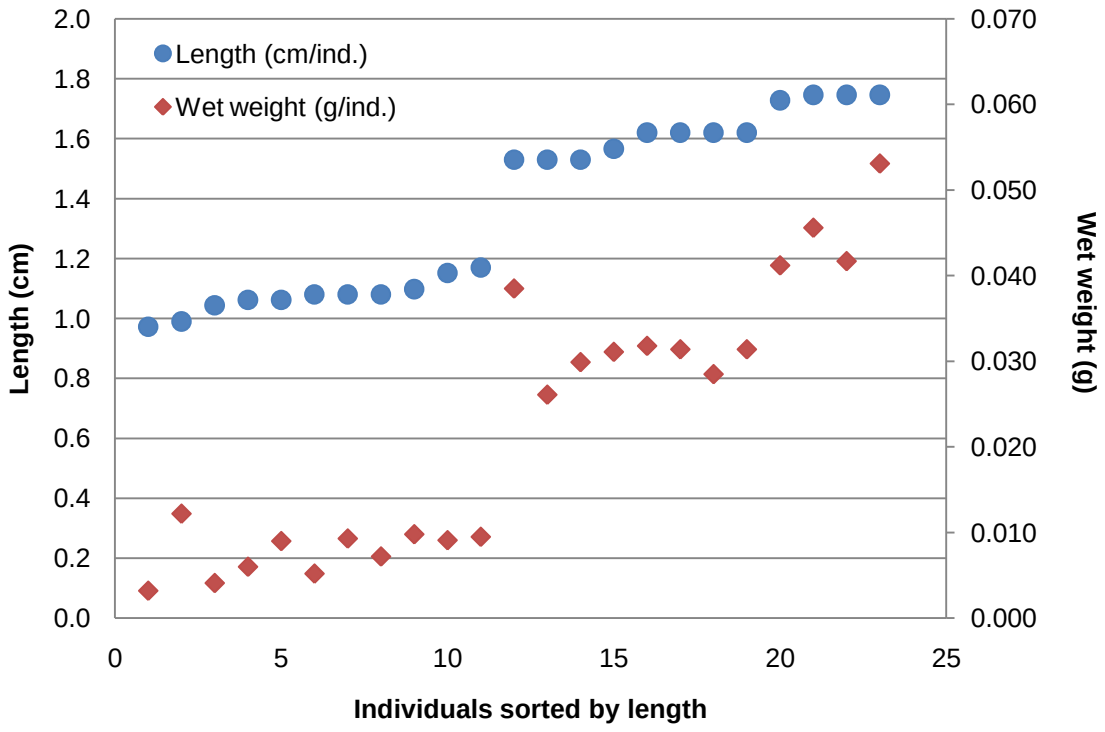


**Figure 3.1**: The length and wet weight of the 23 analyzed cod larvae sorted by length.

**Table 3.1**: Length and weight of cod individuals, with average and standard deviation.

| Small larvae, ID number | Length (cm) | Wet weight (g) | Large larvae, ID number | Length (cm) | Wet weight (g) |
|---|---|---|---|---|---|
| **S1** | 1.0 | 0.003 | **B1** | 1.5 | 0.039 |
| **S2** | 1.0 | 0.012 | **B2** | 1.5 | 0.026 |
| **S3** | 1.0 | 0.004 | **B3** | 1.5 | 0.030 |
| **S4** | 1.1 | 0.006 | **B4** | 1.6 | 0.031 |
| **S5** | 1.1 | 0.009 | **B5** | 1.6 | 0.032 |
| **S6** | 1.1 | 0.005 | **B6** | 1.6 | 0.031 |
| **S7** | 1.1 | 0.009 | **B7** | 1.6 | 0.029 |
| **S8** | 1.1 | 0.007 | **B8** | 1.6 | 0.031 |
| **S9** | 1.1 | 0.010 | **B9** | 1.7 | 0.041 |
| **S10** | 1.2 | 0.009 | **B10** | 1.7 | 0.046 |
| **S11** | 1.2 | 0.010 | **B11** | 1.7 | 0.042 |
| | | | **B12** | 1.7 | 0.053 |
| **Average** | **1.1** | **0.008** | | **1.6** | **0.036** |
| **Std. dev.** | **0.1** | **0.003** | | **0.1** | **0.008** |

### 3.1.2 DGGE analysis of cod larvae GI microbiota

DNA was extracted from the gut of the cod larvae, and PCR with universal primers was used to amplify a ~180 base pair (bp) fragment of 16S rRNA gene (encompassing with variable region 3) from all the different bacterial species present in the samples. The fragments were determined to be of expected length by agarose gel electrophoresis (results not included). The PCR products were analyzed on a DGGE gel (Figure 3.2). There are a total of 47 bands in the gel, of which only one is present in all of the samples. Some of the bands were sequenced, and these are marked in the gel.

A section of the DGGE gel (enclosed in frame in Figure 3.2) was analyzed by the use of the software program GEL2k (Norland, 2002). The selected section had higher clarity and a lower degree of smearing; this applies to all of the chosen sections in the rest of the DGGE gels. The peak areas for all bands were determined and normalized by dividing on the total peak area for all bands in the lane, giving fractional peak areas. The fractional peak areas were used for calculation of band richness (number of bands in each lane, k), Shannon diversity index (H') and Pielou's evenness index (J') (Table 3.2). Further, the average and standard deviation for each group was calculated (Table 3.2). Band richness varied between 12 and 23, and the average band richness values are similar between the two groups. The J' values and the H' values are significantly different between the two groups ($p < 0.05$), where the small individuals show a greater diversity and evenness than the larger individuals.

The exploratory NMDS analysis (Figure 3.3) produces a coordinate system where the distance between samples reflects the similarity between them. The produced coordinate system clearly separates the two groups, with an overlapping zone between them. The stress value of the analysis is 0.229, and thus >0.2, and gives an uncertain result.

The hypothesis testing with ANOSIM (Table 3.3) produces a significant R value of 0.335. This means there is a significant difference in the GI microbiota composition between the large and small individuals.

The sequenced bands are, as mentioned above, marked and numbered in the DGGE gel. Band 4 is the most dominating in all of the large individuals and in most of the 11 small individuals. This band was by sequencing determined to most likely represent a bacterium from the class *ε-proteobacteria* and within the order *Campylobacterales* (Table 3.4). Some of the closest matches from GenBank were uncultured bacteria sampled from petroleum contaminated ground water (AB030593), a river estuary (DQ234254) and anaerobic bioreactor treating brewery waste (EF515495). Three of the bands were only observed in the small larvae (Band 1, 2 and 5). Bands 2 and 5 were confirmed to have the same sequence, as expected. All were determined to most likely be from the class *Vibrionales* and within the genus *Aliivibrio*. The closest matches in GenBank were all bacteria isolated from different fish species. The DNA sequencing data obtained from the DGGE gel are summarized in Table 3.4.

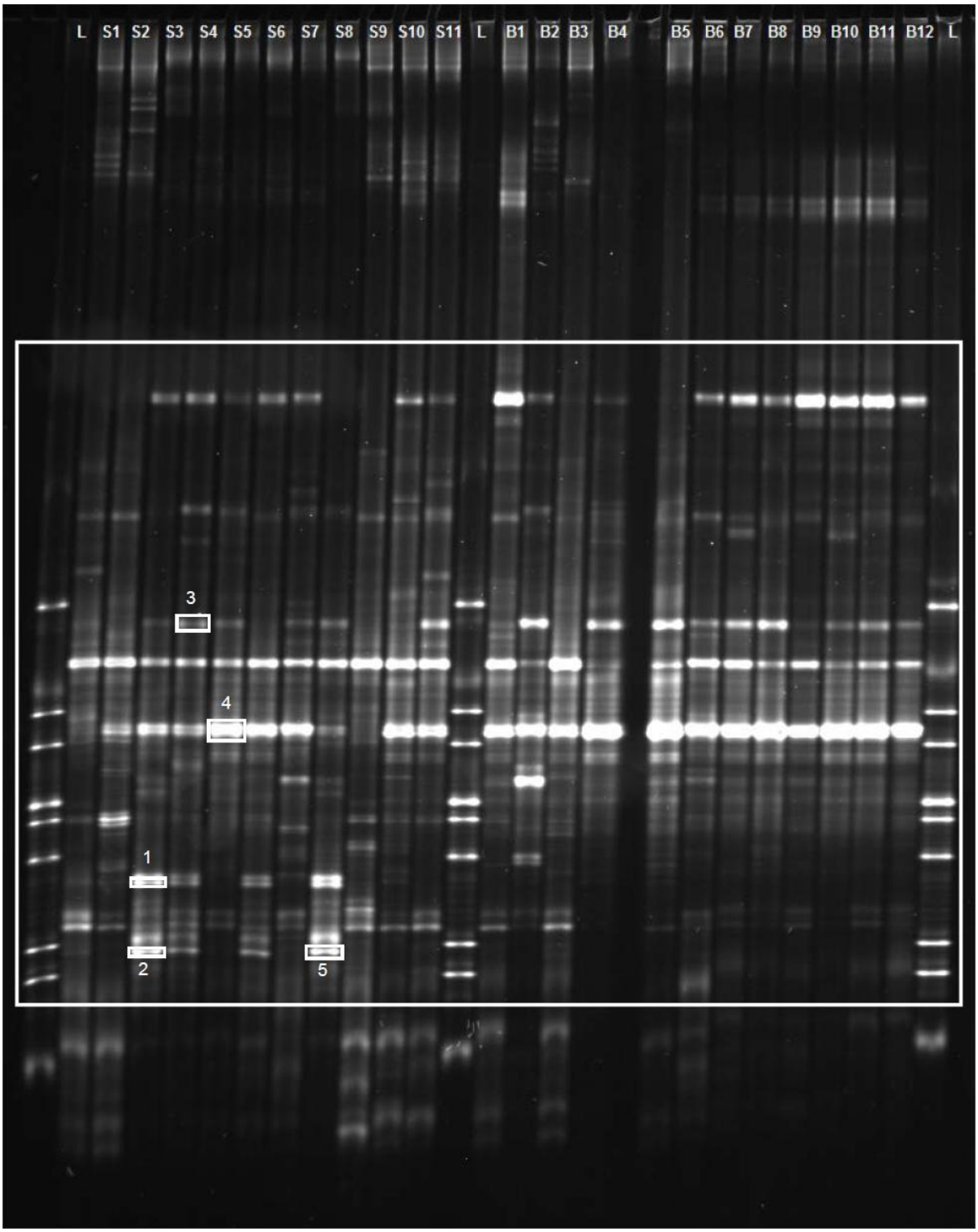


**Figure 3.2**: The DGGE gel (8 % acrylamide, 35-55 % denaturing gradient) obtained from the descriptive study with cod. The analyzed area is enclosed in frame. The lanes are marked with sample names, where S refers to small larvae, B to large larvae and L to ladder/standard. The sequenced bands are marked and numbered.

**Table 3.2**: The diversity indices found from the DGGE from the descriptive study with cod. Band richness (k), Shannon index (H') and evenness (J') were calculated. S1 to S11 are samples obtained from small cod larvae, and B1 to B12 are samples from large cod larvae, and the average and standard deviation of the two groups are calculated.

| Sample | Band richness (k) | Shannon index (H') | Evenness (J') |
|---|---|---|---|
| **S1** | 16 | 2.18 | 0.79 |
| **S2** | 16 | 2.19 | 0.79 |
| **S3** | 17 | 2.37 | 0.84 |
| **S4** | 17 | 2.48 | 0.88 |
| **S5** | 12 | 1.82 | 0.73 |
| **S6** | 17 | 2.19 | 0.77 |
| **S7** | 18 | 2.17 | 0.75 |
| **S8** | 16 | 2.24 | 0.81 |
| **S9** | 12 | 1.90 | 0.76 |
| **S10** | 20 | 2.16 | 0.72 |
| **S11** | 20 | 2.28 | 0.76 |
| **Average** | **16** | **2.18** | **0.78** |
| **Std. dev.** | **2.6** | **0.19** | **0.05** |
| **B1** | 23 | 2.23 | 0.71 |
| **B2** | 15 | 1.92 | 0.71 |
| **B3** | 19 | 1.85 | 0.63 |
| **B4** | 13 | 1.58 | 0.62 |
| **B5** | 18 | 2.07 | 0.71 |
| **B6** | 15 | 1.87 | 0.69 |
| **B7** | 15 | 1.89 | 0.70 |
| **B8** | 14 | 1.80 | 0.68 |
| **B9** | 14 | 1.61 | 0.61 |
| **B10** | 15 | 1.74 | 0.64 |
| **B11** | 17 | 1.86 | 0.66 |
| **B12** | 14 | 1.60 | 0.61 |
| **Average** | **16** | **1.84** | **0.66** |
| **Std. dev.** | **2.8** | **0.19** | **0.04** |

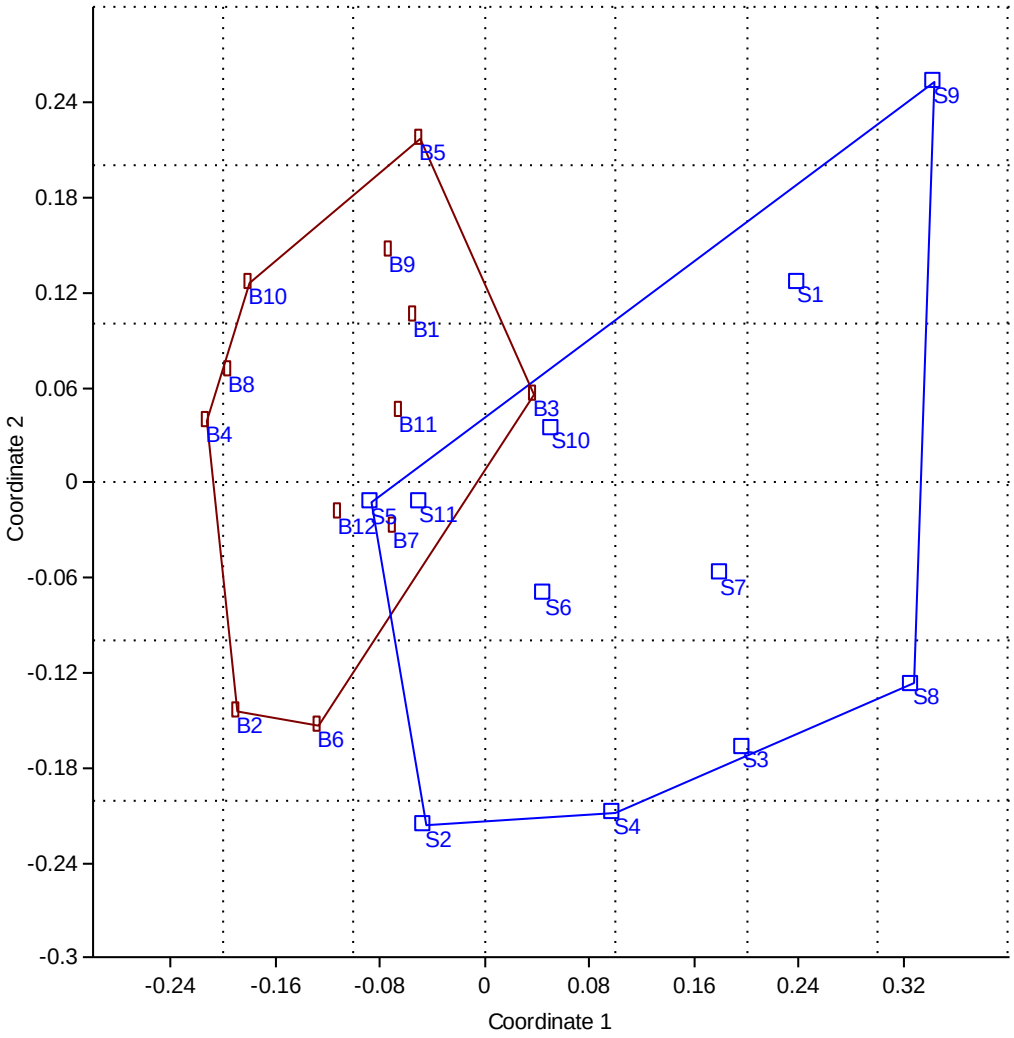


**Figure 3.3**: NMDS of DGGE results from descriptive study with cod larvae with Bray-Curtis dissimilarity measure. The points are marked (S1 to S11 from small cod larvae, and B1 to B12 from large cod larvae) and in addition there are drawn lines between the outer points in the two groups. The analysis has a stress value of 0.229.

**Table 3.3**: ANOSIM of the GI microbiota between the two different groups (S – small larvae and B – large larvae) of cod larvae.

| | **Groups** | **ANOSIM R value** | **p value** |
|---|---|---|---|
| **Between groups** | S and B | 0.335 | < 0.0001 |

**Table 3.4**: DNA sequencing results for selected DGGE bands (Figure 3.2). "% in lane" refers to percent of the total content in the DGGE lane, and "Observed in" refers to in which samples the band can be observed.

| DGGE bands | Sample origin | % in lane | Observed in | Classification[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **Class** | **Order** | **Family/Genus** | **Examples of possible close relatives** | **Similarity (%)** | **GenBank accesion number** |
| **1** | S3 | 10.7 | 7 S | *γ-proteobacteria* | *Vibrionales* | *Aliivibrio* | *Aliivibrio logei*; 15382 | 100 | AY292932 |
| | | | | | | | *Aliivibrio fischeri*; SI1E | 96.1 | AY292949 |
| | | | | | | | *Aliivibrio salmonicida*; PB3-7rrnA | 100 | EU091321 |
| **2, 5** | S3, S8 | 16.5, 10.8 | 6 S | *γ-proteobacteria* | *Vibrionales* | *Aliivibrio* | *Aliivibrio wodanis* | 100 | AY628647 |
| | | | | | | | *Aliivibrio logei*; SR181 | 100 | AY292934 |
| | | | | | | | *Aliivibrio fischeri*; SI1E | 100 | AY292949 |
| | | | | | | | *Aliivibrio salmonicida*; PB1-8rrnA | 100 | EU091323 |
| **3** | S4 | 8.4 | 11 B / 8 S | *Flavobacteria* | *Flavo-bacteriales* | *Polaribacter* | *Polaribacter sp.* J2- | 91.3 | HM010401 |
| | | | | | | | *Flavobacteriaceae bacterium* G1B2 11 | 88.6 | AY285943 |
| **4** | S5 | 42.3 | 12 B / 9 S | *ε-proteobacteria* | *Campylo-bacterales* | *Arcobacter* | uncultured *epsilon proteobacterium* 1053 | 100 | AB030593 |
| | | | | | | | uncultured *Arcobacter* sp.; DS172 | 100 | DQ234254 |
| | | | | | | | uncultured bacterium; 29b10 | 100 | EF515495 |

[a] Classification data was obtained by the use of Classifier and SeqMatch at RDP (See 2. Materials and methods, Section 2.3.5).

## 3.2 Descriptive study: Comparison of GI microbiota associated with large and small mangrove killifish juveniles

### 3.2.1 Weight and length of the mangrove killifish juveniles

The growth of the mangrove killifish in the descriptive study was described by regular measurements of length throughout the rearing period. The average length of DAN and PAN-RS strains increased linearly with time, but the growth of DAN was a bit slower than for PAN-RS and demonstrated a two phase pattern with slower growth after three weeks (Figure 3.4). The average length at day 40 of the all the 30 DAN juveniles was 11.0 ± 0.5 mm SL. The average length of all the 25 PAN-RS juveniles at day 35 was 11.2 ± 0.5 mm SL. Figure 3.5 shows the difference in average length between the 7 smallest and the 7 largest a) DAN and b) PAN-RS individuals.

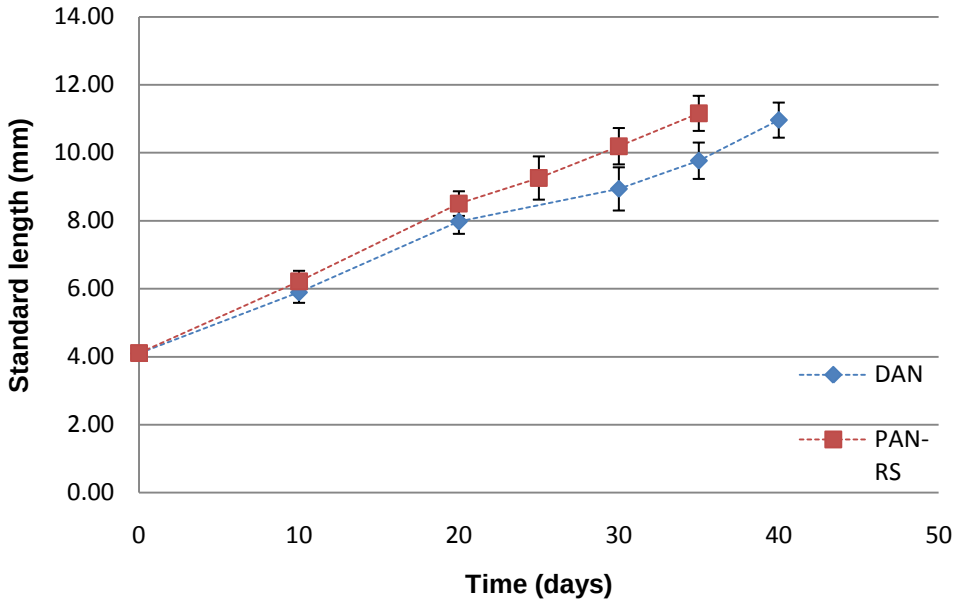


**Figure 3.4**: The growth, described by length, of the two different strains of fish, DAN and PAN-RS, with standard deviation.

As shown in Figure 3.4, mangrove killifish of the strain PAN-RS has a higher growth rate, and reaches juvenile stage approximately 5 days before the DAN strain. Due to this higher growth rate the PAN-RS strain was used in the later Experimental test. For the seven smallest and the seven biggest juveniles significant differences in length was observed from day 30 for DAN and 25 for PAN-RS post hatch onwards (Figure 3.5a and 3.5b).

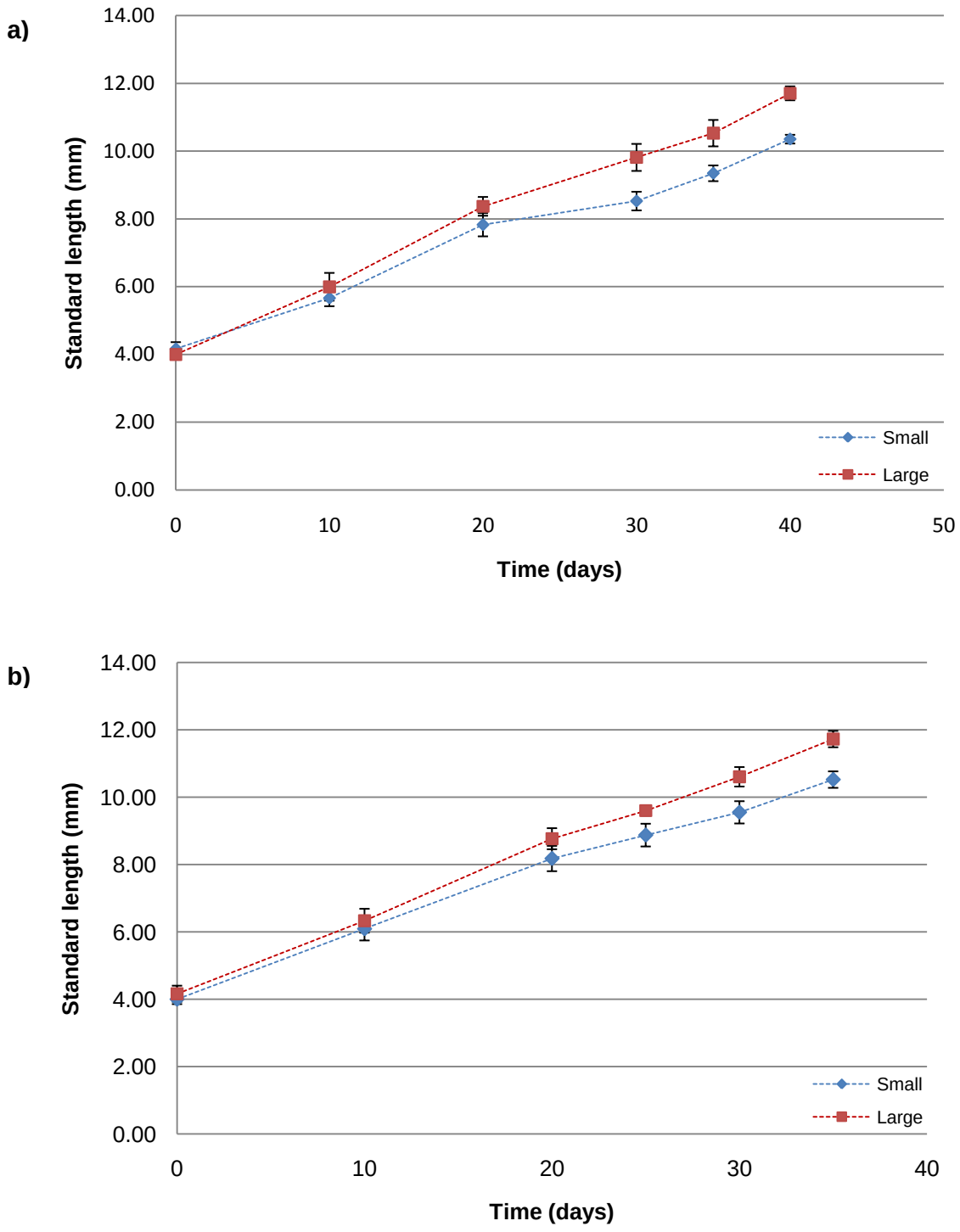


**Figure 3.5**: The length of the 7 smallest and 7 biggest juveniles of the strain a) DAN and B) PAN-RS in the descriptive study, with standard deviation.

The mangrove killifish juveniles were kept until all of the individuals reached the juvenile stage, which was 40 DAH for DAN and 35 DAH for PAN-RS. The length and wet weight of all individuals were determined (Figure 3.6). For the 7 largest and 7 smallest individuals of DAN and PAN-RS strains the intestines were sampled for further analysis of the GI microbiota.

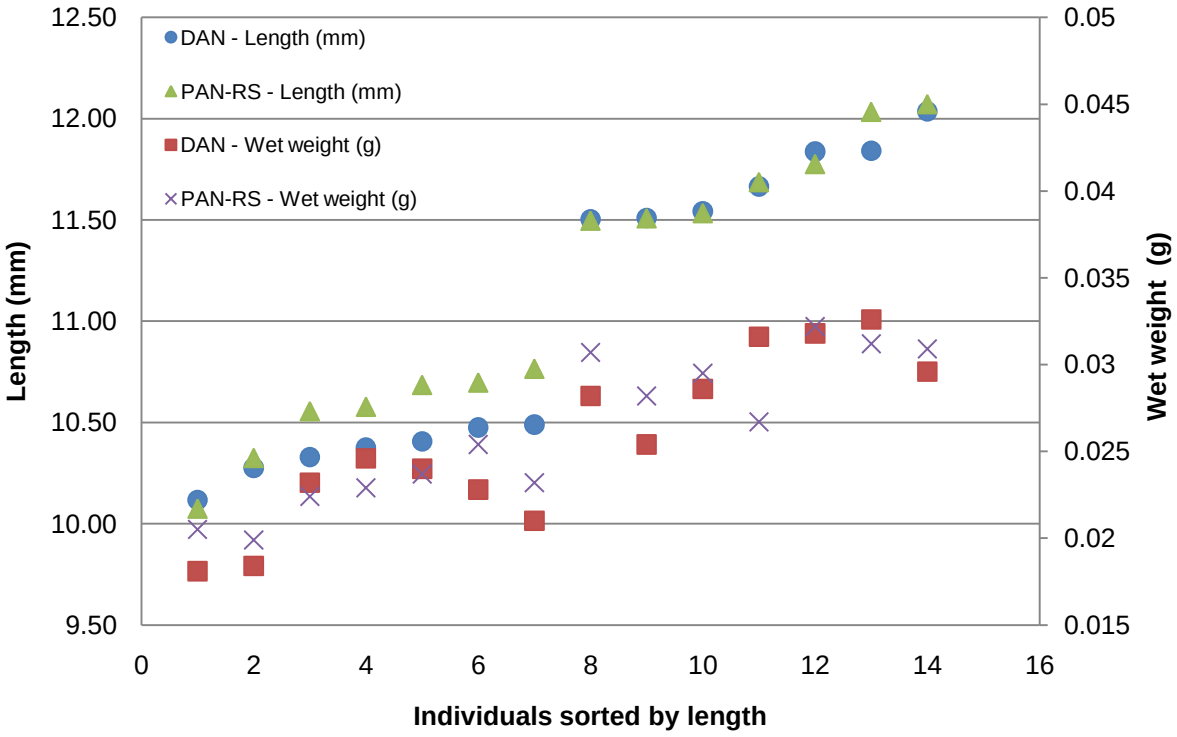


**Figure 3.6**: The length (mm) and wet weight (g) of the 7 largest and 7 smallest individuals of both DAN and PAN-RS at the day of sampling of the GI microbiota (day 40 for DAN and day 35 for PAN-RS).

### 3.2.2 DGGE analysis of DAN mangrove killifish juveniles' GI microbiota

DNA was extracted from the gut of the DAN mangrove killifish juveniles, feed and a water sample. PCR with universal primers was used to amplify a ~180 base pair (bp) fragment of 16S rRNA gene (encompassing with variable region 3) from all the different bacterial species in the samples. The fragments were determined to be of expected length by agarose gel electrophoresis (results not included). The PCR products were analyzed on a DGGE gel (Figure 3.7). There are four types of samples on the gel, S refers to the smallest and B to the largest juveniles, A refers to feed samples from 13, 14, 21 and 27 DAH and W refers to a water sample from the water used for the rearing and *A. franciscana* hatching.

The gel has a total of 60 bands, and these are in general evenly distributed and without any clearly dominating bands (Figure 3.7). From Figure 3.7 it can clearly be observed that S- and

B-samples are partially similar, except from B6, B7 and to some extent B2. The water and feed samples have larger visible variation.

The average band richness values vary between 26 and 35, where the large individuals show the largest variation with a standard deviation of 4.8, and the small individuals the lowest with a standard deviation of 1.3 (Table 3.5). The Shannon index and evenness varied between respectively 2.53 - 3.39 and 0.80 - 0.95, and there is no significant difference between the small and large individuals with regards to both Shannon index and evenness ($p > 0.05$).

The NMDS analysis has a stress value <0.02, and the analysis is therefore reliable and can be interpreted with less precaution (Figure 3.8). The NMDS show a clear clustering of the feed samples, while the GI microbiota of the small and large juveniles is clearly overlapping. Three samples from the large juveniles stand out, and is not a part of the overlapping area (B2, B7 and B8). The water sample does not have a clear grouping.

The ANOSIM confirms the NDMS, with a clear difference between the feed and the juveniles (Table 3.6). The comparison of the feed and small juveniles (R=0.995) and the feed and large juveniles (R=0.897), show a strong significant difference. The comparison between the large and small juveniles reflects the overlapping seen in the NMDS, and produces an R of 0.208 which indicates a weak difference between the two groups.

7 bands from the gel were sequenced (Table 3.7), and among these Band 5 and 6 are of special interest. This is because these are only present at high intensity in the two largest juveniles in the test (B6 and B7), and is therefore one of the factors contributing to these samples standing out in the NMDS. The bands were both found to most likely represent bacteria from the class *γ-proteobacteria*, and within the genera *Listonella* or *Vibrio*. The DNA sequencing data obtained from the DGGE gel are summarized in Table 3.7.

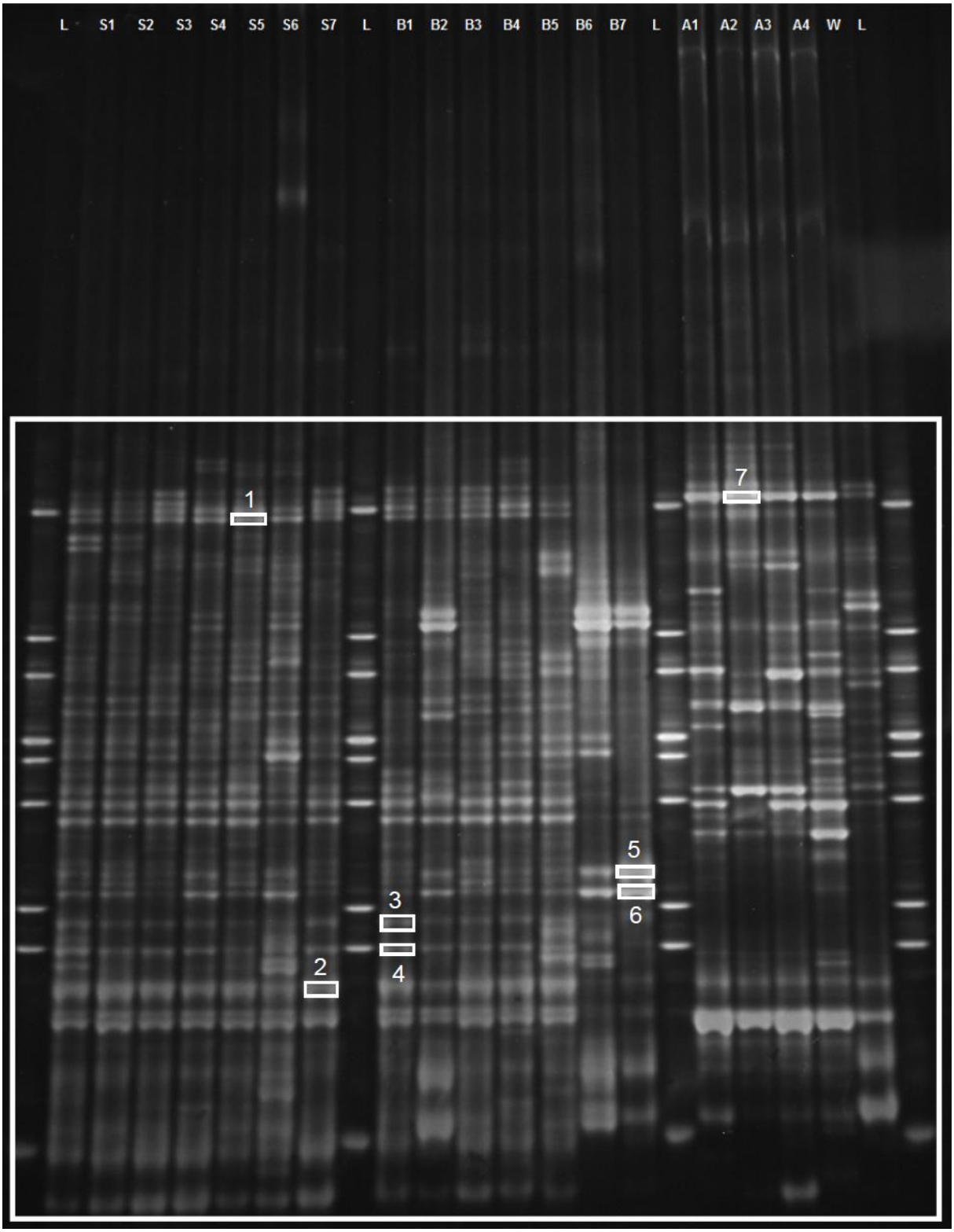


**Figure 3.7**: DGGE gel (8 % acrylamide, 35-55 % denaturing gradient) obtained from the descriptive study with DAN mangrove killifish. The analyzed area is indicated, and lanes are marked with sample names, where S refers to the smallest and B to the largest juveniles. A refers to feed samples from 13, 14, 21 and 27 DAH. W refers to water sample. The sequenced bands are marked and numbered.

**Table 3.5**: The diversity indices found from the DGGE from the descriptive study with DAN mangrove killifish. Band richness (k), Shannon index (H') and evenness (J') were calculated, together with the average and standard deviation of the three groups. S refers to the smallest and B to the largest juveniles. A refers to feed samples from 13, 14, 21 and 27 DAH and W to rearing water sample.

| Samples | Band richness (k) | Shannon index (H') | Evenness (J') |
|---|---|---|---|
| **S1** | 36 | 3.29 | 0.92 |
| **S2** | 36 | 3.39 | 0.95 |
| **S3** | 33 | 3.13 | 0.89 |
| **S4** | 35 | 3.30 | 0.93 |
| **S5** | 34 | 3.14 | 0.89 |
| **S6** | 35 | 3.27 | 0.92 |
| **S7** | 33 | 3.12 | 0.89 |
| **Average** | **35** | **3.23** | **0.91** |
| **Std. dev.** | **1.3** | **0.11** | **0.02** |
| **B1** | 29 | 2.92 | 0.87 |
| **B2** | 27 | 2.94 | 0.89 |
| **B3** | 33 | 3.25 | 0.93 |
| **B4** | 35 | 3.25 | 0.91 |
| **B5** | 35 | 3.25 | 0.91 |
| **B6** | 24 | 2.71 | 0.85 |
| **B7** | 24 | 2.53 | 0.80 |
| **Average** | **30** | **2.98** | **0.88** |
| **Std. dev.** | **4.8** | **0.29** | **0.05** |
| **A1** | 27 | 2.87 | 0.87 |
| **A2** | 22 | 2.66 | 0.86 |
| **A3** | 24 | 2.67 | 0.84 |
| **A4** | 30 | 3.01 | 0.88 |
| **Average** | **26** | **2.80** | **0.86** |
| **Std. dev.** | **3.5** | **0.17** | **0.02** |
| **W** | 22 | 2.79 | 0.90 |

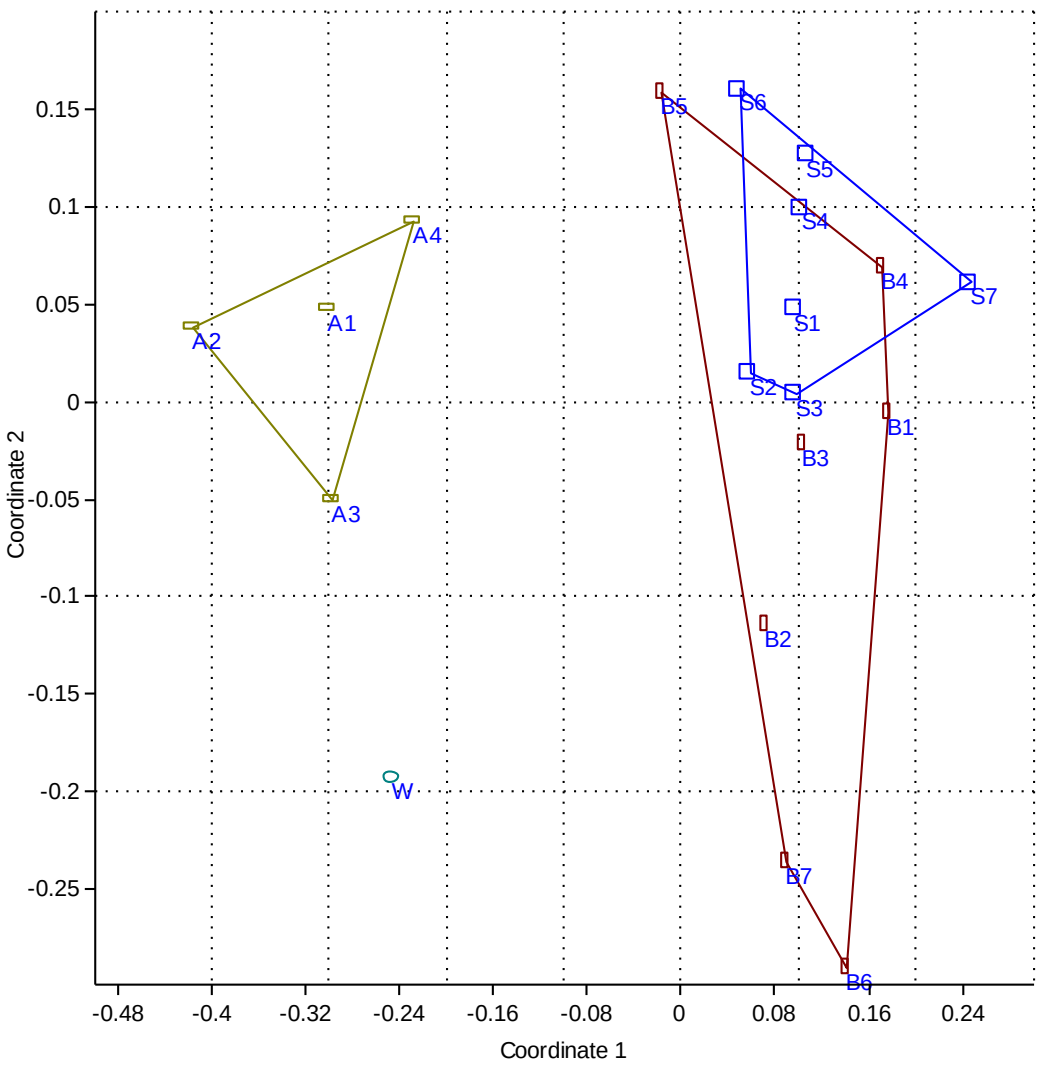


**Figure 3.8**: Non-metric multidimensional scaling of DGGE results from descriptive study with DAN mangrove killifish juveniles with Bray-Curtis similarity measure. The points are marked and in addition there are drawn lines between the outer points in the three groups. The figure is marked with sample names, where S refers to the smallest and B to the largest juveniles. A refers to feed samples from 13, 14, 21 and 27 DAH. W refers to water sample. The analysis has a stress value of 0.195.

**Table 3.6**: ANOSIM of the similarity of the GI microbiota between the small (S) and large (B) DAN juveniles, and of the bacterial composition of the feed (A).

| | **Groups** | **ANOSIM R value** | **P value** |
|---|---|---|---|
| **Total** | - | 0.598 | <0.0001 |
| **Between groups** | B and S | 0.208 | 0.031 |
| | A and B | 0.897 | 0.003 |
| | A and S | 0.995 | 0.004 |

**Table 3.7**: DNA sequencing results for selected DGGE bands (Figure 3.7). "% in lane" refers to percent of the total content in the DGGE lane, and "Observed in" refers to in which samples the band can be observed.

| DGGE bands | Sample origin | % in lane | Observed in | Classification[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **Class** | **Order** | **Family/Genus** | **Examples of possible close relatives** | **Similarity (%)** | **GenBank accesion number** |
| **1** | S5 | 10.9 | 7S/7B/ 4A/1W | *Bacilli* | *Bacillales* | *Staphylococcus* | uncultured bacterium; nbu450h11c1 | 98.7 | GQ047539 |
| | | | | | | | uncultured bacterium; ncd292f05c1 | 98.7 | HM263913 |
| | | | | | | | uncultured bacterium; ncd226b12c1 | 98.7 | HM267959 |
| **2** | S7 | 5.6 | 6S/6B/ 4A/0W | *Actinobacteria* | *Actinomycetales* | *Microbacteriaceae* | *Agrococcus sp.* QSSC2-2 | 100 | AF170739 |
| | | | | | | | uncultured *Cryobacterium sp*.; PIC-D10 | 100 | DQ418530 |
| | | | | | | | *Klugiella xanthotipulae* (T); 44C3; C3 coryneform | 100 | AY372075 |
| | | | | | | | *Microbacterium aurum* (T); DSM 8600 | 100 | Y17229 |
| **3** | B1 | 7.0 | 6S/6B/ 0A/0W | *γ-proteobacteria* | *Alteromonadales Enterobacteriales Oceanospirillales Vibrionales Xanthomonadales* | | | | |
| **4** | B1 | 6.2 | 7S/5B/ 0A/0W | *Actinobacteria* | *Actinomycetales* | *Microbacteriaceae* | *Agromyces ramosus* (T); DSM 43045 | 100 | X77447 |
| | | | | | | | *Clavibacter michiganensis*; P 250/01 | 100 | AJ310416 |
| | | | | | | | *Leifsonia aquatica* (T); JCM 1368 | 100 | D45057 |
| **5** | B7 | 9.7 | 6S/7B/ 1A/0W | *γ-proteobacteria* | *Vibrionales* | *Listonella/Vibrio* | *Vibrio ordalii*; ADL-2063-ACU-03 | 100 | AY628631 |
| **6** | B7 | 19.1 | 7S/7B/ 0A/0W | *γ-proteobacteria* | *Vibrionales* | *Listonella/Vibrio* | *Vibrio aestuarianus*; 01/064 | 98.7 | AJ845011 |
| | | | | | | | *Listonella anguillarum*; M3 | 98.7 | AY035897 |
| **7** | A2 | 5.1 | 2S/3B/ 4A/1W | *Bacilli* | *Lactobacillales* | *Enterococcus* | *Enterococcus pseudoavium*; NCFB 2138T | 94.2 | Y18356 |
| | | | | | | | *Enterococcus casseliflavus*; LMG 13518 | 94.2 | AJ301832 |

[a] Classification data was obtained by the use of Classifier and SeqMatch at RDP (See Section 2.3.5).

### 3.2.3 DGGE analysis of PAN-RS mangrove killifish juveniles' GI microbiota

DNA was extracted from the gut of the PAN-RS mangrove killifish juveniles, feed and water samples. PCR with universal primers was used to amplify a ~180 bp fragment of the 16S rRNA gene, (encompassing with variable region 3) from all the bacterial species in the samples. The fragments were determined to be of expected length by agarose gel electrophoresis (results not included). The PCR products were analyzed on a DGGE gel (Figure 3.9). There are four types of samples, where S refer to the smallest and B to the largest juveniles, A refer to feed samples taken at 1, 5, 11, 20 and 27 DAH and w to water samples taken from the rearing cups of 6 randomly picked individuals at 35 DAH.

A total of 61 bands were observed in the DGGE gel (Figure 3.9). Some of the samples (S1, S2, S3, S7 and B3) are smeared and have a strong band not observed in the other samples. The band seems to correspond with the eukaryotic band observed by Bakke *et al*. (2011) and the samples were therefore excluded from further analysis. This lead to only three analyzed S-samples, which lowers the strength of the test and further statistical analysis.

The band richness varied between 25 and 32 (Table 3.9) and analysis of variance (ANOVA) showed a significant difference between all the groups ($p < 0.05$), but there was no significant difference between S and B. The Shannon index and evenness varied between respectively 1.54 - 2.21 and 0.43 – 0.67, and ANOVA showed no significant difference between all the groups ($p > 0.05$).

The NMDS had a low stress value of 0.099 (Figure 3.10), and show a clear clustering. The water and feed samples cluster on different sides of the larval samples. The larval samples also cluster together, with two of the three analyzed S samples standing out (S4 and S5).

The ANOSIM show a significant difference between all of the groups, except between B and S which have a $p = 0.058$ (Table 3.9). Still this p value is close to 0.05, so this was interpreted to be marginally significant.

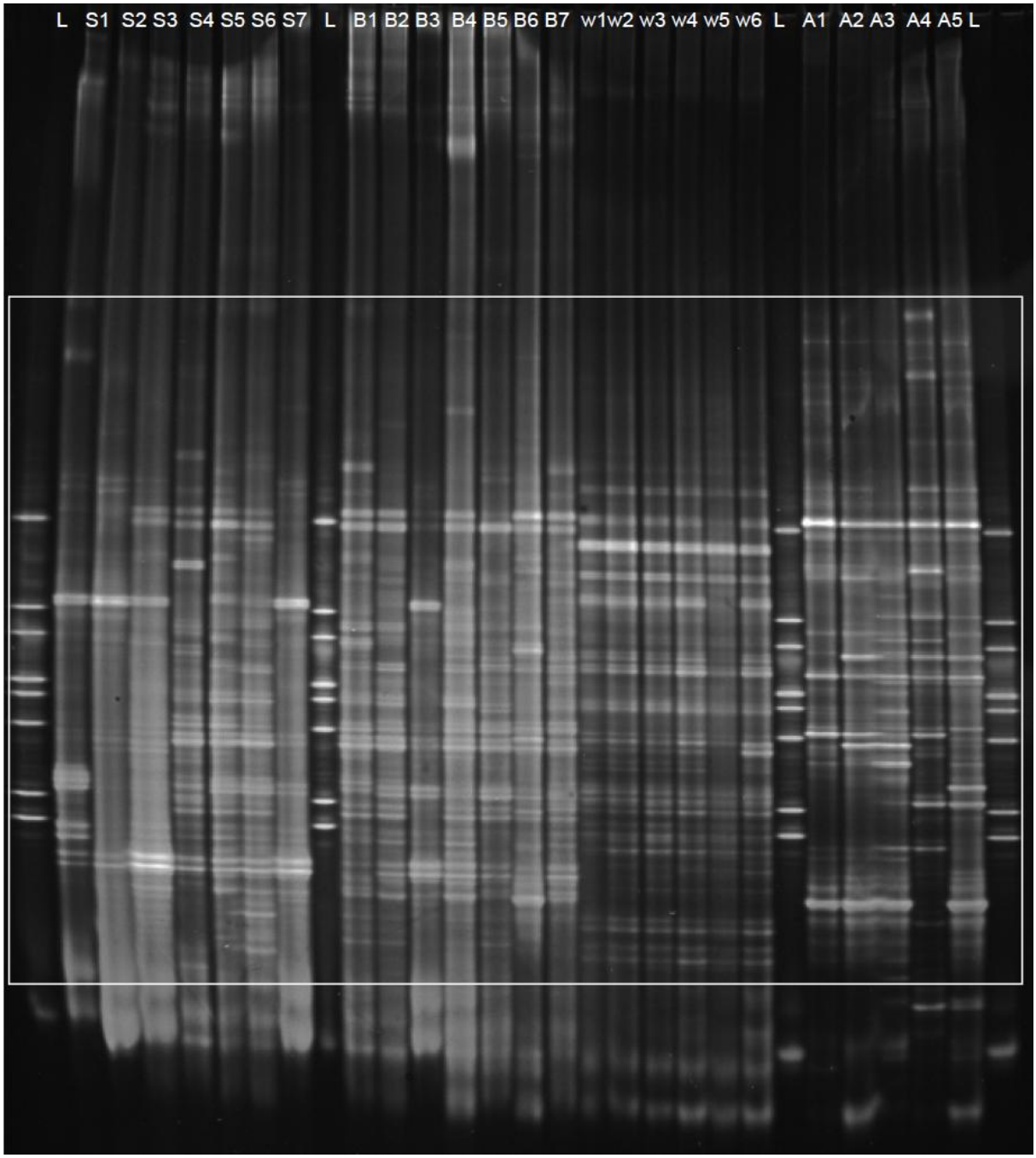


**Figure 3.9**: DGGE gel (8 % acrylamide, 35-55 % denaturing gradient) obtained from the descriptive study with PAN-RS mangrove killifish. The analyzed area is indicated, and lanes are marked with sample names. S refers to the smallest fish and B to the largest. A refers to feed samples from 1, 5, 11, 20 and 27 DAH. W refer to water sample from rearing cups at 35 DAH, from 6 random individuals, and L means ladder/standard.

**Table 3.8**: The diversity indices found from the DGGE from the descriptive study with PAN-RS mangrove killifish. Band richness (k), Shannon index (H') and evenness (J') were calculated, together with the average and standard deviation of the three groups. S refers to the smallest and B to the largest juveniles. A refers to feed samples from 1, 5, 11, 20 and 27 DAH and W to rearing water samples.

| Samples | Band richness (k) | Shannon index (H') | Evenness (J') |
|---|---|---|---|
| **S4** | 27 | 1.80 | 0.55 |
| **S5** | 31 | 1.46 | 0.43 |
| **S6** | 31 | 1.61 | 0.47 |
| **Average** | **30** | **1.62** | **0.48** |
| **Std.dev.** | **2.3** | **0.17** | **0.06** |
| **B1** | 27 | 2.05 | 0.62 |
| **B2** | 32 | 1.65 | 0.48 |
| **B4** | 29 | 1.54 | 0.46 |
| **B5** | 30 | 1.76 | 0.52 |
| **B6** | 25 | 1.95 | 0.61 |
| **B7** | 29 | 1.79 | 0.53 |
| **Average** | **29** | **1.79** | **0.54** |
| **Std.dev.** | **2.4** | **0.19** | **0.07** |
| **w1** | 25 | 2.11 | 0.66 |
| **w2** | 26 | 1.97 | 0.60 |
| **w3** | 26 | 1.67 | 0.51 |
| **w4** | 25 | 1.95 | 0.61 |
| **w5** | 24 | 1.73 | 0.55 |
| **w6** | 28 | 1.99 | 0.60 |
| **Average** | **26** | **1.90** | **0.59** |
| **Std.dev.** | **1.4** | **0.17** | **0.05** |
| **A1** | 28 | 2.04 | 0.61 |
| **A2** | 28 | 1.92 | 0.58 |
| **A3** | 27 | 1.76 | 0.54 |
| **A4** | 27 | 2.21 | 0.67 |
| **A5** | 31 | 1.75 | 0.51 |
| **Average** | **28** | **1.94** | **0.58** |
| **Std.dev.** | **1.6** | **0.19** | **0.06** |

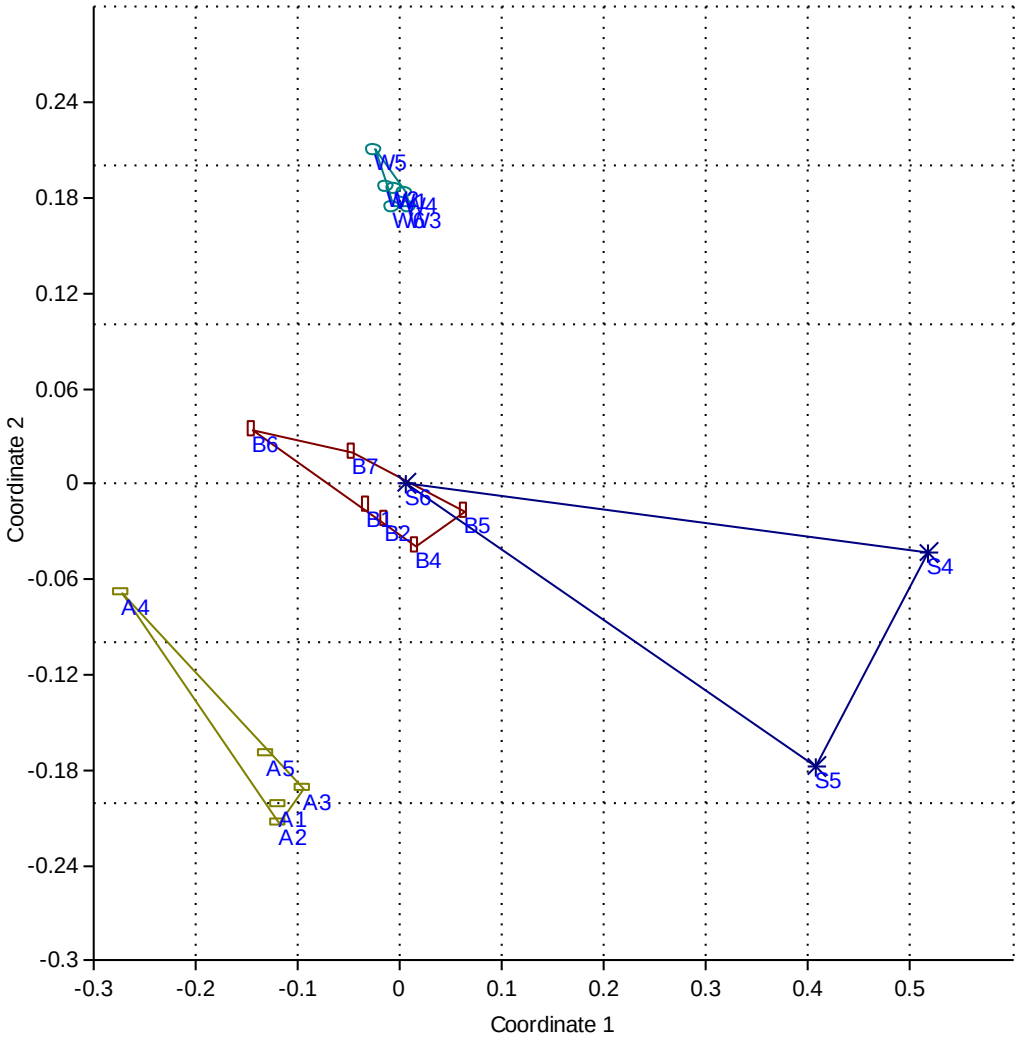


**Figure 3.10**: Non-metric multidimensional scaling of DGGE results from descriptive study with PAN-RS mangrove killifish juveniles with Bray-Curtis similarity measure. The points are marked and in addition there are drawn lines between the outer points in the groups. S refers to the smallest fish and B to the largest. A refers to feed samples from 1, 5, 11, 20 and 27 DAH. W refers to water sample from rearing cups at 35 DAH, from 6 random individuals, and L means ladder/standard. The analysis has a stress value of 0.099.

**Table 3.9**: ANOSIM of the GI microbiota between the small (S) and large (B) PAN-RS juveniles, and of the bacterial composition of the feed (A) and water (W).

| | **Groups** | **ANOSIM R value** | **P value** |
|---|---|---|---|
| **Total** | - | 0.802 | < 0.0001 |
| **Between groups** | B and S | 0.482 | 0.058* |
| | A and B | 0.757 | 0.002 |
| | A and S | 0.856 | 0.018 |
| | W and B | 0.880 | 0.003 |
| | W and S | 0.901 | 0.013 |
| | W and A | 1.000 | 0.001 |

* p > 0.05.

## 3.3 Experimental test with PAN-RS mangrove killifish

### 3.3.1 Growth of the larvae

GI microbiota from the 7 smallest and 7 largest PAN-RS individuals in the descriptive study were pooled in two different batches (Sb and Bb), and cultured before encapsulation in *A. franciscana* and further used to feed three groups of PAN-RS larvae. 30 PAN-RS larvae were divided in 3 groups:

Bf - Larvae with bacteria from large PAN-RS juveniles added to the rearing water and feed.

Sf - Larvae with bacteria from small PAN-RS juveniles added to the rearing water and feed.

Cf - Larvae without any bacteria added.

The fish were fed 5 times, and kept for a total of 20 days. There was no significant difference in length at 20 DAH between the three groups, but the enriched larvae had a bigger variation, and lower average length than the control group (Figure 3.11).

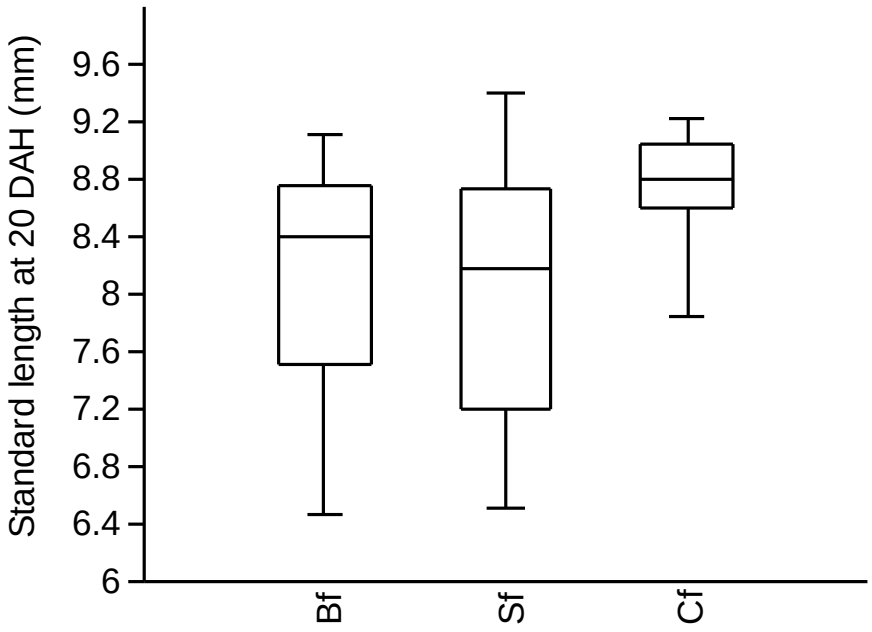


**Figure 3.11**: The length of PAN-RS larvae in the Experimental test. The larvae was divided in different groups based on the different bacteria added to the rearing water and feed: Bacteria from large PAN-RS juveniles added to the rearing water and feed (Bf), bacteria from small PAN-RS juveniles added to the rearing water and feed (Sf) and control group without any bacteria added (Cf). DAH: days after hatching.

### 3.3.2 DGGE analysis of bacterial, water and feed samples from the Experimental test

DNA was extracted from the cultured bacteria (Sb and Bb), *A. franciscana* with encapsulated bacteria (A), from water in rearing cups at 20 DAH (Sw/Bw/Cw) and from all the larvae in the three groups (Bf/Sf/Cf). The PCR products from these were run on two different DGGE gels, the division and the sample names are explained (Table 3.10).

**Table 3.10**: Sample names for the Experimental test, and an explanation of their origin.

| DGGE gel | Sample names and explanations | |
|---|---|---|
| **Figure 3.12** | Sb | Cultured bacteria from small PAN-RS larvae in the descriptive study, number refers to the treatment number. |
| | Bb | Cultured bacteria from large PAN-RS larvae in the descriptive study, number refers to the treatment number. |
| | Sw | Rearing water from two of the larvae treated with Sb at 20 DAH. |
| | Bw | Rearing water from two of the larvae treated with Bb at 20 DAH. |
| | Cw | Rearing water from two of the larvae in the control group at 20 DAH. |
| | A | *A. franciscana* used for feed, number refers to the number of the feeding (in total 5 feedings, samples from feeding 1, 3 and 5 were analyzed) and S/B/C to type of bacteria encapsulated. |
| **Figure 3.14** | Bf | Larvae treated with Bb in rearing water and feed. |
| | Sf | Larvae treated with Sb in rearing water and feed. |
| | Cf | Larvae treated with no bacteria in rearing water and feed. |

From observation of the DGGE gel (Figure 3.12), and the NMDS (Figure 3.13) and ANOSIM results (Table 3.12) it was confirmed that the cultured bacteria (Sb and Bb) are very similar. Only Bb5 stands out. Seeing that the two groups were similar, the rest of the samples were compared in groups to investigate the influence of the treatment. In the NMDS and ANOSIM analyses the groups are divided with this in mind. The water samples from the three different groups clearly show that the experiment is flawed, with the three groups having very similar bacterial composition, even the control group. It was expected that the Sw and Bw samples should be similar, since there was not any clear difference between the Bb and Sb, but the similarity with the Cw show that the control group also were supplied with bacteria. From the DGGE gel (Figure 3.12) and the diversity indices (Table 3.11) it is observed that the strategy for keeping the *A. franciscana* bacteria-free was successful in two of the three analyzed control samples (A1C and A5C). The two samples display four and two weak bands in the DGGE, which implies a very low concentration of bacteria present. These samples were excluded from further analyses, to keep them from lowering the resolution in the NMDS and ANOSIM.

12 bands in the gel were excised, reamplified and sequenced. These bands were all determined to most likely belong to one of the following orders: *γ-proteobacteria*, *Actinobacteria* and *Flavobacteria*. The DNA sequencing data obtained from the DGGE gel are summarized in Table 3.13.

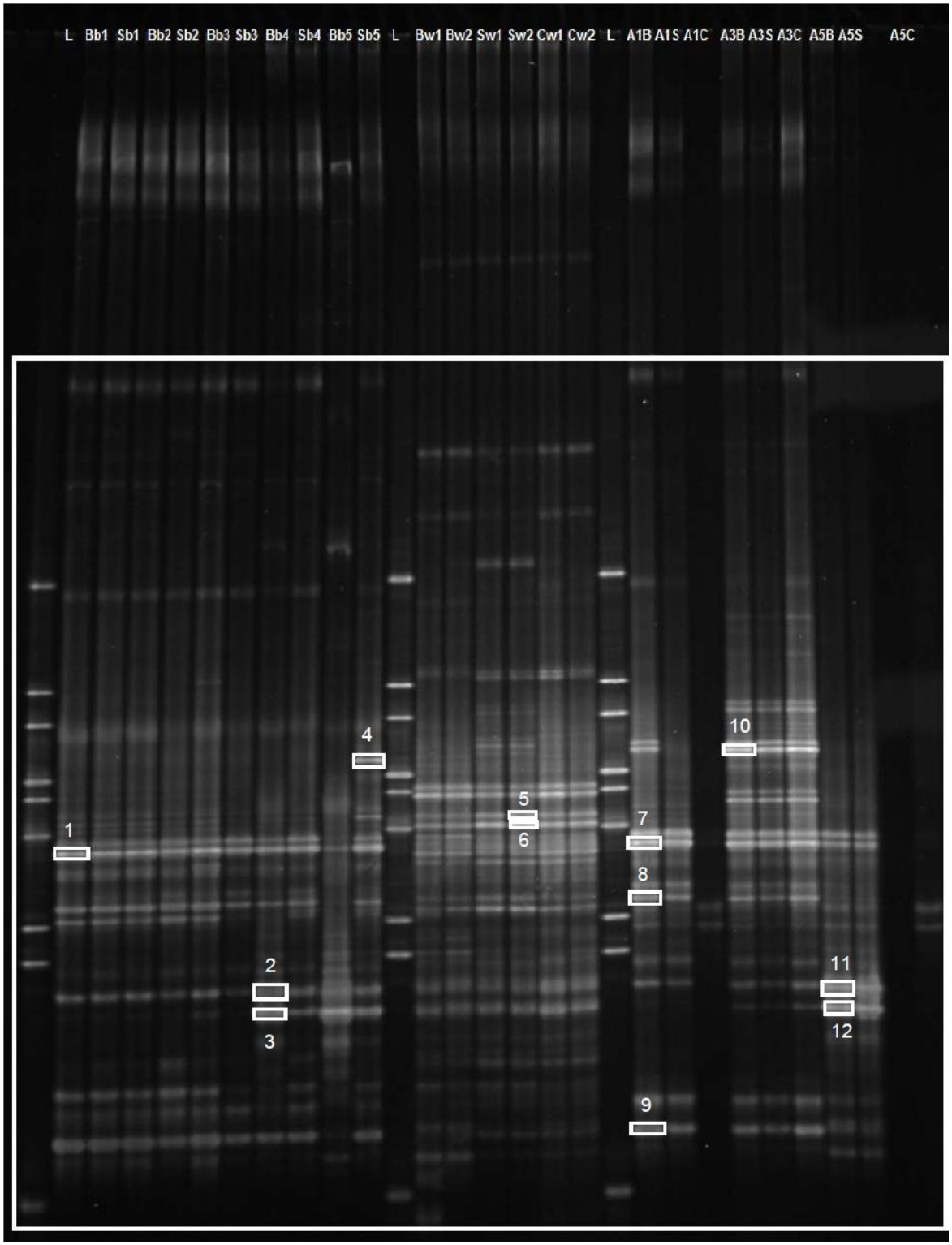


**Figure 3.12**: DGGE gel (8 % acrylamide, 35-55 % denaturing gradient) obtained from the Experimental test with PAN-RS mangrove killifish. The analyzed area is indicated, and lanes are marked with sample names (Table 3.10). The sequenced bands are marked and numbered.

**Table 3.11**: The diversity indices found from the first DGGE gel in the Experimental test. Band richness (k), Shannon index (H') and evenness (J') were calculated, together with the average and standard deviation of the four groups, sample names are explained in Table 3.10.

| | Band richness (k) | Shannon index (H') | Evenness (J') |
|---|---|---|---|
| **Bb1** | 20 | 2.65 | 0.88 |
| **Bb2** | 21 | 2.68 | 0.88 |
| **Bb3** | 24 | 2.81 | 0.88 |
| **Bb4** | 21 | 2.52 | 0.83 |
| **Bb5** | 21 | 2.64 | 0.87 |
| **Average** | **21** | **2.66** | **0.87** |
| **Std. Dev.** | **1.5** | **0.10** | **0.02** |
| **Sb1** | 22 | 2.67 | 0.86 |
| **Sb2** | 21 | 2.67 | 0.88 |
| **Sb3** | 17 | 2.39 | 0.84 |
| **Sb4** | 20 | 2.57 | 0.86 |
| **Sb5** | 25 | 2.74 | 0.85 |
| **Average** | **21** | **2.61** | **0.86** |
| **Std. Dev.** | **2.9** | **0.14** | **0.01** |
| **A1B** | 20 | 2.68 | 0.89 |
| **A1S** | 15 | 2.28 | 0.84 |
| **A1C** | 4 | 1.25 | 0.90 |
| **A3B** | 24 | 2.85 | 0.90 |
| **A3S** | 18 | 2.64 | 0.91 |
| **A3C** | 30 | 3.03 | 0.89 |
| **A5B** | 16 | 2.40 | 0.87 |
| **A5S** | 15 | 2.38 | 0.88 |
| **A5C** | 2 | 0.66 | 0.96 |
| **Average** | **16** | **2.24** | **0.89** |
| **Std. Dev.** | **8.8** | **0.78** | **0.03** |
| **Bw1** | 27 | 3.04 | 0.92 |
| **Bw2** | 29 | 3.06 | 0.91 |
| **Sw1** | 36 | 3.25 | 0.91 |
| **Sw2** | 33 | 3.22 | 0.92 |
| **Cw1** | 30 | 3.03 | 0.89 |
| **Cw2** | 27 | 2.95 | 0.90 |
| **Average** | **30** | **3.09** | **0.91** |
| **Std. Dev.** | **3.6** | **0.12** | **0.01** |

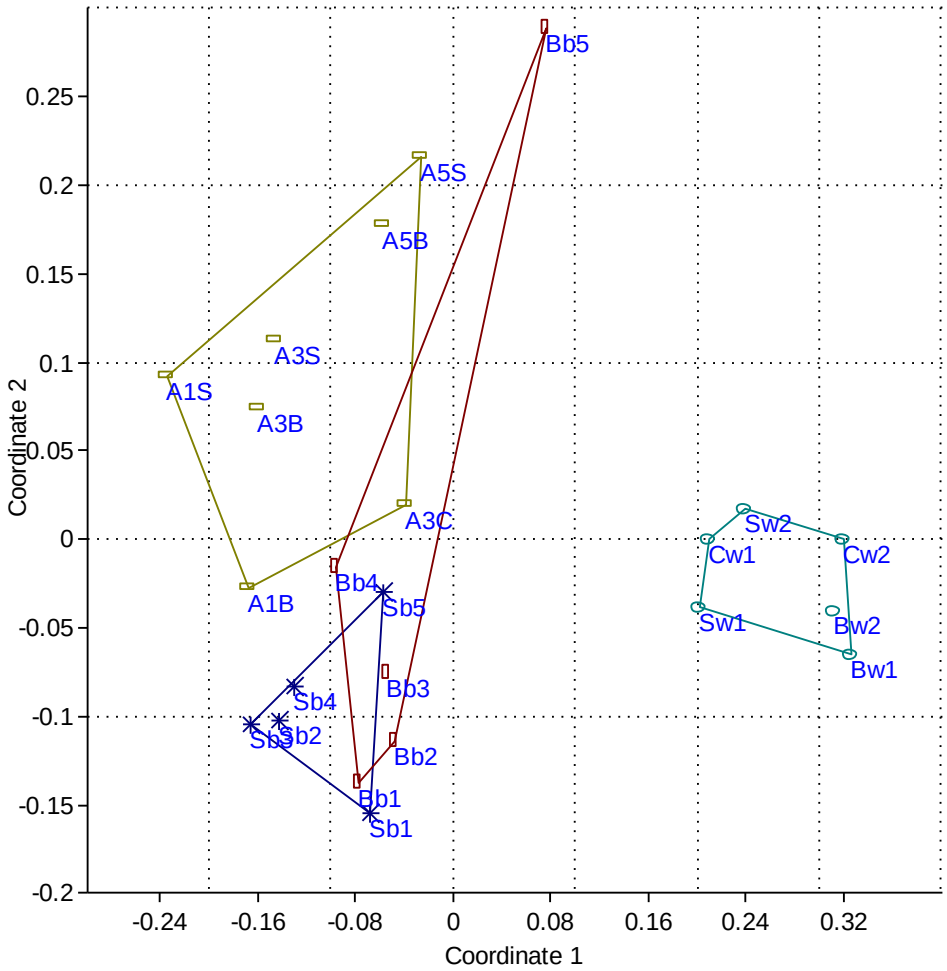


**Figure 3.13**: Non-metric multidimensional scaling of DGGE results from Experimental test with Bray-Curtis similarity measure. The points are marked and named according to Table 3.10, and in addition there are drawn lines between the outer points in two of the groups. The groups are feed samples (A - yellow), bacteria cultures from the GI microbiota of small individuals (Sb – blue), bacteria cultures from the GI microbiota of large individuals (Bb – red) and water samples (Sw, Bw and Cw – cyan). The analysis has a stress value of 0.184.

**Table 3.12**: ANOSIM of the microbial composition of the cultured bacteria from the small larvae (Sb) and large larvae (Bb), and of the bacterial composition of the feed (A) and water (W).

| | Groups | ANOSIM R value | P value |
|---|---|---|---|
| **Between groups** | Bb and Sb | 0.016 | 0.346* |
| | A and Bb/Sb | 0.515 | <0.0001 |
| | W and Bb/Sb | 0.859 | 0.0001 |
| | W and A | 0.931 | 0.0004 |

* p > 0.05

**Table 3.13**: DNA sequencing results for selected DGGE bands (Figure 3.13). "% in lane" refers to percent of the total content in the DGGE lane, and "Observed in" refers to in which samples the band can be observed.

| DGGE bands | Sample origin | % in lane | Observed in | Classification[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **Class** | **Order** | **Family/Genus** | **Examples of possible close relatives** | **Similarity (%)** | **GenBank accesion number** |
| **1, 7** | Bb1, A1B | 17.2, 12.3 | 5 Sb / 5 Bb / 8 A / 6 W | *γ-proteobacteria* | *Vibrionales* | *Vibrio* | *Vibrio agarivorans*; 351A, CECT 5084 | 100 | AJ310648 |
| | | | | | | | *Vibrio natriegens* (T); ATCC 14048T | 100 | X74714 |
| | | | | | | | *Vibrio rotiferianus* (T); R-14939 | 100 | AJ316187 |
| **2, 3, 11, 12** | Bb4, Bb4, A5B, A5B | 7.6, 16.0, 15.3, 16.4 | **2,11**: 2 Sb / 4 Bb / 5 A / 6 W **3, 12**: 1 Sb / 2 Bb / 2 A / 5 W | *Actinobacteria* | *Actino-mycetales* | *Microbacteriaceae* | *Frigoribacterium sp*. PIC-C17 | 100 | DQ227784 |
| | | | | | | *Leifsonia* | *Leifsonia poae* (T); VKM Ac-1401 | 100 | AF116342 |
| | | | | | | | *Salinibacterium amurskyense* (T); KMM3673 | 100 | AF539697 |
| **4** | Sb5 | 11.6 | 1 Sb / 0 Bb / 0 A / 0 W | *γ-proteobacteria* | *Altero-monadales "Vibrionales"* | | | | |
| **5** | Sw2 | 6.5 | 5 Sb / 4 Bb / 2 A / 6 W | *Flavobacteria* | *Flavobacteriales* | *Fluviicola* | uncultured bacterium; D13S-38 | 73.2 | EU617867 |
| | | | | | | *Owenweeksia* | uncultured marine bacterium; SJC1.17 | 73.8 | DQ071103 |
| | | | | | | Unclassified *Cryomorphaceae* | uncultured bacterium; SGUS1259 | 85.6 | FJ202110 |
| **6** | Sw2 | 9.9 | 1 Sb / 3 Bb / 1 A / 6 W | *Flavobacteria* | *Flavobacteriales* | Unclassified *Cryomorphaceae* | uncultured bacterium; MethaneSIP2-10-12 | 73.8 | GU584375 |
| **8** | A1B | 9.7 | 5 Sb / 5 Bb / 7 A / 6 W | *γ-proteobacteria* | *Vibrionales* | *Vibrio* | *Vibrio agarivorans*; 351A, CECT 5084 | 100 | AJ310648 |
| | | | | | | | *Vibrio vulnificus* (T) ATCC 27562 T | 100 | X76333 |
| | | | | | | | *Vibrio ichthyoenteri*; LMG 19664T | 100 | AJ437192 |
| **9** | A1B | 9.2 | 5 Sb / 5 Bb / 7 A / 6 W | *γ-proteobacteria* | *Vibrionales* | *Vibrio* | *Vibrio alginolyticus*; H050815-1 | 100 | EF219054 |
| | | | | | | | uncultured *Vibrio sp*.; HG103 | 100 | FM878645 |
| **10** | A2B | 10.3 | 3 Sb / 1 Bb / 5 A / 5 W | *γ-proteobacteria* | *Pseudo-monadales* | *Moraxellaceae/ Psychrobacter* | *Psychrobacter glacincola;* NF1 | 100 | AJ430829 |
| | | | | | | | *Psychrobacter aquaticus*; CMS 51 | 100 | AJ830004 |

[a] Classification data was obtained by the use of Classifier and SeqMatch at RDP (See Section 2.3.5).

### 3.3.3 DGGE analysis of GI microbiota of PAN-RS larvae from the Experimental test

The gut samples from the PAN-RS larvae in the Experimental test were run on another DGGE gel (Figure 3.14). This DGGE has a different denaturing gradient (35-55 %) than the other DGGE gels (35-50 %) in this thesis. This is because it was difficult to obtain an interpretable gel with a narrower gradient, the reason for this is not known. The gel is also observed to have worse resolution than the other gels; this is clearly seen by comparing the sample A3S, which was run on both gels in the Experimental test. On the other gel (Figure 3.12) it had a band richness of 18 and a Shannon index of 2.64, while in this gel (Figure 3.14) the sample had a band richness of 5 and a Shannon index of 1.42. This clearly shows the difficulty with comparison between gels and why this should be avoided.

The gel had a total of 47 bands, and from observation one can see that the samples (Figure 3.14) show a great deal of variation, also within the three groups.

The results from the other DGGE gel (Figure 3.12) in the Experimental test showed that the design setup was unsuccessful. The similarity between the Bb and Sb samples gave the expectation that the Sf and Bf larvae would have similar GI microbiota. Furthermore, the unsuccessfulness in feeding Cf larvae bacteria-free *A. franciscana* gave the expectation that the Cf larvae also would have a developed GI microbiota.

These expectations are confirmed from the diversity indices (Table 3.14). The Bf and Sf samples show similar average band richness, Shannon index and evenness. The Cf samples are not significantly different with regards to average Shannon index and evenness, compared to the two other groups ($p > 0.05$).

The overlapping between the three groups can clearly be seen in both the NMDS analysis (Figure 3.15) and ANOSIM (Table 3.15). The 2 dimensional NMDS analysis had a high stress value (0.264), which indicates that the configuration is very close to arbitrary. The analysis was therefore changed to a 3 dimensional (3D) analysis which is more difficult to interpret, but gives a lower stress value (0.217). The 3D analysis is shown in Figure 3.15, with the three coordinates in two different 2 dimensional plots. This shows that the three groups are clearly overlapping, especially Sf and Bf. This is also confirmed in the ANOSIM, where there is a weak difference between Sf - Cf and Bf - Cf, but not between Sf - Bf.

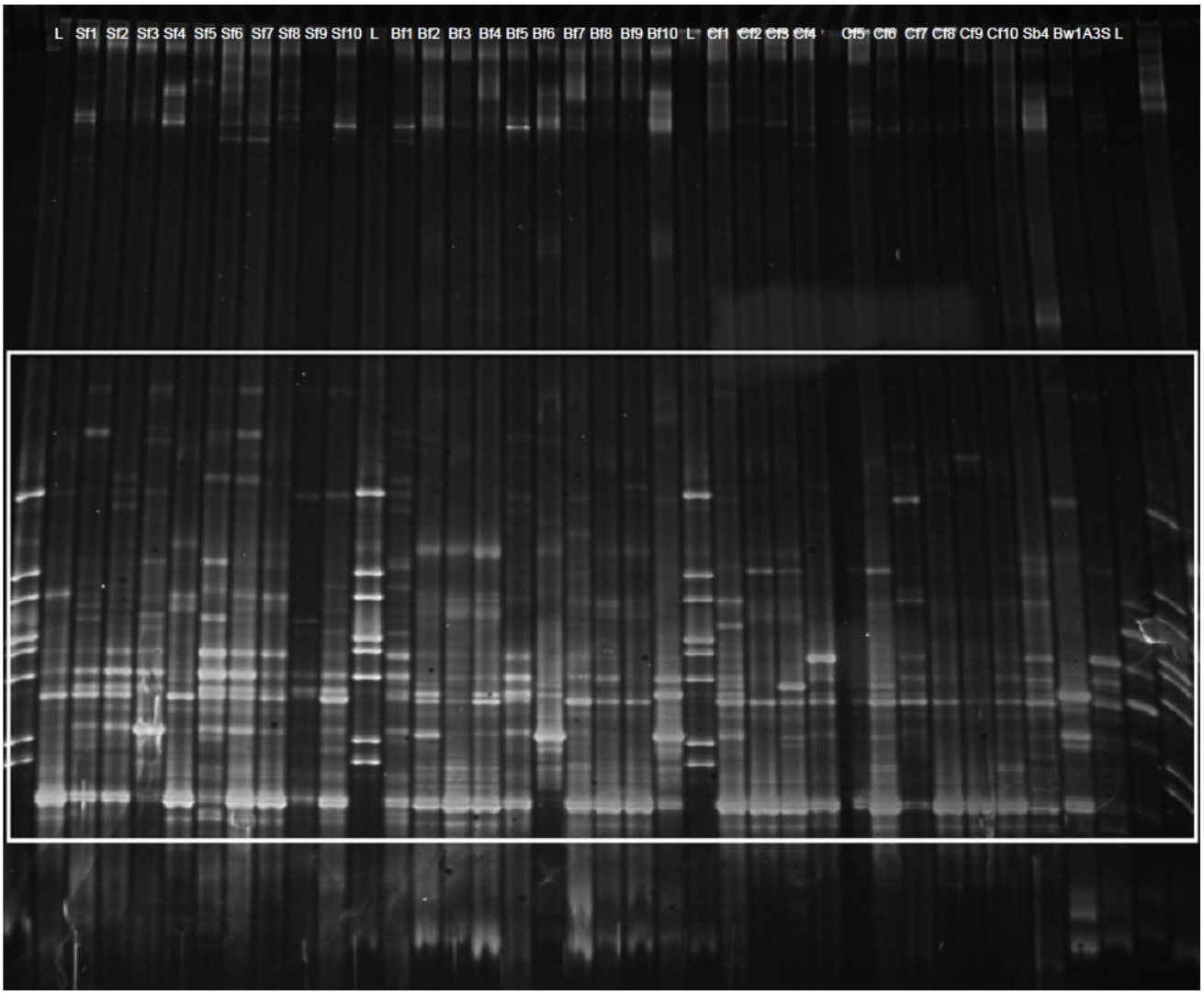


**Figure 3.14**: DGGE gel (8 % acrylamide, 35-55 % denaturing gradient) obtained from the experiment test with PAN-RS mangrove killifish. The analyzed area is indicated, and lanes are marked and named according to Table 3.10. L refers to ladder/standard.

**Table 3.14**: The diversity indices found from the second DGGE gel in the Experimental test. Band richness (k), Shannon index (H') and evenness (J') were calculated, together with the average and standard deviation of the three groups. Sample names are explained in Table 3.10.

| Samples | Band richness (k) | Shannon index (H') | Evenness (J') |
|---|---|---|---|
| **Bf1** | 14 | 1.62 | 0.61 |
| **Bf2** | 19 | 2.23 | 0.76 |
| **Bf3** | 21 | 2.28 | 0.75 |
| **Bf4** | 18 | 2.44 | 0.84 |
| **Bf5** | 17 | 1.95 | 0.69 |
| **Bf6** | 19 | 2.62 | 0.89 |
| **Bf7** | 18 | 2.46 | 0.85 |
| **Bf8** | 16 | 2.24 | 0.81 |
| **Bf9** | 9 | 1.75 | 0.80 |
| **Bf10** | 15 | 2.17 | 0.80 |
| **Average** | **16.6** | **2.18** | **0.78** |
| **Std. dev.** | **3.4** | **0.32** | **0.08** |
| **Sf1** | 19 | 2.49 | 0.85 |
| **Sf2** | 19 | 2.40 | 0.81 |
| **Sf3** | 19 | 2.36 | 0.80 |
| **Sf4** | 17 | 2.35 | 0.83 |
| **Sf5** | 17 | 2.24 | 0.79 |
| **Sf6** | 15 | 2.40 | 0.89 |
| **Sf7** | 15 | 2.20 | 0.81 |
| **Sf8** | 14 | 2.06 | 0.78 |
| **Sf9** | 14 | 1.80 | 0.68 |
| **Sf10** | 12 | 2.08 | 0.84 |
| **Average** | **16.1** | **2.24** | **0.81** |
| **Std. dev.** | **2.5** | **0.21** | **0.05** |
| **Cf1** | 20 | 2.60 | 0.87 |
| **Cf2** | 12 | 1.87 | 0.75 |
| **Cf3** | 15 | 2.00 | 0.74 |
| **Cf4** | 8 | 1.54 | 0.74 |
| **Cf5** | 19 | 2.40 | 0.82 |
| **Cf6** | 11 | 2.09 | 0.87 |
| **Cf7** | 12 | 1.70 | 0.68 |
| **Cf8** | 12 | 1.63 | 0.65 |
| **Cf9** | 10 | 1.78 | 0.77 |
| **Cf10** | 14 | 2.31 | 0.88 |
| **Average** | **13.3** | **1.99** | **0.78** |
| **Std. dev.** | **3.8** | **0.36** | **0.08** |
| **Sb4** | 9 | 1.69 | 0.77 |
| **Bw1** | 10 | 1.83 | 0.79 |
| **A3S** | 5 | 1.42 | 0.88 |

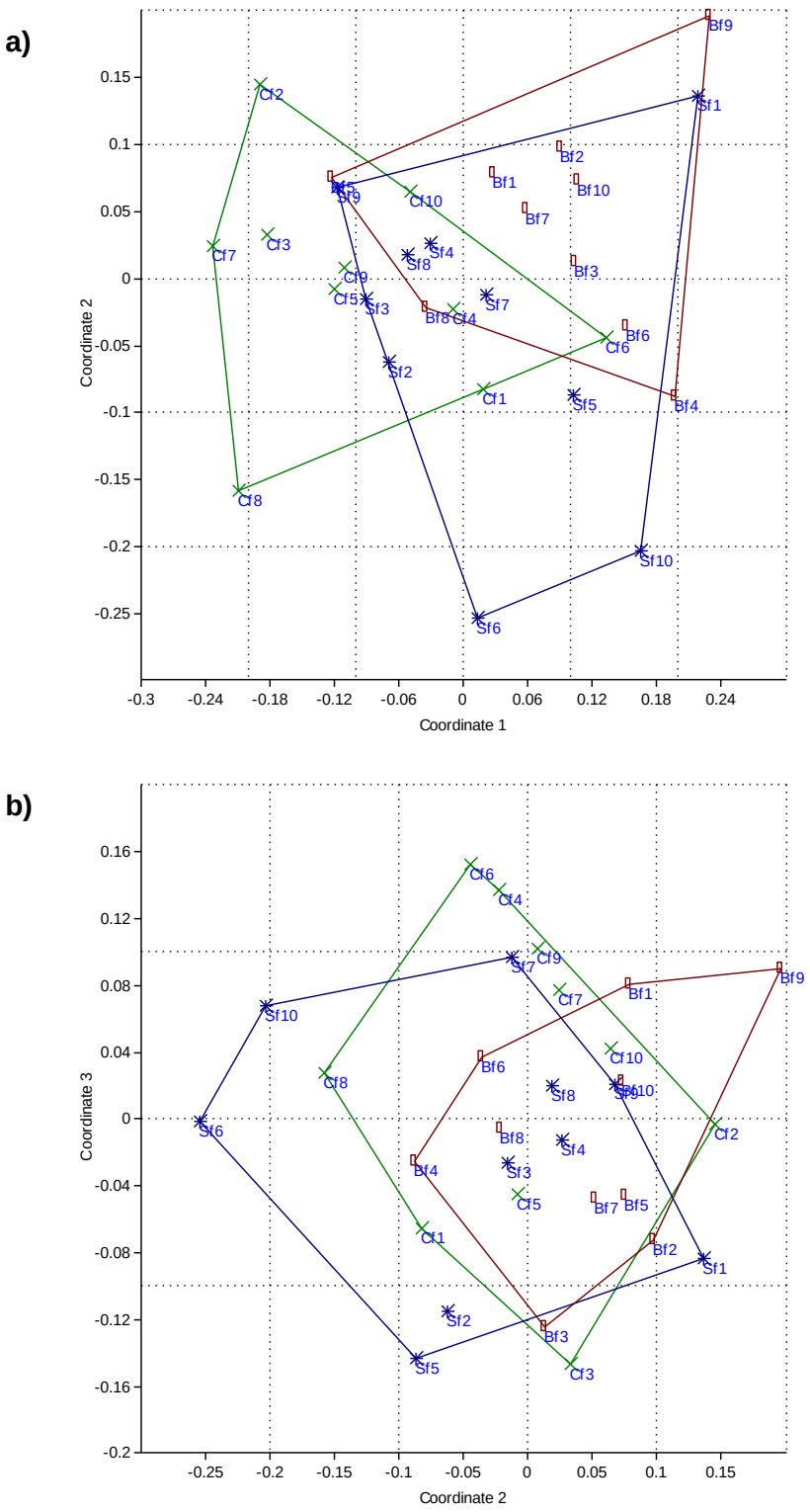


**Figure 3.15**: 3D non-metric multidimensional scaling of DGGE results from Experimental test with Bray-Curtis similarity measure. Coordinates 1 and 2 shown in a) and coordinates 2 and 3 in b). The points are marked (Table 3.10) and in addition there are drawn lines between the outer points in the groups. The groups are Sf (Blue), Bf (red) and Cf (green). The analysis has a stress value of 0.217.

**Table 3.15**: ANOSIM of the GI microbiota of PAN-RS larvae fed with *A. franciscana* with bacteria grown from small PAN-RS larva encapsulated (Sf) and the PAN-RS larvae fed with *A. franciscana* with bacteria grown from large PAN-RS larva encapsulated (Bf), and C to the PAN-RS larvae fed with *A. franciscana* with no bacteria encapsulated (Cf).

| | **Groups** | **ANOSIM R value** | **P value** |
|---|---|---|---|
| **Total** | - | 0.143 | 0.005 |
| **Between groups** | Bf and Sf | 0.079 | 0.103* |
| | Cf and Bf | 0.246 | 0.006 |
| | Cf and Sf | 0.115 | 0.043 |

* $p > 0.05$

# 4. Discussion

This project had three objectives mentioned in Section 1.6; all of these were investigated by culture-independent methods. The GI microbiota of mangrove killifish and Atlantic cod larvae was examined by PCR-DGGE, and sequencing of DGGE bands. This was done to look for differences in the GI microbiota between fast and slow growing fish larvae and juveniles. It was also attempted to test experimentally if exposure to the culturable microbiota from slow or fast growing individuals respectively, could reproduce size differences.

## 4.1 Evaluation of methods

PCR-DGGE was the main method used in this project, and the protocol mainly produced gels of good quality, with good band separation and little smiling effect. Still DGGE, like all PCR-based community analysis techniques, is vulnerable to biases from DNA extraction and amplification. The method is only semi-quantitative, and there is a problem with heterogeneity of the 16S rRNA gene in some bacteria (Muyzer, 1998). Gels can also be difficult to reproduce, and comparison between gels should be avoided. This is clearly seen when comparing the sample A3S, which were analyzed on two DGGE gels (Figure 3.12 and Figure 3.14). The pattern of the sample is different between the gels, with only the most dominating bands from Figure 3.12 being seen in Figure 3.14. This leads to different band richness, Shannon index and evenness.

PCR was the most challenging step in the process of producing reliable DGGE gels. The little amount of DNA extracted from the guts of the fish larvae, made it necessary to use many PCR cycles. There was a contamination by bacterial DNA from some of the PCR reagents. The bacterial DNA originates from polymerases used in the PCR reaction, and is known to be a problem when using universal primers and having a low amount of bacterial DNA in the sample (Tseng *et al*., 2003). All of these problems were resolved by using a different polymerase, presumably with a lower DNA content. Another problem occurred, which can be observed in the DGGE gel of the descriptive study of PAN-RS (Figure 3.9). Several of the samples have a band which seems to correspond with the eukaryotic band observed by Bakke *et al*. (2011). Some conserved regions of the 16S rRNA gene (*e.g.* the “518-region”), have counterparts in the 18S rRNA gene in eukaryotes. The eukaryotic fragment can thus be amplified when the bacterial DNA concentration is low compared to the eukaryotic DNA concentration. It was attempted to get a sequence of the mentioned bands to confirm that the band was eukaryotic, but the sample did not produce a readable sequence. Bakke *et al*. (2011) got the eukaryotic band when not following a nested protocol, and resolved the issue by using a nested PCR protocol that was also used in this thesis. It is difficult to determine why this problem occurred. A reason could be the very low DNA

concentrations of bacterial DNA and the many cycles of PCR, which may have led to a more unspecific reaction and amplification of eukaryotic DNA.

The Experimental test was designed to show the same as Turnbaugh *et al*. (2006). They used mice to show that colonization of germ-free mice with an 'obese microbiota' results in a significantly greater increase in total body fat than colonization with a 'lean microbiota'. There were many challenging aspects when trying to transfer this experimental strategy to fish larvae. Fish does, as mentioned in Section 1.2, live in a far more exposed environment and it is more difficult to control the environment of a fish than a mouse. This caused problems associated with cultivation of bacteria, and decapsulation and encapsulation with *A. franciscana*. The cultivation of bacteria from the fast and the slow growing mangrove killifish juveniles, did not give a significant difference in bacterial composition between the two groups (Table 3.12), and the Experimental test was in this aspect unsuccessful. This can be seen in the DGGE gel (Figure 3.12), where the cultured bacteria for both the GI microbiota of the small and large PAN-RS individuals gave similar DGGE band patterns. Further, NMDS (Figure 3.13) show that the cultured bacteria (Sb and Bb) cluster together, except from one of the samples (Bb5).

Why the cultured bacteria from the two groups (fast and slow growing) did not show differences, but their inoculums did (see below), can be explained by the cultivation protocol. The GI microbiota from the PAN-RS individuals were mixed and plated on marine agar. Generally ≤ 5 % of marine microorganisms can be isolated using conventional microbiological methods, such as liquid enrichment and plating on solid media. This is due to unknown growth requirements, and probably less than 1 % of the marine microbial species have been successfully cultivated under standard laboratory conditions (Jensen and Fenical, 1994; Amann *et al*., 2005). Additionally, many of the microorganisms which grow on plates grow so slowly that they are quickly outcompeted by fast growing colonies if the plates are inoculated with too high densities (Akselband *et al*., 2006). The plates in this study were overgrown at day 1 and day 2 after plating. This is not enough time for the slow growing bacteria to form colonies. An additional negative factor is that fast growing bacteria (r-strategists) which were dominating the cultured bacteria are more likely to be harmful to the fish, as they are more likely to be opportunistic pathogens than more slow-growing K-strategists (Vadstein *et al*., 2004). Smalla *et al.* (1998) investigated the change in microbial composition of inoculums and their respective cultures grown on BIOLOG GN plates by PCR-DGGE/TGGE. A decrease in diversity was observed in the cultures, and the dominating fragments belonged to *γ-proteobacteria*. The sequencing results (Table 3.13) show that the fast growing bacteria dominating the cultured bacteria is *γ-proteobacteria*, mostly *Vibrio* (Band 1, 7, 8, 9), and are thus in accordance with the finds by Smalla *et al*. (1998). A large

fraction of the bacteria that are pathogenic to fish belong to the *γ-proteobacteria* (Vadstein *et al*., 2004).

The rearing water samples (Figure 3.12) confirm that the attempt to keep the control group "bacteria-free" failed, with the control group being similar to the other rearing water samples. It can be observed in the DGGE gel (Figure 3.12), that two of the analyzed treatments were successful at keeping the control bacteria free (A1 and A5), whilst one where not (A3). This shows that the decapsulation and encapsulation protocols were partly successful.

## 4.2 Descriptive studies

This project was dependent upon growing fish individuals with a sufficient difference in size. For the cod larvae this was done by picking out some large and small larvae at the same age reared in tanks. For the mangrove killifish the larvae were reared individually, and the 7 smallest and largest were selected for further analysis of the GI microbiota. Significant differences in length and weight were obtained between the two groups in the different species and strains (Figure 3.1 and Figure 3.6).

The GI microbiota of large and small Atlantic cod larvae were evaluated by PCR-DGGE (Figure 3.2), and statistical analysis (Table 3.3) revealed a significant difference between the two different groups, and support the hypothesis. Further, it was observed that the small cod larvae had a bigger diversity and evenness than the larger larvae (Table 3.2).

There was a common dominating band in 7 of the small individuals and in 12 of the large in the DGGE gel with samples from cod larvae (Band 4, Figure 3.2 and Table 3.4). This band was most likely in the order *Campylobacter* and the closest hits within GenBank where sequences from uncultured bacteria assumed to be within the genus *Arcobacter*. *Arcobacter* are aerotolerant relatives of the *Campylobacter*, which have been found to be an abundant (but unculturable) component of the microbiota of hatchery reared Chilian oyster (*Tiostrea chiliensis*) (Romero *et al*., 2002). *Arcobacter* (putative) were found by McIntosh *et al*. (2008) to be a major component of the live feed rotifer's microbiota. They did not detect these bacteria in cod larvae, and concluded that the bacterium was unable to overcome the non-specific defense mechanisms present in the larval digestive tract or that they lacked mechanisms believed to be necessary for colonization of fish mucosal surfaces. This is thus not in accordance with the findings in the present study.

Bands 1, 2 and 5 (Figure 3.2) were only observed in the microbiota of the small cod larvae. The bands were all classified to the order *Vibrionales*, and within the genus *Aliivibrio* (Table 3.4). This genus was classified as a group within the *Vibrio* genus until 2007 (Urbanczyk *et al.*, 2007). Two of the species in the *Aliivibrio* genus are associated with diseases: *Aliivibrio*

*wodanis*, which is associated with winter ulcer in farmed Atlantic salmon (*Salmo salar*), and *Aliivibrio salmonicida*, which is associated with cold water vibriosis in farmed Atlantic salmon, sea farmed rainbow trout (*Oncorhynchus mykiss*) and captive Atlantic cod (Lunder *et al*., 2000; Hjerde *et al*., 2008). The presence in only the small larvae is interesting, and can be an influencing factor for the difference in growth rate.

The descriptive study with mangrove killifish had some differences in setup compared with the descriptive study with cod, including lower salinity and individual rearing. The mangrove killifish were thus not exposed to competition from other individuals or to interactions with respect to the GI microbiota from other individuals due to defecation. Still the individuals were supplied with rearing water and feed with the same microbiota, so differences in the GI microbiota diversity is due to selection at individual level and random events.

Although mangrove killifish have been used extensively for research in some fields, there have not been any previous studies which have looked at the species' microbiota. However, the growth of the PAN-RS and DAN strain in the descriptive study is in accordance with the growth observed by Grageda *et al*. (2005), with the PAN-RS strain growing faster than DAN (Figure 3.4).

The DGGE gel of the DAN mangrove killifish (Figure 3.7) revealed a GI microbiota without any dominating bacteria, and with a high number of bands. The ANOSIM (Table 3.6) confirms that there is significant difference between the fast and slow growing individuals, and it can be seen from the gel (Figure 3.7) that there are 3 of the large individuals which stand. These are showing lower band richness, Shannon index and evenness than the other individuals (Table 3.5). This is also visualized in the NMDS analysis (Figure 3.8), where the three mentioned individuals are placed outside the main clustering. Two of these three individuals are the biggest of all the large individuals. This may imply that some of the bands which are present with a stronger intensity in these samples may have a positive effect for the growth rate of the individuals. Two of these bands where sequenced (Band 5 and 6, Figure 3.7 and Table 3.7), and found to be within the order *Vibrionales*, and with similarity to the genera *Listonella* and *Vibrio*. A contradiction to the assumed positive effect of these bacteria is that one of the closest matches to Band 6, using RDP SeqMatch search (with 98.7 % similarity), is the two bacterial species *Listonella anguillarum* and *Vibrio aestuarianus*, which are associated with disease in fish and bivalves, respectively (Egidius, 1987; Garnier *et al*., 2007).

The results from the DGGE gel of the GI microbiota of the PAN-RS mangrove killifish (Figure 3.9) was obstructed by the fact that the PCR products from several of the samples were contaminated with a eukaryotic 18S rRNA product, as discussed above. Thus only 3

individuals from the group of small individuals were analyzed, and 6 within the group of large individuals. This gives the analysis of the PAN-RS considerably less strength. Still, among the three analyzed small individuals two stand out (S4 and S5), while one (S6) cluster with the large individuals (Figure 3.10). This is in correspondence with the observations for the DAN strain, where a partial overlapping also was seen (Figure 3.8). The two PAN-RS individuals with divergent GI microbiota are not very similar, and do not cluster – but in comparison to the other samples, they are more similar to each other. The ANOSIM (Table 3.9) is obstructed by the low strength due to the low number of samples analyzed, but still shows a strong significant difference between microbiota of the feed, rearing water and the fish. The GI microbiota of the large and small individuals was marginally significant ($p = 0.058$) with an R-value of 0.482.

The water samples of the PAN-RS were similar to each other, as can easily be seen in the gel (Figure 3.9) and in the NMDS analysis (Figure 3.10). The samples were taken from 6 different rearing cups at 35 DAH. Different succession could have taken place, but it seems that the treatment of the fish have been equal, as intended, which have led to similar bacterial composition of the rearing water.

There is little knowledge about the development and establishment of the GI microbiota in fish. Nayak (2010) point out that the GI microbiota is believed to reflect the microbial composition of the rearing water, the diet and the surrounding environment. It is generally believed that the microbes in feed is the important factor for the colonization of the gastrointestinal tract, but in some fish species it has been shown that bacteria is actively taken up from the rearing water during the early larval stages and could thus play an important role in the colonization of the gastrointestinal tract (Reitan *et al.*, 1998). This is in agreement with the NMDS analysis of the PAN-RS individuals (Figure 3.10), where it is clearly demonstrated that the bacterial composition of both the rearing water and the feed are important factors for the establishment of the fish's GI microbiota. In the Experimental test cultured bacteria was added to PAN-RS mangrove killifish larvae, and the larvae had a more varying length than the larvae in the control group (Figure 3.11). This show that bacteria added in the rearing water and in the feed had an effect on the growth of the mangrove killifish larvae.

Ley *et al*. (2006) showed that the differences in GI microbiota between obese and lean mammals are related to the relative abundance of the two predominant bacterial divisions *Firmicutes* and *Bacteriodetes*. Lean individuals have a higher abundance of *Bacteriodetes* and a lower of *Firmicutes*. The difference is thus not related to species-specific differences, but rather a division-wide dissimilarity. This thesis examined only a limited number of bands

by sequencing, and can thus give an idea of the composition of the GI microbiota, but it is difficult to show any trends at the taxonomic level of divisons. Still there was observed some differences at species level between GI microbiota of slow and fast growing cod and mangrove killifish, as discussed above. Examples of this are the *Aliivibrio* sp. (Bands 1, 2 and 5, Figure 3.2), which were only observed in the small cod larvae, and *Vibrio/Listonella* sp. (Bands 5 and 6, Figure 3.7) which were only observed at high intensity in the large DAN juveniles.

It is difficult to interpret whether or not the differences found in GI microbiota is due to the difference in size or vice versa. The big size difference between the large and small cod (the larger group is 45 % bigger than the smaller), implies that the development, especially with regards to the gastrointestinal system (Kjørsvik *et al.*, 1991; Pedersen and Falk-Petersen, 1992) and immune system (Shrøder *et al*., 1998), could be at different stages. This could contribute to the observed differences between the microbiota of the two groups. The further developed large larvae will have a higher ability to fight opportunistic pathogens, which could influence on microbial composition. The mangrove killifish larvae showed a smaller size difference than the cod larvae, with the larger group of DAN being 13 % bigger than the smaller and the PAN-RS being 12 % bigger than the smaller. The difference in development should thus not be such an important factor for the mangrove killifish as for the cod (Figure 3.6). Moreover, all individuals of mangrove killifish had passed metamorphosis. As previously mentioned in Section 1.1 and above, have studies in mammals shown a difference between the GI microbiota in lean and obese individuals, where the GI microbiota of the obese individuals have a increased capacity for energy harvest (Ley *et al*., 2006; Turnbaugh *et al*., 2006; Turnbaugh *et al*., 2009). This could also be the case for the large fish individuals, where a GI microbiota with increased capacity for energy harvest could be the cause for the growth differences. Still it is important to bear in mind the large differences between the environment of the mammals and fish, and also the difference between being lean/obese and having a fast/slow growth rate.

Still it would be interesting to examine if the differences in GI microbiota is caused by the differences in developmental stage or if the composition of the GI microbiota is a factor influencing the growth rate. This could be done by investigating the GI microbiota of small and large larvae through the developmental stages, and compare the development of the GI microbiota.

## 4.3 Future prospects

This thesis has shown interesting results, and more work could be done to examine the hypothesis further. A difference in the GI microbiota between small and large individuals were seen, and it would be interesting to examine the correlation between the magnitude of differences in gut flora and coefficient of variation in body size of fish, (*e.g.* with CABFAC factor analysis), to see if some of the differences can be explained by size. It would also be interesting to investigate the succession of bacteria in cod larvae at different developmental stages, examine to what extent the difference in microbiota could be explained by the difference in development.

The experimental design of the Experimental test was flawed, due to lack of microbial control. Forberg *et al*. (2011) have established a protocol for keeping gnotobiotic cod, which could be employed for a better control of the microbial environment. Microinjection could be a possibility for transfer of a non-cultured GI microbiota.

Future research could look further into the functionality of fish-microbe interactions. The controlling factors in the development of the GI microbiota and its role in fish larvae is a interesting field of study, which could be further investigated by the use of other methods than DGGE. Examples of such methods are characterization of the microbial composition by cloning and pyrosequencing of 454 amplicons (See Section 1.5) and characterization of low molecular compounds in the intestine by GC-MS.

# 5. Conclusion

The descriptive studies of cod and two strains of mangrove killifish (DAN and PAN-RS) revealed difference in GI microbiota between fast growing and slow growing individuals. Significant differences were found between the two groups in cod and in the mangrove killifish strain DAN. The PAN-RS strain also showed differences, but they were only marginally significant. This can partially be explained by the lower number of individuals analyzed in the descriptive study of PAN-RS.

The descriptive study with PAN-RS mangrove killifish included feed and water samples. The GI microbiota of the PAN-RS juveniles had similarities with the microbial composition of both the feed and the water, and showed that the GI microbiota is affected by both.

In the Experimental test it was attempted to examine if exposure to the culturable microbiota from slow or fast growing individuals respectively, could reproduce the size differences found in the descriptive study. The cultured bacteria from fast and slow growing mangrove killifish PAN-RS larvae did show differences with regards to the microbial composition, due to experimental difficulties. Thus it was not expected to find any size difference between the fish larvae supplied with the different cultured bacteria. No significant differences in size were found, but the fish larvae supplied with the bacteria had a larger variation in size than the control group.

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# Appendices

## Appendix 1: OD measurements and calculations

The bacterial concentration was determined by measuring the OD at 600 nm. A dilution of the bacterial suspensions (1:100) was used. See Table A.1.1 for measurements and calculations of bacterial concentrations.

**Table A.1.1**: OD measurements of bacterial suspensions for encapsulation.

| Treatment | DAH | Batch | OD measurements of diluted bacterial suspensions (Abs) | Approx. bacterial concentration (cells/mL) | Amount added to the fish water (µL) | Amount added to the *A. franciscana* encapsulation solution (µL) |
|---|---|---|---|---|---|---|
| **1** | 0 | B | 0,129 | 1,3*10^10 | 4,6 | 769 |
| | | S | 0,263 | 2,6*10^10 | 2,3 | 385 |
| **2** | 4 | B | 0,154 | 1,5*10^10 | 4 | 667 |
| | | S | 0,268 | 2,7*10^10 | 2,2 | 370 |
| **3** | 7 | B | 0,108 | 1,1*10^10 | 5,5 | 900 |
| | | S | 0,098 | 1,0*10^10 | 6 | 1000 |
| **4** | 12 | B | 0,168 | 1,7*10^10 | 2,2 | 588 |
| | | S | 0,266 | 2,7*10^10 | 3,5 | 370 |
| **5** | 16 | B | 0,129 | 1,3*10^10 | 4,6 | 769 |
| | | S | 0,202 | 2,0*10^10 | 3,0 | 500 |

## Appendix 2: Modified protocol for DNA extraction

The modified protocol for marine bacteria used for the DNA extraction, with the Qiagen DNeasy blood and tissue kit.

1. Add 180 µL enzymatic lysis buffer to eppendorf tube with the sample (see DNeasy protocol).
2. Incubate at 1 h at 37 °C.
3. Add 40 µL proteinase K and 180 µL ATL buffer, vortex, incubate at 55 °C for 1-2 h.
4. Add 200 µL AL buffer, vortex and incubate at 70 °C for 10 min.
5. Add 300 µL 96 % ethanol and vortex.
6. Centrifuge at 15 000 rpm for 3 minutes.
7. Transfer the solution to the DNeasy column (placed in a 2 mL tube) and centrifuge at 8000 rpm in 1 min. Discharge the filtrate.
8. Add 500 µL AW 1 buffer and centrifuge at 8000 rpm for 1 min. Discharge the filtrate.
9. Add 500 µL AW 2 buffer and centrifuge at 15 000 rpm for 3 min.
10. Transfer the column to an eppendorf tube, and 50 µL AE buffer (or DNA free water) directly to the membrane, incubate for 1 minute at room temperature and centrifuge at 8000 rpm for 1 min.
11. Elute once more with 50 µL AE buffer (or DNA free water) to obtain a total of 100 µL extract.
12. Quantify amount of DNA with Nanodrop. Go to PCR or freeze at -20 °C.

## Appendix 3: Primer sequences, PCR solutions and regimes

The different primer sequences for the primers used in the PCR reactions, and an overview of the different amount of PCR cycles, the different template amounts and amounts of PCR product put on DGGE.

**Table A.3.1**: Primer sequences with their respective sequence and reference.

| Primer | Sequence (5'-3') | Reference |
|---|---|---|
| **Eub8F** | AGA GTT TGA TCM TGG CTC AG | Weisburg *et al*., 1991 |
| **984yR** | GTA AGG TTC YTC GCG T | Wang and Qian, 2009 |
| | cgcccgccgcgcgcggcgggcggggcggggcacgggggg | |
| **338F-GC** | ACT CCT ACG GGA GGC AGC AG | Muyzer *et al*., 1993 |
| **518R** | ATT ACC GCG GCT GCT GG | Muyzer *et al*., 2003 |
| **338F M13 rev (-29)** | CAG GAA ACA GCT ATG ACC<br>ACT CCT ACG GGA GGC AGC AG | |

**Table A.3.2**: The different DGGE gels and their gradient, and the different treatment of the samples.

| Gel | DGGE gel gradient | Sample ID | PCR cycles External primers | PCR cycles Internal primers | Template amount External primers (µL) | Template amount Internal primers (µL) | Amount PCR product on DGGE (µL) |
|---|---|---|---|---|---|---|---|
| **Figure 3.2** | 35-50 % | S1, S2, S9, S10, S11, B1, B3, B5 | 25 | 30 | 1 | 1 | 5-10 |
| | | S3, S4, S5, S6, S7, S8, B2, B4, B6, B7, B8, B9, B10, B11, B12 | 25 | 25 | 1 | 1 | 5-10 |
| **Figure 3.7** | 35 – 50 % | S1, S2, S3, S4, S5, S6, S7, B1, B2, B3, B4, B5 | 20 | 25 | 2 | 2 | 10 |
| | | B6, B7, A1, A2, A3, A4, W | 20 | 20 | 2 | 2 | 5-10 |
| **Figure 3.9** | 35 – 50 % | S1, S2, S3, S7, B3, W1-6 | 20 | 25 | 1 | 2 | 5-10 |
| | | A1, A2, A3, A4, A5 | 20 | 20 | 1 | 1 | 5 |
| | | S4, S5, S6, B1, B2, B4, B5, B6,B7 | 20 | 30 | 1 | 2 | 10 |
| **Figure 3.12** | 35-50 % | All | 20 | 20 | 1 | 1 | 5-10 |
| **Figure 3.14** | 35-55 % | All | 25 | 25 | 1 | 1 | 2.5 -5 |

## Appendix 4: Media and solutions used in DGGE

The different medias and solutions used in the DGGE procedure.

**Table A.4.1:** Recipe for 50 x TAE-buffer.

| Reagent | Amount | Final concentration |
|---|---|---|
| Tris-HCl | 242 g | 2 M |
| Acetic acid glacial | 57.1 mL | 1 M |
| EDTA (0,5 M, pH 8,0) | 100 mL | 50 mM |
| $dH_2O$ | to 1000 mL | |

The buffer was sterilized by autoclaving.

**Table A.4.2:** Recipe for different denaturing gradients, by mixing 0 % and 80 % denaturing solution.

| Denaturing percentage | 0 % | 80 % | TEMED + 10% APS | Total volume (24 mL) |
|---|---|---|---|---|
| 35 | 13.5 mL | 10 mL | 16 µL + 87 µL | 24 |
| 50 | 9 mL | 15 mL | 16 µL + 87 µL | 24 |
| 55 | | | | |

**Table A.4.3:** Recipe for denaturing solution 0 %.

| Reagent | Amount |
|---|---|
| Acrylamide solution (40 %, BioRadLab Inc., Ca., USA) | 50 mL |
| TAE (50x) | 2.5 mL |
| $dH_2O$ | to 250 mL |

The solution was stored at 4 °C and protected from light.

**Table A.4.4:** Recipe for denaturing solution 80 %.

| Reagent | Amount |
|---|---|
| Acrylamide solution (40 %, BioRadLab Inc., Ca., USA) | 50 mL |
| TAE (50x) | 2.5 mL |
| Formamide (deionized with DOWEX RESIN AG 501X8, 3.5 g/100 mL formamide) | 80 mL |
| Urea | 84 g |
| $dH_2O$ | to 250 mL |

The solution was stored at 4 °C and protected from light.

**Table A.4.5:** Recipe for SYBR gold staining solution

| Reagent | Amount |
|---|---|
| SYBR Gold | 3 µL |
| TAE (50x) | 600 µL |
| $dH_2O$ | 30 mL |