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Effect of gastrointestinal microflora on the growth rate of Atlantic Cod (*Gadus Morhua*) larvae

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Marine Coastal Development
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Trinh Thi Truc Ly

Abstract

The gastrointestinal tract of fish larvae is inhabited by complex and diverse groups of microbes. Both internal and external factors affect the composition of the microbiota. Until now little information is available on the correlation between gut microbiota and the growth of fish larvae. This study was carried out to test whether the growth rate of cod larvae is partially explained by the composition of their intestinal microbial communities. In this experiment, the gut microbiota of small and large cod larvae sampled at 7, 10, 14, 17, 21, 24, 28, 31, 39 and 42 days post hatching were investigated using a PCR/DGGE (Denaturing Gradient Gel Electrophoresis) strategy. The results showed significant differences in the intestinal microbiota between small and large larvae on 40% of the ages stages studied. Therefore, the composition of gut microbiota does not generally seem to contribute to the growth rate of the larvae. We further found that the variation in gut microbiota of cod larvae was less impacted by their size than by age for larvae up to 28 dph, but for the older larvae size and age influenced the microbiota equally. Negative correlations between Bray-Curtis similarity in comparisons of two-and-two larvae versus the difference in age and size between the larvae were found in this study.

Abbreviations

ANOSIM: analysis of similarity

ANOVA: analysis of variances

APS: ammonium persulphate

DGGE: denaturing gradient gel electrophoresis

DNA: deoxyribonucleic acid

DPH: day post hatching

DW: dry weight

H': Shannon diversity index

J': evenness index

L: large larvae

MH: myotome height

MS222: tricaine methane sulphonate

NMDS: Non-metric multidimensional scaling

PCR: polymerase chain reaction

RDP: ribosomal database project

S: band richness

S: small larvae

SD: Standard deviation

SL: standard length

TAE: tris base, acetic acid and EDTA

TEMED: tetramethylethylenediamine

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1. Introduction

The aquaculture industry has grown considerably and become the biggest protein producer in Norway. Today a lot of effort has been put on establishing cod and other marine fish as new commercial species for aquaculture (FAO, 2004-2012) in order to make the Norwegian aquaculture industry more robust. In this area, bottlenecks exist at all their life stages, however for most of marine aquaculture species the major bottleneck is production of high quality juveniles, manifested as low growth rates, high mortality, malformation and low reproducibility during larval stages (Vadstein 1993; Ringo 1999; Bengtson 2007). Different factors have been proposed to cause the observed symptoms such as temperature, water quality, stocking density, salinity, oxygen and nutrition/food during larval rearing (Sun 2009). Currently, an increasing number of studies indicate that the intestinal microbial communities in fish larvae can be considered a major cause of the observed problems (Vadstein 2004; Sun 2009; Nayak 2010).

The gastrointestinal tract of animals, including fish, provides a habitat for a complex and diverse ecosystem of both aerobic and anaerobic microorganisms, and has been investigated by many scientists during the last decade (Burr 2005). There is evidence that fish possess a large number of bacterial populations in their digestive tract that typically come from surrounding water, sediment and feed. The intestinal microbial communities in fish do not only play an important role in the nutrition of fish, but also influences the establishment of pathogenic microorganisms in the fish intestine and have effects in preventing diseases (Denev 2009). However, until now the contribution of individual gastrointestinal microbes in the growth of cod larvae is poorly understood. Therefore, the investigations of the intestinal microbiota are important for the cod juvenile production or finfish aquaculture.

1.1. Atlantic cod (*Gadus Morhua*)

Atlantic Cod (*Gadus Morhua*) is an important marine fish species which belongs to the family of Gadidae. They can be found along the eastern and northern coasts of North America, along the coast of Greenland, and also along the coasts of Europe. These fish are living in temperate climatic zones with a range in temperature from 0-20^{o}C, depending on the time of year, location and size of fish (Campbell, 2005).

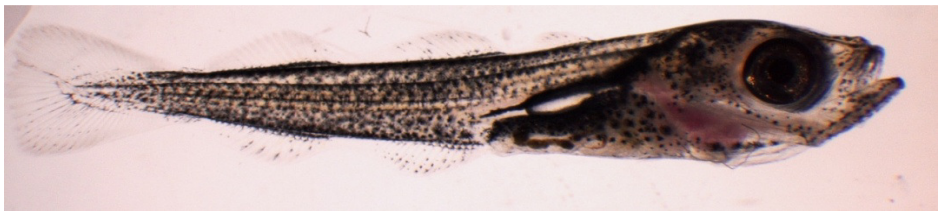

Figure 1.1: Cod larvae after 42 day post hatch (Sealab, 2013)

Atlantic cod pass through a series of four main life stages during their development. They start their life as pelagic eggs, which are buoyant, stay close to the surface water, and drift with the current. Cod eggs are hatched after 8 to 60 days depending on the temperature (Hardy 1978). In the second step in the development, the larval stage, the fish is most vulnerable. This stage has significant impacts on the quality of juveniles (Fahay, et al., 1999). There are three factors that have important influences in the probability of viable larvae including larval itself, physiochemical environment and microbial community in the environments (Vadstein 2004). Transformation to the juvenile stage occurs when the size of larvae is greater than 20 mm, when fin rays are formed completely (Fahay 1983). In natural environments, juveniles occupy in the coastal and offshore waters in the summer and deeper waters in the winter. The final stage is adult fish which tend to move in school and primarily inhabit the bottom of water column (Fahay et al., 1999). The reproduction of these fish takes place in warmer waters during the winter and early spring near the bottom of sea through external fertilization (Campbell, 2005).

Atlantic cod is one of the most important commercial species in North Atlantic. However, in the early 1990's, the wild stocks of cod have collapsed due to overfishing of older cod resulting in smaller population of fertile females, and also due to the harvesting of young fish before they had had a chance to mature and reproduce (Campbell, 2005).

Together with the declining of wild stocks, considerable efforts have been placed into developing the farming of Atlantic cod, which was demonstrated in Figure 1.2. This figure shows the global aquaculture production of cod from 1950 until 2010. However, in Norway, the commercial aquaculture production of cod fish has decreased dramatically since 2008. Presently, the aquaculture industry of cod fish in Norway is facing numerous challenges. The reasons were economic issues due to the financial crisis, problems with profitability due to expensive and complicated production. The aquaculture of cod furthermore appears to suffer the problem with lacking of stable production of high quality larvae and juveniles (FAO, 2004-2012).

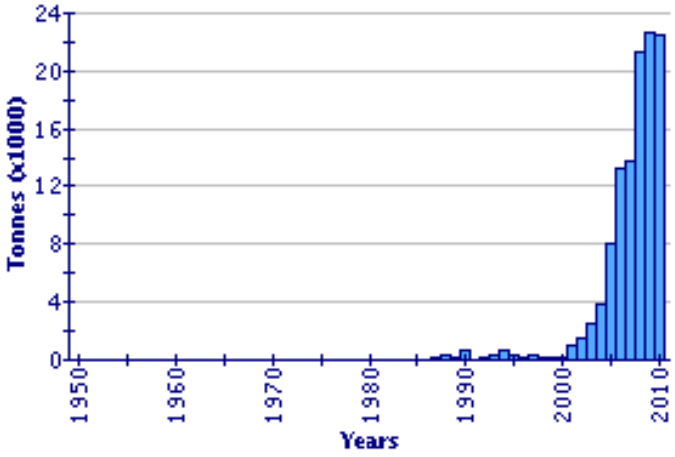

Figure 1.2: Global aquaculture production of Gadus morhua (FAO Fishery Statistic)

1.2. Establishment of intestinal bacteria in marine fish larvae

For terrestrial animals, the gastrointestinal microbiota from parents is probably transferred to offspring during and immediately after birth (Mändar and Mikelsaar 1996). In contrast to terrestrial animals, aquatic animals, including fish, spend their life in aqueous environment, so they are continuously exposed to a high number of bacteria present in water, including both pathogenic and opportunistic bacteria. Therefore, these bacteria will surely affect to the microflora on the external areas of fish (Hansen and Olafsen 1999; Austin 2002). Besides, digestive tract of fish will receive a large number of microorganisms from their food and aqueous habitat, so the establishment of gut microflora seems to be influenced by the numbers and ranges of microorganisms in different sources, including the eggs, live feed, water environment (Austin 2002). Thus, the fish gastrointestinal tract possesses a diverse and complex range of different microorganisms (Cahill 1990).The physiological complexity of gastrointestinal tract of marine fish increase during their development (Hansen and Olafsen 1999). Ringo (1999) reported that the development of larval intestine could enhance bacterial attachment site or niches, leading to the increase in the complexity of the bacterial community

Highly diverse groups of bacteria have been found on the surface of marine fish eggs few hours after fertilization, leading to the formation of the epiflora on eggs. Both non-pathogenic and pathogenic bacteria can be found on the surface of fish eggs in both culture and natural conditions. The microbiota which inhabits on eggs appears to reflect the microbial composition of ambient water, which may damage developing eggs or their hatching (Hansen and Olafsen 1999; Olafsen 2001). However, it is still unknown whether a natural epiflora may provide any protection against the domination by potentially harmful bacteria.

According to Hansen et al. (1999) and Olafsen (2001), fish larvae start “drinking” before the yolk-sac is consumed and microorganisms go into the gastrointestinal tract of fish larvae immediately at the time of hatching before first active feeding. It is therefore evident that the bacteria is colonized and established in fish gut very soon after birth as a primary transient intestinal microflora which develops in to persistent flora at juvenile stages or after metamorphosis (Hansen and Olafsen 1999; Balcázar, Blas et al. 2006). This is due to the fact that some larvae need to consume seawater to osmoregulate, and then bacteria can be ingested into gastrointestinal tract along with the water. Once active feeding starts, the microbial compositions in the gastrointestinal tract may be affected by ambient water microbiota and feed microbiota (Hansen and Olafsen 1999; Birkbeck 2002).At larval and juvenile stages the fish rely on their innate immune system or non-specific defense mechanism to protect themselves against pathogens. (Hansen and Olafsen 1999).

Nayak (2010) reported that there are several factors contributing to the establishment of microbial community in the gastrointestinal tract of fish, including water, sediment, feed, and rearing conditions. The author also supposed that feed had great impact on the intestinal microbiota of individual larvae. However, Bakke et al. (2013) demonstrated the diet seems not to entail major changes the gastrointestinal microbes of cod larvae. Birkbeck and Verner-Jeffrey (2002) reported that a stable microbiology was observed in juvenile fish due to the completion of the gastrointestinal tract development and the formation of microenvironments in the gastrointestinal tract. It seems to be generally accepted that the bacterial composition changes with host age, nutritional status and environmental conditions (Olafsen 2001; Eddy and Jones 2002). Early exposure to high bacterial densities may important for immune tolerance. Kanther (2010) demonstrated that gut microbiota has a significant contribution in the development of the immune system. In the absence of the gut microflora, the development of normal intestinal morphology, normal immune system maturation and function are impaired.

1.3. Roles of gastrointestinal microbiota in fish

The roles of gut microbiota have been studied in various fish species of teleosts. Complex intestinal microbial communities are believed to provide some benefits to their host. The gut microflora has been suggested to play the crucial roles in the nutrition, in immunity and in health management of fish host. The colonization of potentially opportunistic bacteria that cause serious diseases in marine aquaculture can be restrained by the formation of a stable indigenous microflora or harmless bacteria in the rearing environment. In other words, the larvae are

protected from the infection of other potentially pathogenic strains, including obligate and opportunistic bacteria. It is known that the presence of non-opportunistic strains which are capable of producing antimicrobial compounds such as bacteritoxin, organic acids… or actively competing available energy and adhesion sites may limit the growth of opportunistic bacteria (Hansen and Olafsen 1999; Verschuere 2000).

The nutritional importance of fish gastrointestinal microbiota has been established by their ability in producing some beneficial bioactive substances such as vitamins, amino acids, digestive enzymes and metanolites (Nayak 2010). In zebrafish the intestinal microbiota has been shown to stimulate uptake of fatty acids and lipid droplet formation in the intestinal epithelium. Moreover, the different members of the intestinal microbiota were found to promote fatty acid absorption via distinct mechanisms (Semova, Carten et al. 2012). Various studies reported that some gastrointestinal bacteria have the ability to synthesize biotin which may contribute by enhancing the growth of catfish (*Ictalurus punctatus*) (Robinson and Lovell 1978; Lovell and Buston 1984). Moreover, Ringo et al. (Ringø, Jøstensen et al. 1992a; Ringø, Sinclair et al. 1992b) have been demonstrated the intestinal microbial components of turbot larvae have the ability to produce eicosapentaenoic acid (EPA). EPA, together with DHA, is an important fatty acid in larval fish nutritions, leading to the increase of larval survival rate, so it has significant contribution to the development of aquaculture (Sargent 1999).

The gastrointestinal tract serves as a major habitat of diverse population of non-pathogenic, bacteria which can contribute significantly to the overall health in a host by acting as a protective barrier against pathogens (Nayak 2010). It was proved that the microflora of digestive tract of the fish and shellfish plays an important role in the formation of resistance to infectious diseases, which can produce some types of antibacterial agents preventing pathogenic bacteria from getting into organisms. Sugita et al. (1996) examined the antibacterial activity of some bacterial strains from seven fresh cultured fish (such as common carp, goldfish, and rainbow trout). They found that approximately 3.2 % of tested intestinal strains exhibited antimicrobial activity against 18 different bacterial strains, including *Aeromonas* species and pathogenic bacteria. The authors suggested that the intestinal bacteria with antimicrobial ability may inhibit the growth of pathogenic bacteria in the intestine of fish. Furthermore, some bacteria strains were isolated from the intestine of adult marine flatfish, turbot and dab have the capacity to suppress the establishment of the fish pathogen *Vibrio Anguillarum* (Olsson, Westerdahl et al. 1992). These intestinal microbes can hinder the invasion or colonization of pathogens by competing available nutrients and secreting a range of antimicrobial substances.

Furthermore, gastrointestinal microbiota has a significant contribution to the development, functions and maturation of fish gut-associated immune system (Fraune 2010; Kanther 2010; Nayak 2010). The serious problems with the immunity system of germ-free mice, including higher risk of getting infections, fewer lymphocytes; reduce digestive enzyme activity, serum immunoglobulin levels and CD4 T cells, compared to conventionally colonized mice were recorded by Fraune (2010). It has been also reported that bacteria could stimulate B cell proliferation thanks to a classical antigen-specific immune response like protein A of Stapphylococcus aureus and protein L of Peptostreptococcus magnus (Silverman and Goodyear 2002). The contribution of intestinal microflora through enhancing fish immune responses was stated by Ringo and Birkbeck (1999). Additionally, Kanther (2010) suggested that the immune system of zebrafish can be influenced by some microbial cells and their products, which is revealed by altering the proliferation, differentiation and maintenance of hematopoietic immune cells. Turnbaugh and his colleagues (2006) demonstrated that in human and mice, gut microbiota of obese or overweight individuals contributed to the increase of their capacity to harvest more energy from the diet. The authors concluded that the gut microbiota can be considered as one of the factors that contribute to mice obesity. Bajzer and Seeley (2006) explored the differences in intestinal microbial community between obese and lean human, and found that increased body weight in obese individual was due to a higher efficiency in caloric extraction from food. Although intestinal microbiota of fish has been studied extensively, still very little information is known on the correlation between gut microbiota and host fish. Sjulstad (2011) showed that small and large larvae of similar age of both mangrove killifish and Atlantic cod differed with respect to their GI microbiota. For cod we have indications that the microbiota is involved in regulation of genes involved in energy metabolism (Forberg et al. 2011).

1.4. Fish-microbe interactions

Marine species spends all their lifetime in the aquatic environment, both in natural seawater and in aquaculture, which leads to their close contact with high concentrations of varied ambient microflora, including opportunistic and pathogenic bacteria. These bacteria move easily between habitats and fish, thus they can interact with each other in a number of ways when they have colonized the gastrointestinal tract of fish. The interactions taking place between host and microbes can be categorized in three different terms: commensalism, symbiosis and parasitism. Symbiosis refers to a relationship between two different organisms where at least one of the two receives benefits without harming the others, while commensalism is a co-existent of two or

more species without either detriment or obvious benefits. Therewith, pathogenic relationship is a result of host damaging (Hooper and Gordon 2001). However, only a small fractions of microbes are known to be harmful to the fish, the majority of interactions between host-microbes are non-pathogenic (Vadstein 2004).

The mucus layers on the surface of gills, skin and gastrointestinal tract can serve as adhesion sites for bacteria (Cahill 1990). The bacteria attached on these layers can be categorized into two major groups: indigenous bacteria can grow and multiply to become established or may bring the infection to host while the growths of transient strains are limited, and do not persist for a long time on the surface or expelled. Generally, the complex relationship between fish and gut microbes was mentioned by Sullam (2012).

Many studies have reported that there are several factors, including both internal and external, contributing to the intestinal microbiology of fish in the bacterial number and composition (Figure 1.3) (Vadstein 2013). This figure shows the sources of bacteria in a rearing tank for cultivation of marine fish larvae. Microbes can be transferred and interacted with the larvae through the live feed cultures (including microalgae) and intake water. The bacteria in the water may colonize the outer surface of the fish while the intestine of the fish is inhabited by varied bacteria coming from both water and feed. It has been shown that the effect of feed on gut microbiota of fish depends on the biochemical composition of the feed, and the fish should be fed for some period to alter as well as establish new microbial communities (Ringø 1995). Furthermore, the raising of microbes inside rearing environment is also associated with organic materials released by the defecation processes of both fish and live feed as internal factors.

In addition, abiotic factors such as rearing water temperature and stress (such as the use of chemicals, antibiotics to manage pathogens) could also influence to the total number of bacteria observed in the larval intestinal tract. It has been demonstrated that low temperature resulted in low numbers of bacteria in the GI tract of turbot larvae (Munro, Barbour et al. 1994) compared to the numbers of bacteria found at a higher temperature. In aquaculture, high stocking density and suboptimal rearing condition could be the reasons for the increase of bacterial load. As the intestinal tract is a main entrance for microorganisms, microbial disease causes high losses during larval and juvenile production of marine species due to the colonization of opportunistic bacteria in gut.

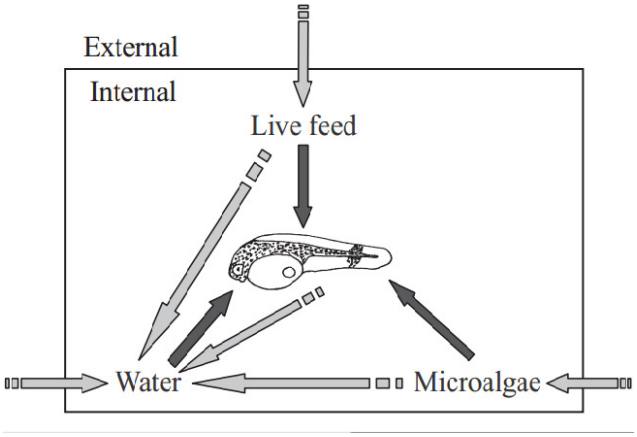

Figure 1.3: Important microbial sources interacting with mucosal surfaces of larval fish. Various external sources of microbes (blue⁄ grey arrows) such as water, live feed and microalgae enter the rearing environment and interact with the fish (red⁄ black arrows). Internally, the rearing environment is enriched by microbes due to defecation by fish or live feed, or indirectly through growth based on organic matter released by defecation by animals or exudation by microalgae. From Vadstein (2013)

1.5. Techniques to study microbial diversity

The ability to quantify the composition and diversity of microbial community is fundamental to the understanding of the structure and function of an ecosystem. In the past, most studies have used conventional techniques to investigate the intestinal microbiota, which is based on phenotypic characteristics and the culturable properties of bacteria on agar media under different conditions. However, considering that many bacteria are morphologically and biochemically similar and only a minor percentage of microflora can be cultured in a nutrient media which are generally very selective, probably only a small number of gut bacteria have been recognized (Amann, Ludwig et al. 1995; Nayak 2010; Su, Lei et al. 2012). Therefore, these methods are laborious, time-consuming and not reliable for distinguishing species or strains.

Due to the development of new molecular techniques, it is now possible to study the structure of microbial communities without culturing the microbes first. Examples of such molecular methods include FISH (fluorescence in situ hybridization), Single Strand Conformation Polymorphism (SSCP), Terminal Restriction Fragment length Polymorphism (T-RFLP), Ribosomal Intergenic Spacer Analysis (RISA), Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Fakruddin 2013). These culture independent approaches have been used widely to characterizing microbial

communities in different environments in the past 20 years (Su, Lei et al. 2012). However, they still have some limitations as summarized in the Table 1.1.

Table 1.1: Disadvantages of some culture – independent methods to investigate the microbial diversity (Fakruddin 2013)

Methods	**Disadvantages**
DNA microarray DNA hybridization	Only detect most abundant species and only accurate in low diversity system
Single Strand Conformation Polymorphism (SSCP)	PCR bias. Some single stranded DNA can form more than one stable conformation
Terminal Restriction Fragment length Polymorphism (T-RFLP)	Dependent on extraction and lysing efficiency PCR biases Choice of restriction enzymes will influence community fingerprint
Ribosomal Intergenic Spacer Analysis (RISA) / Automated Ribosomal Intergenic Spacer Analysis (ARISA)/ Amplified Ribosomal DNA Restriction Analysis (ARDRA)	Requires large quantities of DNA (for RISA) PCR biases
Denaturing and Temperature Gradient Gel Electrophoresis (DGGE and TGGE)	PCR bias Ways of handling can influence the bacterial community One band can represent more than one species (co-migration) Only detect dominant species

A disadvantage of all these methods is that they are not very good for providing taxonomic information. The new high-throughput and cost effective sequencing technique called “barcoded amplicon pyrosequencing” has been developed for characterizing the microbial communities.

Generally, for this method specific primers will target adjacent conserved regions on the 16S rDNA gene to amplify a highly variable region of the 16S rDNA gene, and then gives the possibility to direct sequencing of individual PCR products (e.g. amplified 16S rDNA amplicons) without any separation on gel first (Andersson 2008).

Denaturing Gradient Gel Electrophoresis is an efficient, simple and relatively cheap technique to study dynamics of microbial communities (Muyzer, de Waal et al. 1993; Nayak 2010), which was applied in this study. The theoretical aspects of DGGE were first described by Fischer and Lerman (Fischer and Lerman 1983) and have been used to study the microbiota of a number of fish species, such as Atlantic halibut larvae (Jensen, Øvreås et al. 2004) and early stages of salmon (Romero and Navarrete 2006).

The general principle of DGGE is based on electrophoretic separation of PCR-amplified 16S rDNA gene by using polyacryalmide gels containing a linearly increasing gradient of denaturants. In DGGE, DNA fragments which have the same length but different in nucleotide sequences can be separated on the basis of different electrophoretic mobility. DGGE utilize the properties of DNA as negatively charged molecule and its denaturing characteristics. In the gel DNA fragments have the ability to move on the polyacryalmide gel according to GC content (the most stable DNA migrating further). The DNA fragments move through the gel until it reaches a particular position in the gel with sufficient denaturants, it will practically stop migrating and become denatured. In order to obtain a complete stop of DNA migration, it is important that a transition of a helical DNA to a partially melted molecule must be occurred. Therefore, GC rich sequence or GC clamp with higher bond strength than the rest of DNA fragment is attached to one of the primer to prevent the two DNA strands from complete separation into single strands. This results in the distinct banding patterns in the gel that represent the level of diversity of the sample. Bands at the same position in the gel have the same melting behavior, but not necessarily the same sequence (Muyzer, de Waal et al. 1993). Therefore the band pattern will not be an exact reflection of the microbial diversity of the sample used as template. DGGE profiles can be analyzed and compared by measuring the number and the thickness of bands on the gel.

An increasing of many power techniques are available for the study of microbial ecology and evolution with 16S rRNA gene, which is used as the most commonly molecular marker for bacterial diversity because it is relatively small size (~1500 bp), contains highly conserved regions and is present in all prokaryotes (Tringer 2008). It contains variable regions that make it possible to distinguish between different species and strains, but still enough similarity to identify members belong to the same larger phylogenetic group. There are still some other

molecular markers which are single copy genes such as RNA polymerase beta subunit (rpoB), recombinase A (recA), and heat shock protein (hsp60) which have also been used as conserved genes to differentiate bacterial species (Ghebremedhin, Layer et al. 2008). However, the number of copies of RNA gene in bacterial genomes varies among species. Therefore the amount of PCR product does not necessarily correspond to the abundance of the species.

1.6. Hypothesis and objective of the study

The hypothesis of this thesis is that the growth rate of cod larvae is affected by GI microbiota composition, more specifically:

1. The composition of the microbiota is different for slow and fast growing individual of developing larvae.
2. The microbiota of a larva affects to the growth rate of the host. Some larvae are big because they have dominance of special bacterial species/genera in the microbiota of their intestinal tract

The main goal of the thesis is to investigate potential correlations between the composition of the microbiota and the growth rate of the larvae. This will be achieved by comparing microbial communities in the intestinal tract of slow and fast growing cod larvae, i.e. small and large larvae of the same age. The community composition of the GI microbiota will be characterized by a PCR/DGGE strategy. Identification of intestinal microorganisms will be obtained by DNA sequencing of DGGE bands.

2. Materials and methods

2.1. Experimental design

In the current study, newly hatched yolk-sac larvae were stocked and fed under the conditions set by the established protocol (Table 2.1). A general overview of experimental process was described in the Figure 2.1. Sample of 5 smalll and 5 large individual larvae in each day was taken 10 times during culturing period: at day 7, 10, 14, 17, 21, 24, 28, 31, 39 and 42 after hatching to achieve sufficiently data on differences in GI microbial communities between small and large larvae. Each larva was photographed by using a dissecting microscope to measure their standard length, then they were stored at -20°C until measuring of dry weight and microbial analyses (DNA extraction, PCR-DGGE fingerprinting, and sequencing to identify the bacterial communities in those samples) were conducted. The sampling design was set up to allow statistical comparisons of the microbial communities of equally old but differently sized larvae.

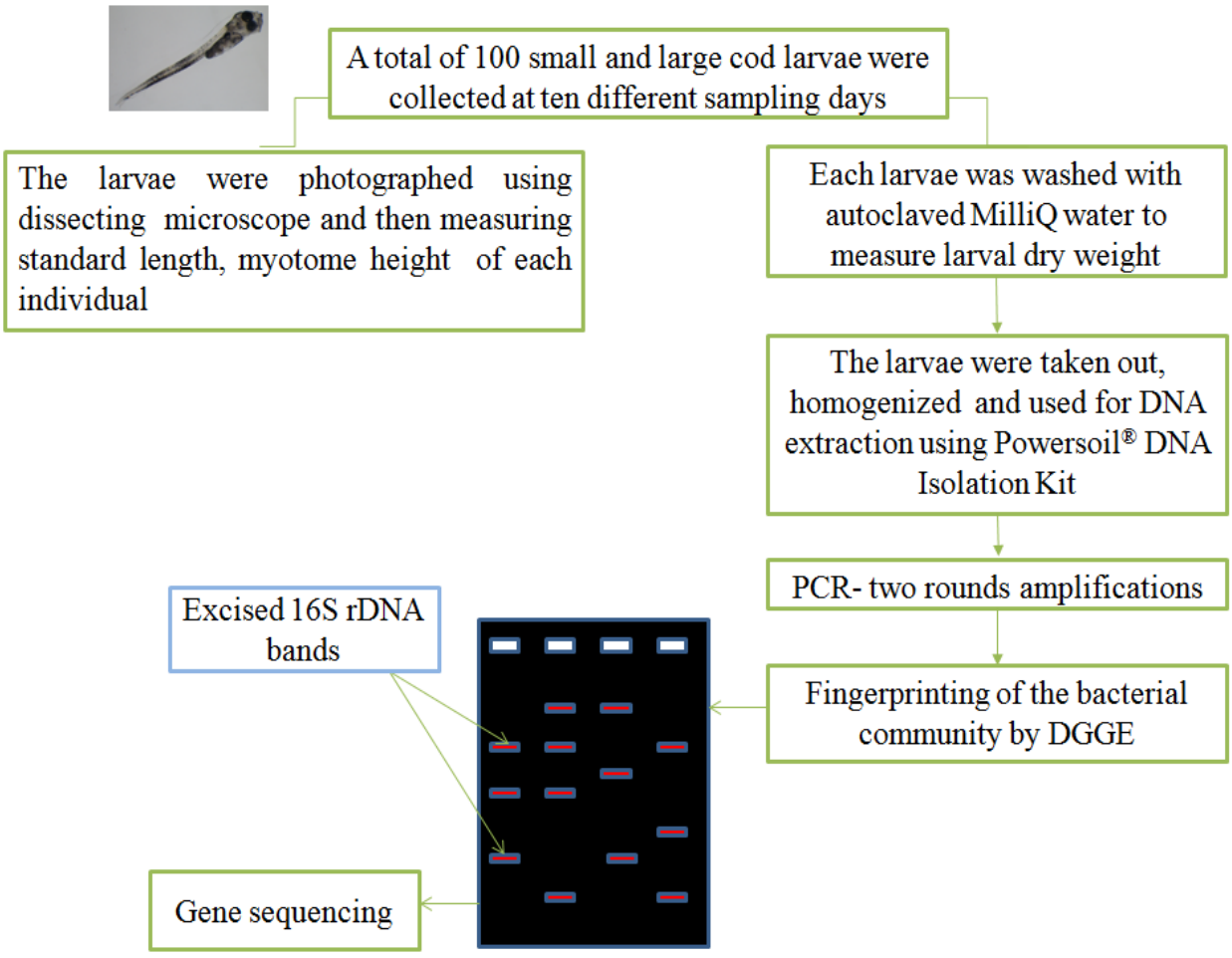

Figure 2.1: The flow scheme of different steps in the analysis of bacterial community

2.2. First feeding experiment and sampling

Cod eggs and sampling used in this experiment came from the brood stock at the Cod Breeding Centre in Tromsø. The eggs were incubated in conical tanks containing seawater maintained at 5 °C until transfer to 150L rearing tanks at an initial density with 100 larvae L^{-1} at 70% hatch on the 9th of April 2013, and were defined as 0 dph. The larvae were reared six weeks post hatch at Sealab and fed automatically by robot. The rearing conditions in the experiment such as temperature, light, aeration, water exchange, feed and also sampling schedule were represented in the Table 2.1. The temperature was 7°C at the first 2 dph, and then slowly increased to reach 12°C at 11 dph. The temperature remained unaltered the rest of the experiment. Cod larvae aged 3 to 24 were fed rotifer. From day 24 to day 27, rotifer quantities used to feed the larvae began to decrease, then being replaced by *Artemia.* The water exchange was gradually increased from 2 to 8 times per days during the experiment.

Table 2.1: The rearing conditions and sampling schedule of the 42 days of experiment

dph	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Temp (°c)	7	7	8			9			10	10	11	11	12										
Light	D			L																			
Aeration	Weak				Increased aeration																		
Water exch	2 per day							4 per day											6 per day				
Rotifer feed (meals d^{-1})				2	4														6				
Artemia feed (meals d^{-1})																							
Sampling day								x			x				x			x				x	

dph	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Temp (°c)	12																			
Light	L																			
Aeration																				
Water exch	8													10						
Rotifer feed (Meals d^{-1})	6	5	3	2	1															
Artemia feed (meals d^{-1})		1	3	4	5	6	6	6	6	6	6	5	4	3	2	1				
Sampling day		x				x			x								x			x

From the rearing tank, a plastic tube was placed at middle depth in the center of the tank, and then samples of 5 biggest and 5 smallest larvae were chosen from more than 100 individual/one sampling at days 7, 10, 14, 17, 21, 24, 28, 31, 39 and 42 after hatching and the larvae were transferred to beakers containing seawater collected from rearing tank to ensure stable

environment for larvae. Sampling was carried out in the middle of two feeding times. The rest of larvae were euthanized, and discharged.

All larvae were photographed individually by dissecting microscope and preserved at -20°C until analyses. Standard length and myotome height were measured from the photos, and the larvae were used for dry weight, DNA extraction, PCR/DGGE fingerprinting.

2.3. Analytical methods

2.3.1. Measurement of length, body height and weight of cod larvae

Larvae from each sampling day were kept alive in cool seawater (by placing beaker containing larvae in ice). Each larva was anaesthetized by using 1 drop of MS 222 to inhibit their moving during taking its photograph under a digitally dissecting microscope for later measurement of its standard length and myotome height.

Thereafter, each larvae was washed with sterile fresh water three times to remove salts before transferring individually to new Eppendorf tubes, and then measured their dry weight after freeze drying for 24 hours and microbalance

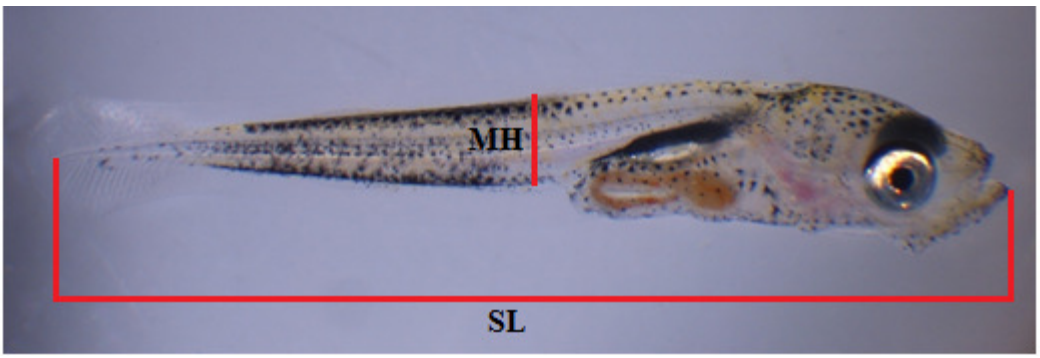

Figure 2.2: Cod larvae at 28dph taken at Sealab. SL: standard length; MH: myotome height

2.3.2. DNA extraction

In the laboratory, DNA was extracted from larvae samples by using PowerSoil® DNA Isolation Kit (see the appendix 1 for detail protocol). Prior to DNA extraction, the larvae were homogenized using a glass rod. An aliquot of 2 µl DNA extract was analyzed by using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) in order to determine the quality and quantity of extracted DNA. If necessary, extracted DNA were diluted to a standard 20-25ng/µl concentration to ensure that the similar amount of DNA was used as the templates in each PCR reaction later. The details are given in Appendix 1.

2.3.3. PCR of 16S rDNA

As described above, total eukaryotic and intestinal bacterial genomic DNA were extracted from the larvae using Powersoil® DNA isolation kit. The DNA preparations were used as templates for amplification of the V3 variable region of the bacterial 16S rDNA gene by using nested PCR reactions with two pairs of universal primers; the external primer pair EUB8F/984yR, and the internal primer pair 338F-GC/518R. The GC clamp, which is a sequence that is rich in Guanine and Cytosine, was attached to the 5'end of the forward internal primer in order to prevent DNA from being completely denatured into two single strands in the DGGE.

The two rounds of amplifications here were used to avoid the co-amplification of eukaryotic DNA (Bakke, De Schryver et al. 2011) .The expected product size for the PCR amplicon was approximately 220 bp. We included non-template controls in all PCR runs.

Table 2.2: Universal primers used for amplification of v3 16S rDNA region

External PCR primers	
EUB 8F	5'- AGA GTT TGA TCM TGG CTC AG -3'
984yR	5'- GTA AGG TTC YTC CGC GT -3'
Internal PCR primers	
338F-GC	5'- cgcccgccgcgcgcggcgggcggggcgggggcacgggggg ACT CCT ACG GGA GGC AGC AG -3'
518R	5'- ATT ACC GCG GCT GCT GG -3'

External PCR:

Each PCR mixture (25 µl) in the first round of amplification contained 1 x reaction buffers, 200 µM each dNTP (Qiagen), 2 mM MgCl2, 0.3 mM of forward and reverse primers, Taq polymerase (QIAGEN) and approximately 20 ng genomic DNA. PCR was performed with an initial denaturation step of 95°C for 3 min, then followed by 20-22 cycles of 95°C for 30s, 50°C for 30s, 72°C for 1 min and the cycles were terminated with the final elongation step of 72°C for 10 minutes.

Internal PCR:

Products from external PCR reactions were used as the template in subsequent reaction with internal primers 338F-GC/518R to obtain a final product suitable for DGGE analysis by using the following cycling parameters: 95°C for 3 min, followed by 20-22 cycles of 95°C for 30s,

53°C for 30s and 72°C for 1min. The reaction was terminated by a final extension step of 72°C for 20 min.

Agarose gel 1% was made (4 grams of agarose in 400 ml of 1x TAE buffer containing 20 µl GelRed (QIAGEN) then used to check PCR products and contaminations. A 150 ml gel was cast into a Thermal Scientific gel caster. The mixture of PCR products (5µl) and 1µl of 6x loading buffer were loaded to the wells. Gels were run for approximately 45 minutes at 140 volts. The DNA bands were visualized and photographed under the UV light. PCR products were accepted when negative control showed no bands on the gel (no amplification).

2.3.4. DGGE

DGGE technique was used to evaluate the microbial diversity in the samples. PCR amplicons were analyzed by DGGE using INGENY phorU system (Ingeny, Netherlands) according to manufacturer's instructions.

The PCR products were analyzed on 8% polyacryalmide gel containing a 35% to 55 % linear gradient of denaturants (Urea and formamide) increasing in the direction of electrophoresis. PCR samples (5-15 µl) mixed with 2-5 µl of loading buffer were loaded in the DGGE wells. After loading all samples, the system was pre-run (100V for 5 min without TAE buffer recirculation) to concentrate the DNA on the bottom of the wells. Finally, electrophoresis was performed in 0.5x TAE (Tris–Acetate–EDTA) running buffer at 60^{o}C for 17 hour at 100 volts.

Detail steps for pouring of gel:

The two glass plates, the spacer and comb were washed well with Deconex soap, and then rinsed with water to remove any traces of soap. Further, they were polished with 95% ethanol and dried by using Kimwipe paper. The plates were then assembled with the spacer and comb in gel cassette.

Acryalmide solutions with the desired denaturating percentages (35% and 55%) were prepared by the mixing of 0%, 80% denaturating acryalmide solution (Appendix 2). The 80% acryalmide solution was filtrated upon addition to remove urea particles. TEMED (Tetramethylethylene-dialmide) and 10% APS (ammonium persulphate) was added the gel solutions to start polymerization. Stacking gel solution with 0% denaturing acrylamide was used for the top part of the gel. Finally the comb was mounted on the top of the gel. The amount of each solution was prepared according to the table 2.2. The gel was allowed to polymerize for at least 2 hours at room temperature.

Table 2.3: Composition of low and high denaturating solutions

Denaturating %	0%	80%	TEMED + 10% APS	Total volume
0%	8ml	0ml	10µl + 40µl	
35%	13,5ml	10,5ml	16µl + 87µl	24ml
55%	7,5ml	16,5ml	16µl + 87µl	24ml

Polymerized DGGE gels were then stained with SYBR® Gold (Invitrogen, Belgium) diluted in 50x TAE for 1 hour at room temperature and then examined under UV transilluminator.

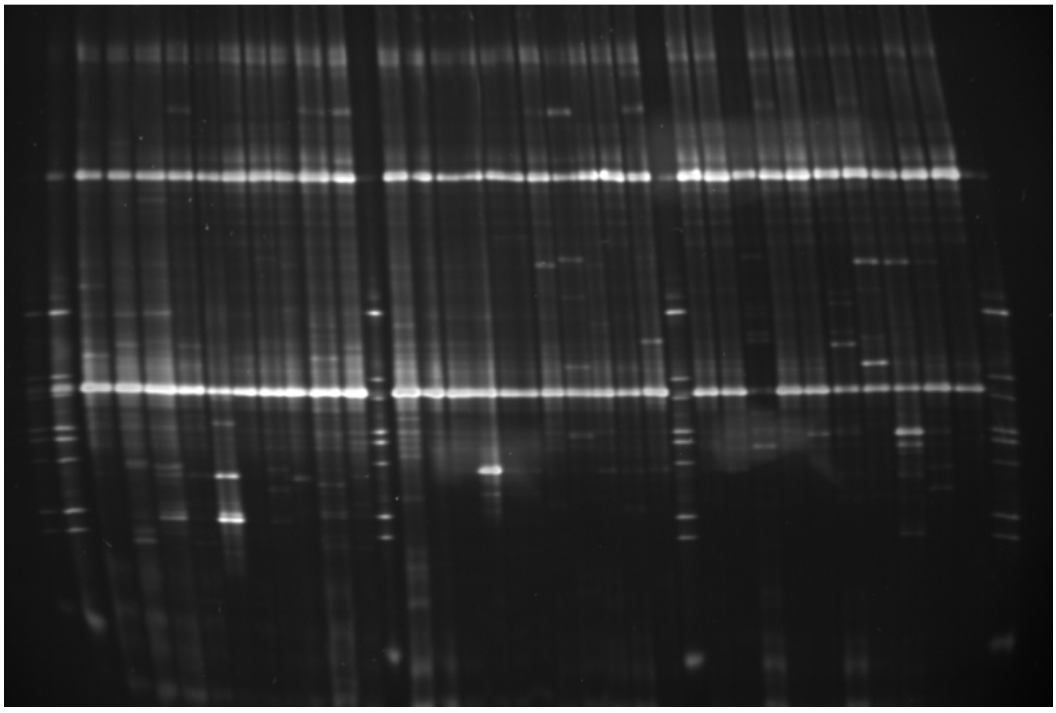

Figure 2.3: Photographed DGGE gel after staining with SYBR® Gold

2.3.5. Reamplification of DGGE bands for DNA sequencing

Samples of individual DGGE bands (dominating bands) were then excised with sterile 1000-µl pipette tips. The excised bands were added in to sterile 1.5ml Eppendorf tubes with 20 µl of nuclease-free water for elution of the DNA from the gel and then stored at 4^{o}C overnight for DNA diffusion into the water. Subsequently, these samples were reamplified by using PCR with the primer pair 338F-GC-M13R/518R, where the 338F-GC-M13R primer is identical to the 338F-GC primer except the inclusion of the sequence of the standard M13 primer (actcctacgggaggcagcag) in the 5' end. Reamplified PCR products of excised bands were purified with the QIAquick PCR Purification kit (Qiagen). The detail steps of reamplification and

purification were given in Appendix 3. DNA sequencing was performed using the M13 sequencing primer by GATC Biotech.

2.3.6. Gel image and statistical analysis

All the bacterial community samples were run on DGGE gels which were then analyzed with image analysis software (Gel2k, v.1.2.0.6; Nordland, 2004, http://folk.uib.no/nimsn/gel2k/). This program facilitates the analysis of gel images by estimating the relatively fractional positions of the peaks along the currently selected lane, and then the recorded in histogram window so that peaks that belong the same lanes in real gel images were grouped together. The peak area data were imported to excel sheet from Gel2k and used for statistical analysis. The relative proportion of species in each sample can be inferred from the relative band intensities which were normalized to percent of the sum of all peak areas within a sample, and then we transformed the percent peak value by square root transformation to reduce the impacts of very strong bands. The transformed percent data of the peaks were used for the determination of diversity indices (band richness (S), Shannon diversity index (H) and evenness index (J)), and also for the construction of non-metric multidimensional ordinations in order to compare the similarity of microbial communities of large and small larvae of different age. The band richness is an indicator of the number of possible species presented in each sample. The evenness index represents how evenly the dominance of each species is distributed in a bacterial community of the sample, which means that the evenness is high when the species are equally abundant. Further, The Shannon diversity index reveals both the abundance and the dominance of species. A large Shannon index indicates high number of species in a given samples and low distribution of each individual across the species present.

2.3.6.1. Characterization of samples: Diversity indices

The variables used as a mathematical measures of species diversity such as species richness, Shannon's diversity index and Pielou's eveness index within a microbial population in a sample were calculated as follows:

Species richness (S) is the total number of species in the community. In this study, they will be the number of bands present in the community.

Shannon's diversity index (H) is another index that accounts for both abundance (numbers of individuals as well as number of taxa) and evenness of species present in the community.

$$H = -\sum_i \frac{n_i}{n} \ln \frac{n_i}{n}$$

Where:

$n_{i:}$ The number of individuals of taxon i or the abundance of taxon i

n: The total number of all individuals

Pielou's eveness index is calculated by dividing the Shannon diversity by the logarithm of number of taxa, which is a measure of the maximum Shannon diversity for a given richness

J'= H/ln(S)

A one-way ANOVA and Turkey multiple comparison in PAST were used to determine whether there are any significant differences in diversity indices between samples.

2.3.6.2. Sample comparisons

Abundance data were analyzed using non-metric multidimensional scaling (NMDS) and statistical comparisons were made using an analysis of similarity (ANOSIM).

Non-metric multidimensional scaling (NMDS) with Bray-Curtis similarity is a distance-based ordination method that was applied for visualization of the similarity of bacterial communities in different samples. This method requires the production of Bray-Curtis similarity matrix which is used to construct the plots. NMDS algorithms aim to place all objects in a coordinate system with 2 or 3 -dimensional space so that similar subjects are closer to each other and dissimilar objects are farther from each other.

NMDS rectifies a linear correlation by maximizing the rank-order correlation between distances in the data and distance in ordination space. The stress value that represents a goodness of fit estimate between ordination and real rank is calculated. The points in the ordination are moved slightly in a direction to minimize the stress. Generally, stress value should be less than 0.2 (Clarke 1999) in order to give the ideal results.

ANOSIM (Clarke 1993) using Bray-Curtis similarity was used to test statistically whether there was a significant difference in bacterial community compositions between two or more groups of samples. This procedure generates two values of R (which is scaled to stay between -1 and +1) and P-value (differences were considered significant if $p<0.05$). R represents for the difference of average rank dissimilarities between and within groups, and indicates how the different the

groups are. An R value near +1 means that there is dissimilarity between groups while an R value near 0 implies no significant difference among the groups (indistinguishable).

Further, in order to assign the taxonomy of the 16S rDNA sequences excised from DGGE bands, the Classifier tools in the Ribosomal Database project (http://rdp.cme.msu.edu/classifier/classifier.jsp) was used for direct comparison between achieved sequences and its online available databases. In case the sequences were not classified by this tool, they were checked with Nucleotide Blast Search (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

In this study, all multivariate data were analyzed with the PAST software package written by Hammer et al. (2001).

3. Results

3.1. Larval standard length, myotome height and dry weight during 42 days experiment

Totally 100 larvae were analyzed from 10 sampling days, including the 50 small (S) and the 50 large (L) individuals. The growth of the cod larvae was described by the measurements of standard length, myotome height and dry weight throughout the rearing period. Figure 3.1; 3.2 and 3.3 shows that there were very clear separations between small and large larvae in length, height and weight (Figure 3.1; 3.2 and 3.3). Therefore, sampled larvae were suitable for evaluating the relationship of intestinal microbial compositions between small and large larvae.

The average length of the small individuals was significantly different from the large ones at all ten sampling days ($p<0.05$). For both large and small individuals the length increased exponentially with time, which is shown by a linear relationship after taking the logarithm of y-axis (Figure 3.1B). The growth of large and small larvae was fairly similar (log (SL_{large}) = (0.0138 ± 0.0002)*age + (0.519 ± 0.0058) and log (SL_{small}) = (0.0104 ± 0.0005)*age + (0.533 ± 0.0119), both with R^2= 0.99). The log-linear plots had high coefficients of determination (R^2), indicating good fits between the model equations and the data.

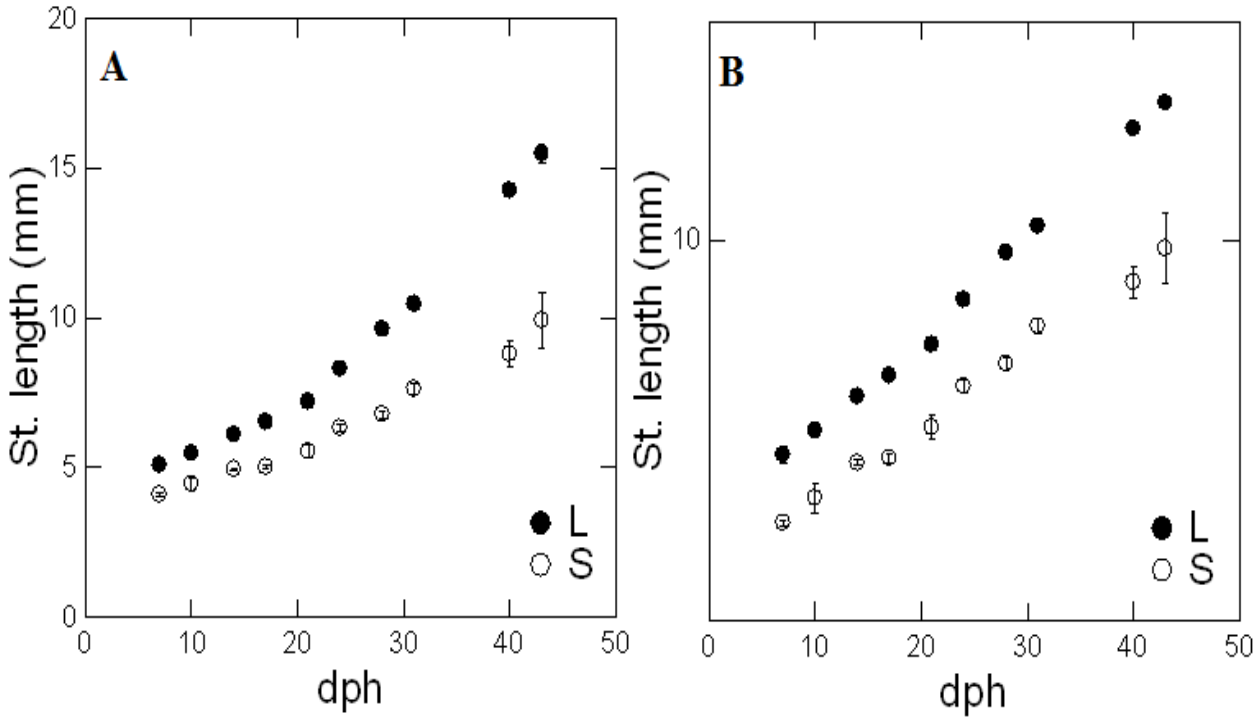

Figure 3.1: Growth of small (S) and large (L) cod larvae during the experimental period presented as their standard length. Relation (B): log (standard length) = a+b*(age). Figure A: original data; Figure B: data with logarithmic y-axix. Error bars are standard error.

There were significant differences in myotome height between small and large larvae at different sampling days ($P < 0.05$), except for small and large individuals at 7 dph with the p-value of 0.89 ($P > 0.05$). An exponential relationship between larval myotome height and their age was shown in Figure 3.1A. For both small and large larvae the height-age curve was linearized by using logarithmic y-axis (Figure 3.1B). The slope of the line for big larvae was slightly higher than for small larvae ($\log (MH_{large}) = (0.0247 \pm 0.0005)$*age – (0.776 ± 0.0127) with $R^2=0.99$, n= 50; and $\log (MH_{small}) = (0.0167 \pm 0.0009)$*age – (0.789 ± 0.0223) with $R^2=0.96$, n=50).

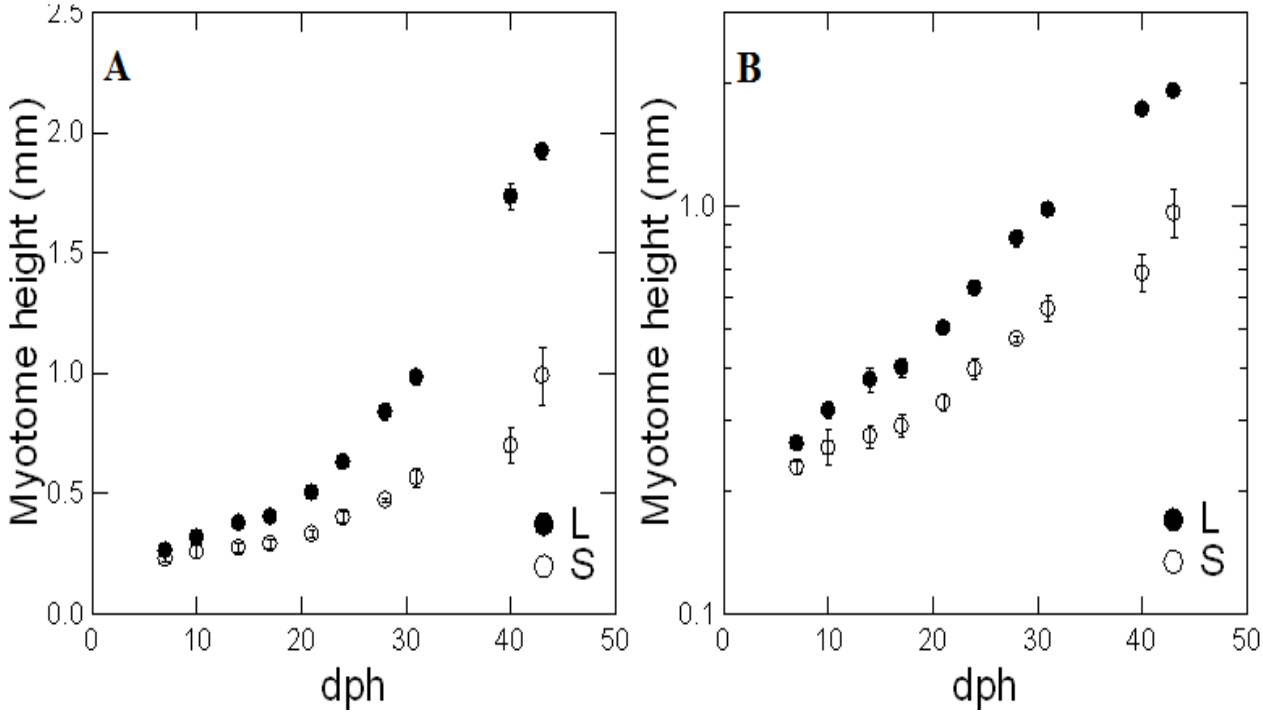

Figure 3.2: Growth of small (S) and large (L) cod larvae during the experimental period presented as their myotome height. A: original data; B: data with logarithmic y-axis. Error bars are standard error.

The average weight of the small individuals was significantly different from the large ones at different sampling days ($P<0.05$), except for small and large individuals at 7 dph ($P = 0.22$). As for larval standard length and myotome height, an exponential relationship between larval weight data and age was observed for both small and large larvae (Figure 3.3A), which is shown by a linear relationship after taking the logarithm of y-axis (Figure 3.3B; $(\log (DW_{large}) = (0.0490 \pm 0.0007)$*age – (1.49 ± 0.02), n= 50; and $\log (DW_{small}) = (0.0409 \pm 0.0013)$*age – (1.74 ± 0.035), n=50).

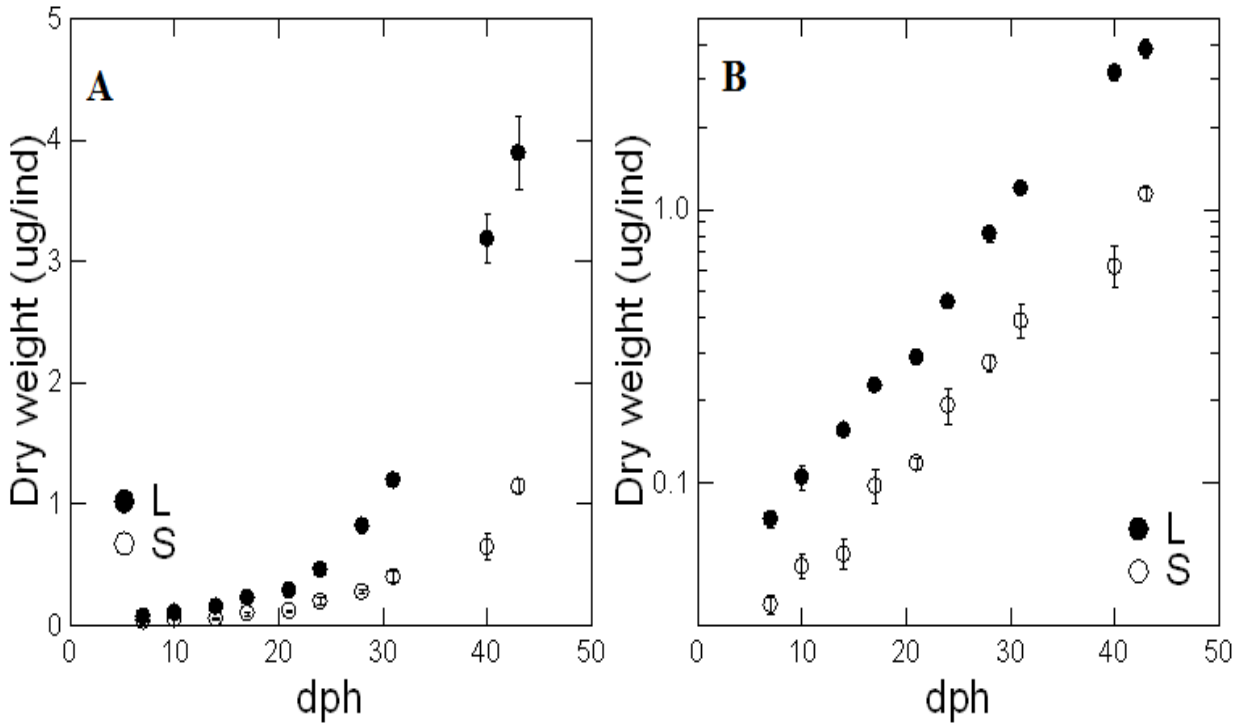

Figure 3.3: Growth of small (S) and large (L) cod larvae during the experimental period presented in their dry weight. A: original data; B: data in logarithmic y-axis. Error bars are standard error.

In conclusion, the ANOSIM show significant differences in average length between small and large larval groups at all ten sampling days. The height and weight of the larvae are also significant different between the two groups, except for the larval groups at 7 dph.

In this study, the growth of larvae was described by the measurement of their length, height and weight. Figure 3.4 shows that these three variables were closely correlated. The power relationships between larval weight and length, and larval weight and height ($DW = (1.126 \pm 0.0251) * (height)^{1.892 \pm 0.0380}$ and $DW = (0.000549 \pm 0.000097) * (length)^{3.244 \pm 0.066}$; Figure 3.4A and B) had a very high coefficients of determinations ($R^2 \sim 0.99$ for both relations), indicating good fits between the model equations and data. These correlations allow good predictions of larval weight from one linear dimension only.

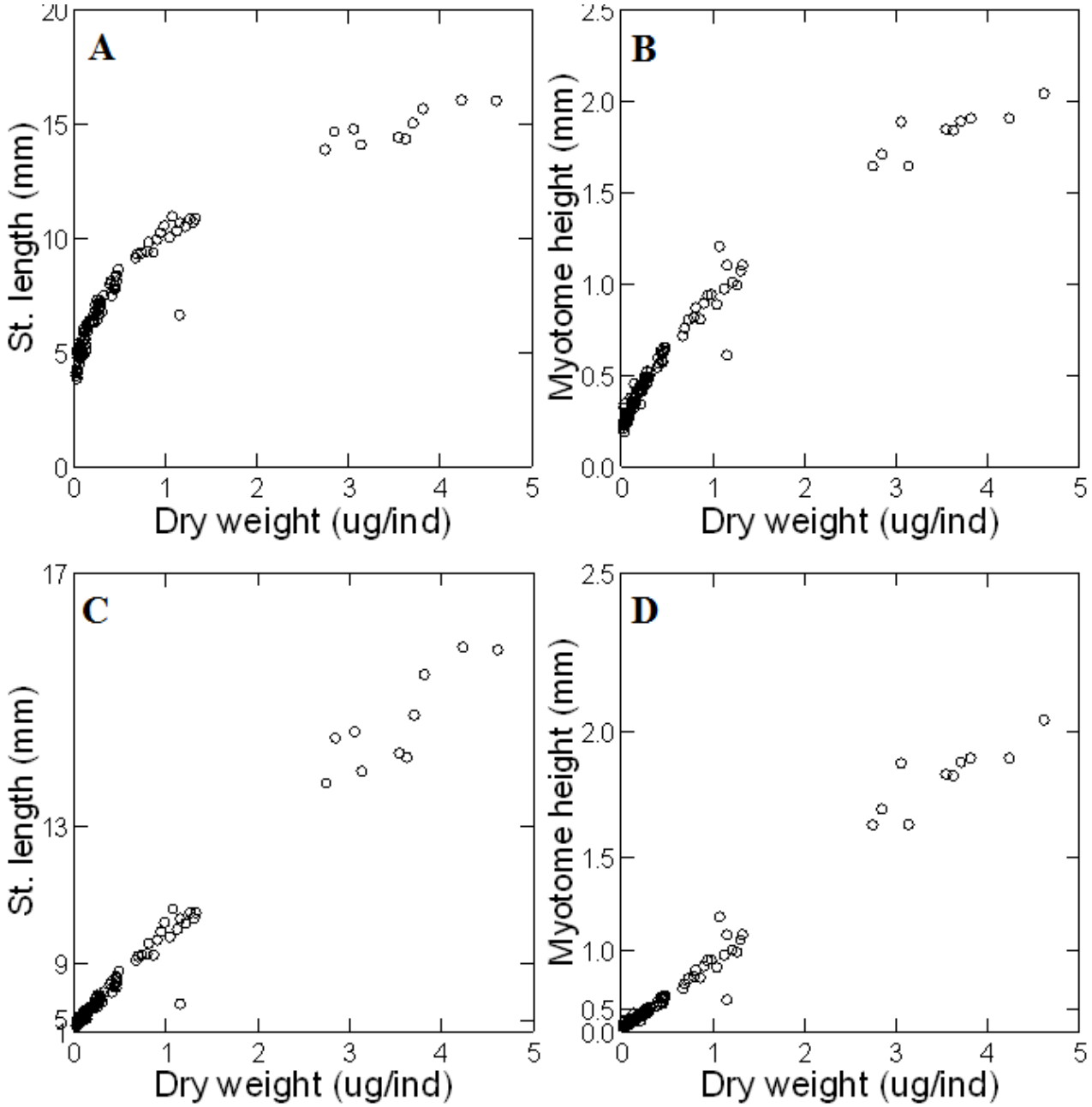

Figure 3.4: Exponential relationship between weight – length, and weight - height of the larvae throughout 42 days of rearing. A&B: untransformed data; C&D: Power transformed weight axis

3.2. DGGE analysis of intestinal microbial communities between small and large cod larvae

A total of 100 individuals of cod larvae at ten sampling days were used to investigate the bacteria communities using PCR/DGGE. Between-gel comparisons should be avoided, and the maximum number of samples to be loaded on one gel is 30. Only samples from 3 different sampling days could be loaded on one gel. Thus, in order to make it possible to compare the microbiota between relevant groups, the samples of some sampling days (day 14, 21, 28, 31 and 39) were run twice. Therefore, the larvae collected from ten different samplings days were run on a total of 5 DGGE gels: Gel 1 for 7, 10 and 14 dph larvae; Gel 2 for 14, 17 and 21 dph larvae; Gel 3 for 21, 24 and 28 dph larvae; Gel 4 for 28, 31 and 39 dph larvae and Gel 5 for 31, 39 and 42 dph larvae. There are two types of samples for each sampling day on each gel, S referring to small, and L referring to large larvae

3.2.1. Intestinal microbial communities in large and small larvae at 7, 10, and 14 dph

The intestinal microbial communities of the fish larvae at 7, 10 and 14 dph were analyzed by PCR/DGGE which is shown in Figure 3.5. Sequenced DGGE bands were marked as red frames and numbered in the gel. Their taxonomy is reported in Table 3.1. From this table, it can be seen that all sequenced bands were classified as bacterial taxa, except band 4 found in a group of large larvae at 7dph, which was similar to Cyanobacteria, and probably represents chloroplast from the algae added to the rearing water.

Sequenced bands from larvae sampled at 7 dph were assigned to Bacilli, Gammaproteobacteria and Actinobacteria. Independent of larval size, two strong bands were dominating most of the DGGE profiles of larvae at 10 and 14 dph. Although positioned in different regions of the gel, both these two bands were found to represent the genus *Arcobacter* (Epsilonproteobacteria).

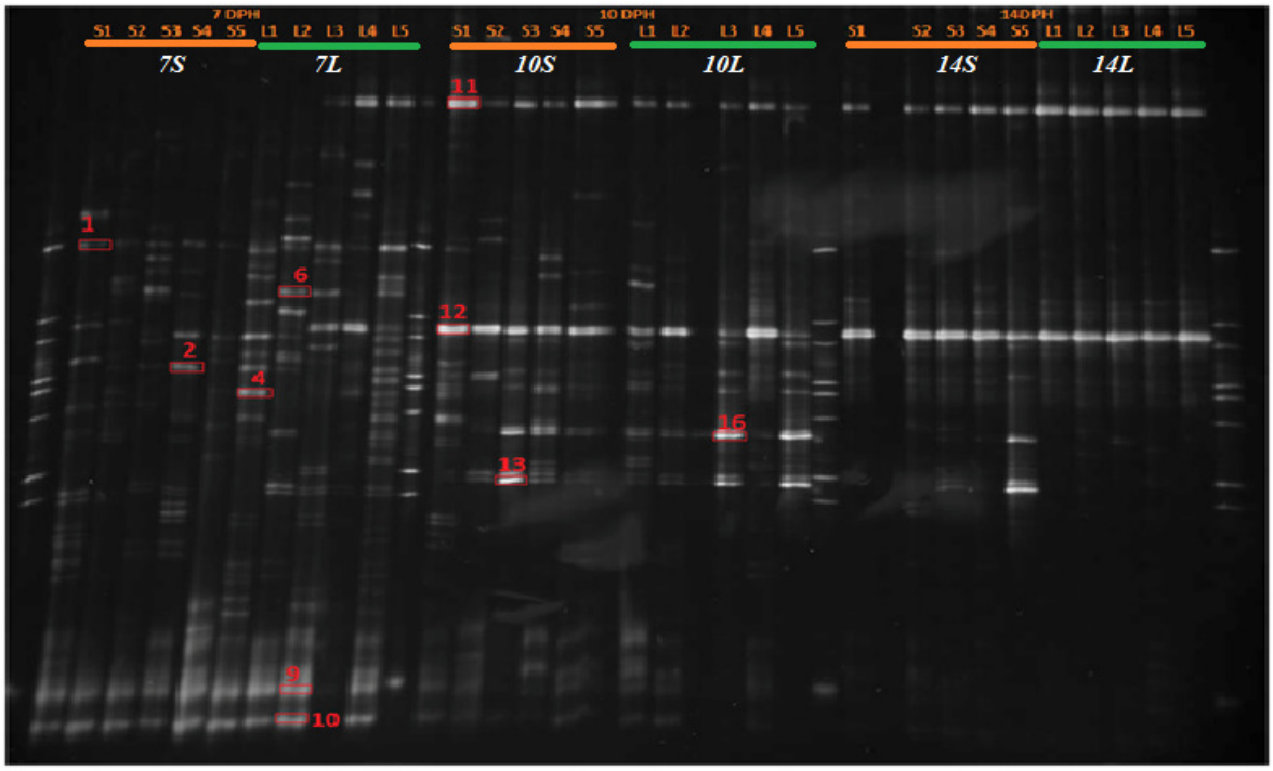

Figure 3.5: Denaturing gradient gel electrophoresis (DGGE) profiles for PCR-amplified 16S rDNA fragments from microbial communities of large (L) and small (S) cod larvae at 7, 10 and 14 dph. The marked and numbered bands are those which were excised and sequenced to assign bacterial taxa

Table 3.1: Taxonomic assignments of DGGE bands for cod larvae microbiota at 7, 10, 14 dph. Band numbers refer to Figure 3.5. S refers small larvae and L refers to large larvae

Band no.	Observed in	Phylum	Class	Genus*
1	7S/ 7L/10S	Firmicutes	Bacilli	*Staphylococcus*
2	7S/ 7L/ 10S/10L	Proteobacteria	Gammaproteobacteria	*Pseudomonas*
4	7L/ 10S	Cyanobacteria	Chloroplast	*Bangiophyceae*
6	7S/ 7L	Proteobacteria	Gammaproteobacteria	*Acinetobacter*
9	7S/ 7L/ 10S/10L	Actinobacteria	Actinobacteria	*Propionibacterium*
10	7S/ 7L/ 10S/10L	Actinobacteria	Actinobacteria	*Propionibacterium*
11	7L/ 10S/ 10L/14S/14L	Proteobacteria	Epsilonproteobacteria	*Arcobacter*
12	7S/7L/10S/10L/14S/14L	Proteobacteria	Epsilonproteobacteria	*Arcobacter*
13	7L/10S/10L/14S	Proteobacteria	Gammaproteobacteria	*Aliivibrio*
16	10S/ 10L	Proteobacteria	Gammaproteobacteria	*Aliivibrio*

**The lowest taxonomy level at confident threshold > 50% - Determined by the Classifier tool.*

The average diversity indices for the intestinal microbial communities of the larvae at 7, 10 and 14 dph (days post hatching) are shown in the Table 3.2. The larvae at day 7 and 10 had higher species richness and Shannon diversity than the larvae at 14 dph. Further, lowest evenness index was recorded for the larvae at day 14.

The average band richness, Shannon diversity and evenness index are similar for small and large individuals at 7 and 10 dph. Statistical analysis (one-way ANOVA) confirmed that there were no significant differences between small and large individuals for neither of these diversity indices ($P > 0.05$, Table 3.3). However, one-way ANOVA test indicate significant differences in richness and Shannon diversity between the small and large larvae at day 14 (with P=0.009 and 0.013, respectively). No significant difference was found in the Evenness between the two groups (P=0.13).

Table 3.2: Average band richness (S), Shannon index (H) and evenness (J) calculated from DGGE profiles of both small (S) and large (L) larvae at 7, 10 and 14 dph

DPH	**7L**		**7S**		**10L**		**10S**		**14L**		**14S**	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
S	21.4	8.56	17.2	2.49	19.2	2.28	18.6	3.78	9	1.87	14.2	2.77
H	2.82	0.48	2.69	0.16	2.72	0.11	2.67	0.23	1.81	0.24	2.27	0.22
J	0.84	0.05	0.86	0.02	0.78	0.06	0.79	0.06	0.69	0.04	0.69	0.03

Table 3.3: ANOVA analysis for testing the differences in diversity indices between largae (L) and small (S) larvae at 7, 10 and 14 dph

		ANOVA P value		
		Band richness	**Shannon index**	**Evenness**
Between groups	**7S vs 7L**	0.323	0.576	0.67
	10S vs 10L	0.769	0.646	0.71
	14S vs 14L	**0.009**	**0.013**	0.13

The 2D NMDS ordination based on Bray-Curtis similarity for the microbial composition of small and large larvae at 7, 10 and 14 dph are shown in Figure 3.6. It can be observed that the points corresponding to the larvae at 14 dph are cluster together, indicating less variable microbiota among individuals compared to the larvae at 7 and 10 dph. Further, the gut microbiota of small and large individuals at 7 and 10 dph are partially overlapping, while the

NMDS shows clear separation between small and large larval groups at 14 dph. One-way ANOSIM confirmed that there was a significant difference in the microbial communities of small and large larvae at 14 dph (P=0.015), but large and small larvae at 7 and 10 dph had similar microbiota (P=0.47 and P=0.89, respectively).

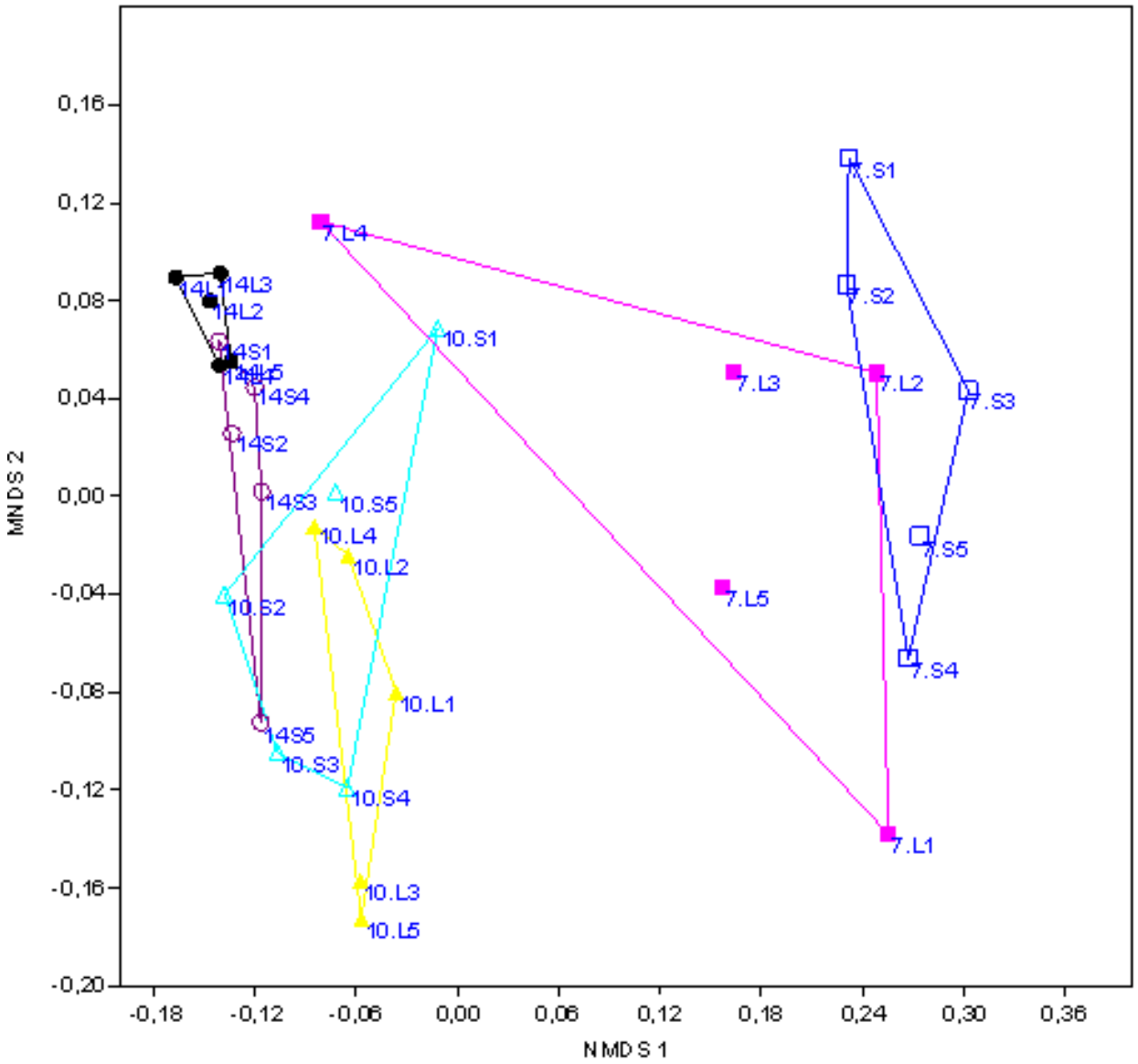

Figure 3.6: NMDS ordination based on Bray-Curtis similarities for microbiota of small (S) and large (L) larvae at 7dph (square and filled squared), 10dph (triangle and filled triangle), and 14dph (circle and filled circle)

The comparisons of the intestinal microbial communities using average Bray-Curtis similarity for larval microbiota within and between groups are showed in Figure 3.7. Within groups, the similarity of large larvae at 14 dph was highest (around 0.76), indicating that less variation in microbiota among individuals. Further, lowest similarities were found for both large and small

individuals at 7 dph (0.312 and 0.431, respectively). Between groups, the average similarity between small and large larvae at 14 dph appeared to be lower than within larval groups. This indicates there is probably difference between groups at 14 dph.

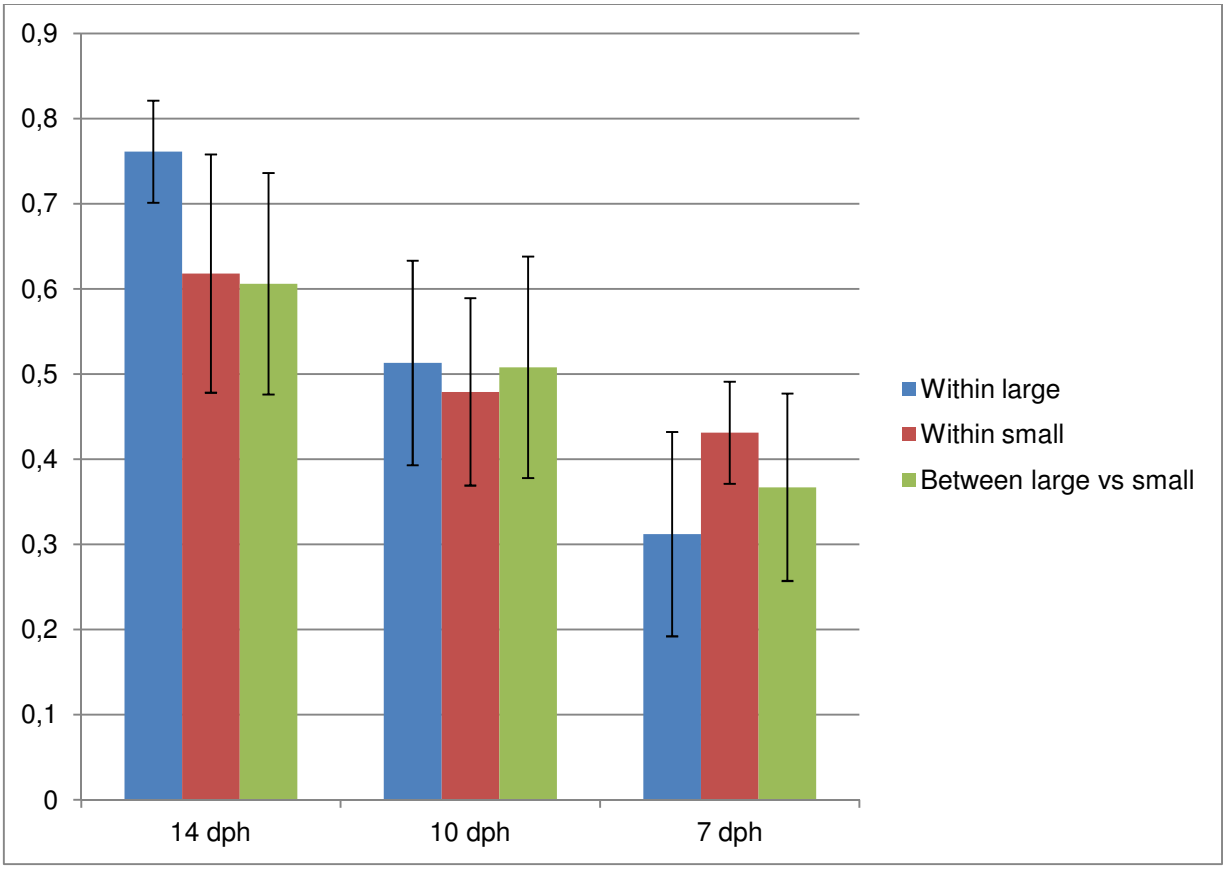

Figure 3.7: Average Bray-Curtis similarities for within and between groups of the microbial compositions of large and small larvae at 7, 10 and 14 dph

3.2.2. Intestinal microbial communities in large and small larvae at 14, 17 and 21 dph

The intestinal microbial communities of the fish larvae at 14, 17 and 21 dph were analyzed by PCR/DGGE. The obtained DGGE gel is shown in Figure 3.8. Five bands marked as red frames and numbered in the gel were sequenced. Their taxonomy assignment is summarized in Table 3.4. Two bacterial classes typically dominated in both small and large larvae at all these three days represented as a class of Epsilonproteobacteria with the genus *Arcobacter* (band number 3 and 4). The other bands corresponding to number 2, 8 and 14 on the gel were assigned to *Pseudoalteromonas and Rubritalea,* respectively.

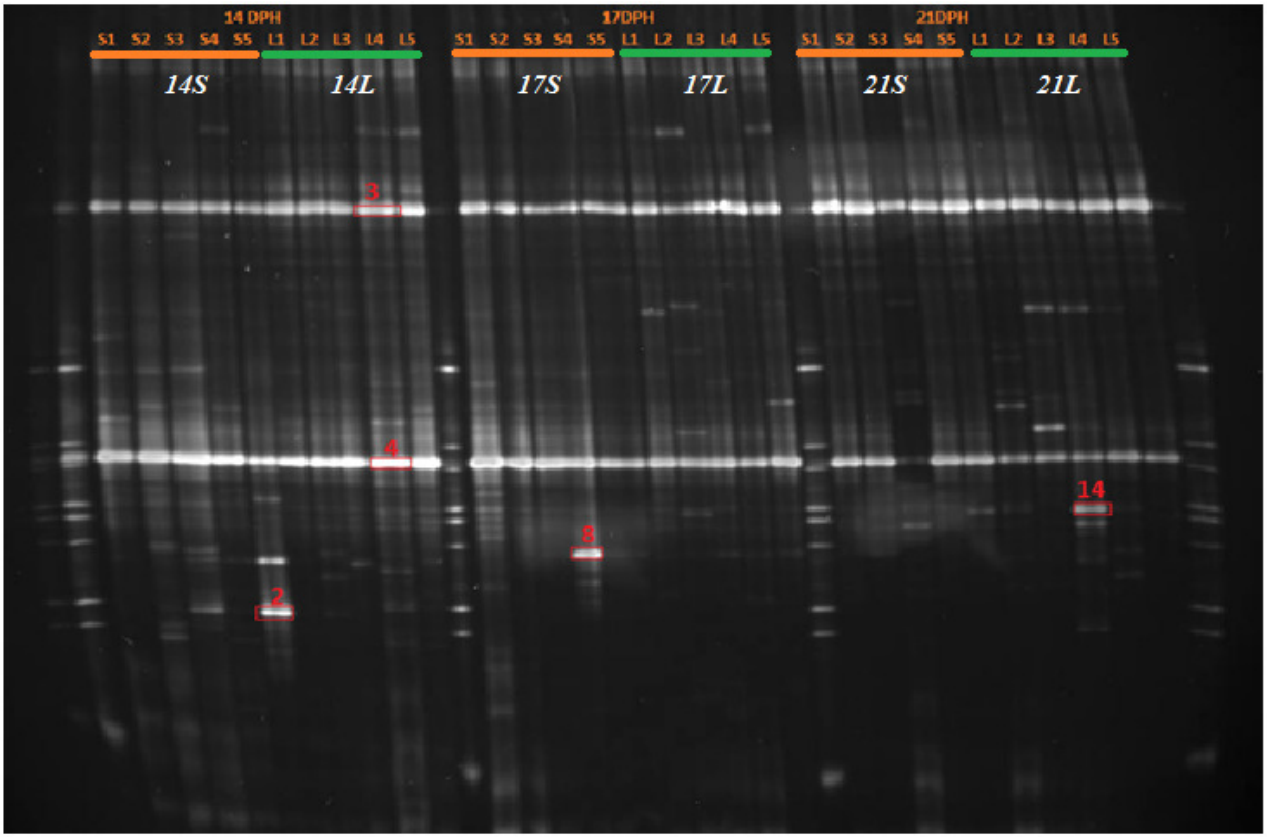

Figure 3.8: Denaturing gradient gel electrophoresis (DGGE) profiles for PCR-amplified 16S rDNA fragments from microbial communities of large (L) and small (S) cod larvae at 14, 17 and 21 dph. The marked and numbered bands are those which were excised and sequenced to assign bacterial taxa

Table 3.4: Taxonomic assignments of DGGE bands for cod larvae microbiota at 14, 17 and 21 dph. Band numbers refer to Figure 3.8. S refers small larvae and L refers to large larvae

Band no.	Observed in	Phylum	Class	Genus*
2	14S	Proteobacteria	Gammaproteobacteria	*Aliivibrio*
3	14S/14L/17S/17L/21S/ 21L	Proteobacteria	Epsilonproteobacteria	*Arcobacter*
4	14S/14L/17S/17L/21S/ 21L	Proteobacteria	Epsilonproteobacteria	*Arcobacter*
8	17S/17L	Proteobacteria	Gammaproteobacteria	*Pseudoalteromonas*
14	21S/21L/14S/14L/17S/7L	Verrucomicrobia	Verrucomicrobiae	*Rubritalea*

**The lowest taxonomy level at confident threshold > 50% - Determined by the Classifier tool.*

The average diversity indices for the intestinal microbial communities of the larvae at 14, 17 and 21 dph are presented in the Table 3.3. One-way ANOVA test (Table 3.6) indicates that there was a significantly difference in species richness between small and large individuals at both 14 and 21 dph (P=0.01 and 0.045, respectively). Generally, a higher value of band richness and

Shannon's index was found in the gut of small larvae at 21dph (17.4 ± 2.51), indicating more diverse microbiota compared to large larvae at this age (13.8 ± 2.68). Whereas the gut microbiota presented in large larvae were similarly diverse to those in small larvae at 17 dph ($P > 0.05$).

Surprisingly, the richness of large individuals at day 14 on this gel was higher than its value of small samples (Table 3.5). This is opposite with the result of small and large samples at 14 dph in DGGE gel 7-10-14 (Figure 3.5). Table 3.6 further shows that there were no significant differences between large and small larvae at 14, 17 or 21 dph with regards to both Shannon and evenness ($P > 0.05$).

Table 3.5: Average band richness (S), Shannon index (H) and evenness (J) calculated from DGGE profiles of both small (S) and large (L) larvae at 14, 17 and 21 dph

DPH	**14L**		**14S**		**17L**		**17S**		**21L**		**21S**	
	Mean	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**
S	23.4	2.51	18.8	1.92	17.2	1.30	16.0	5.34	13.8	2.68	17.4	2.07
H	2.8	0.17	2.56	0.18	2.4	0.08	2.3	0.36	2.24	0.25	2.5	0.11
J	0.68	0.05	0.69	0.06	0.67	0.04	0.68	0.05	0.69	0.04	0.70	0.04

Table 3.6: ANOVA analysis for testing the differences in diversity indices between large (L) and small (S) larvae at 14, 17 and 21 dph

		ANOVA P value		
		Band richness	**Shannon index**	**Evenness**
Between groups	**14S vs 14L**	**0.01**	0.098	0.78
	17S vs 17L	0.64	0.622	0.97
	21S vs 21L	**0.045**	0.081	0.43

Average Bray-Curtis similarities of the intestinal microbial communities within and between larval groups are showed in Figure 3.9. Within groups, the microbial compositions of large larvae at 14, 17 and 21 dph were fairly similar. The average similarity was highest in the small individuals at 21 dph, indicating less variation in microbiota composition among individuals. This was then followed by the lower value of similarity in small ones at 17 dph, and lowest in the individuals at 14 dph. For the larvae at 14 and 21 dph, the similarities between small and

large larvae appeared to be lower than within small and large individuals, indicating that there are differences in microbial communities between groups.

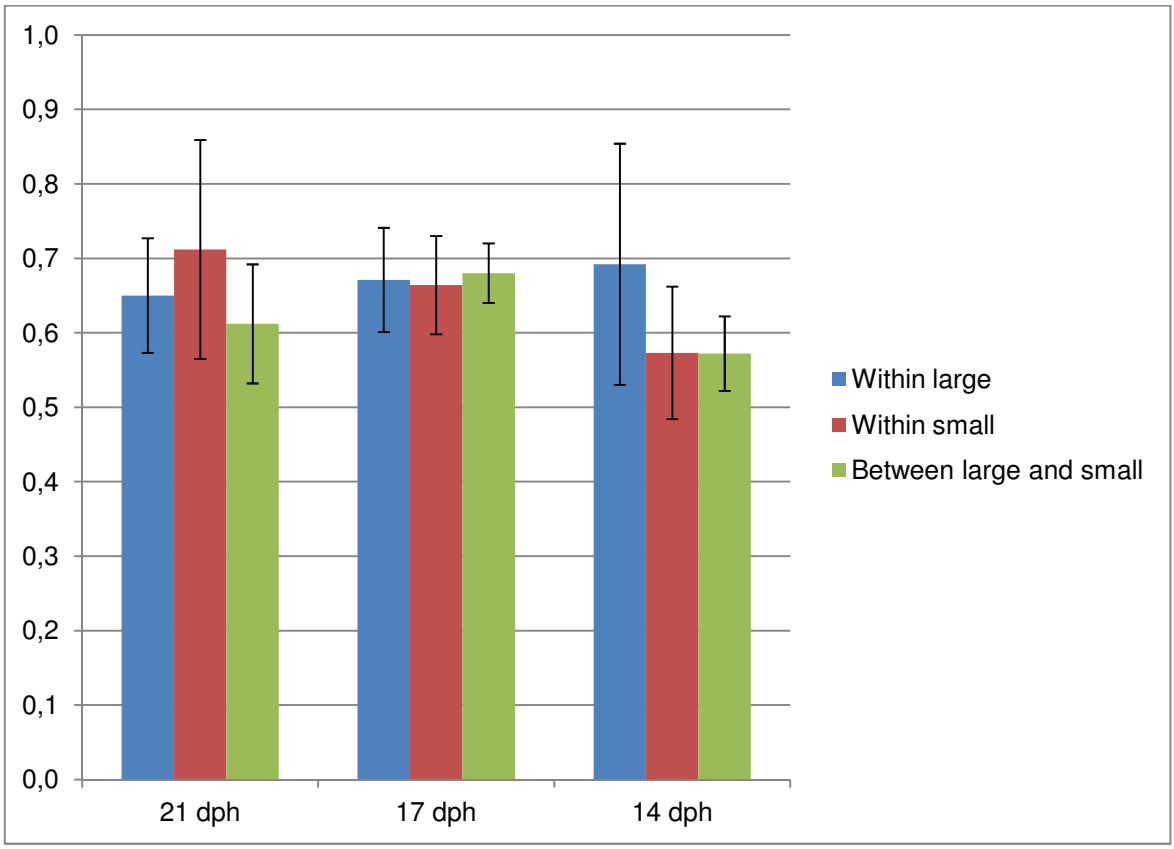

Figure 3.9: Average Bray-Curtis similarities for within and between groups of the microbial compositions of large and small larvae at 14, 17 and 21 dph

The Bray-Curtis similarities of DGGE profiles were used as the base for the distributions of the samples in 2D-NMDS ordination which is showed in Figure 3.10. A clear separation between large and small individuals at 21 dph was observed in the plot, indicating that the gut microbiota of large individuals were different from those of small individuals. Statistical analysis using one-way ANOSIM confirmed this with P= 0.023 (Table 3.10). Further, for the gut microbial community at 17 dph, there is a partial overlap between the points representing large and small larvae seen in NMDS plot. ANOSIM analysis showed that there was no significant difference between these groups (P = 0.107). ANOSIM analysis also indicates that the bacterial communities of large and small individual at 14 dph were significantly different (P= 0.008), which is consistent with the conclusion of the larvae at 14 dph calculated for the 7-10-14 dph gel in the previous section 3.2.1.

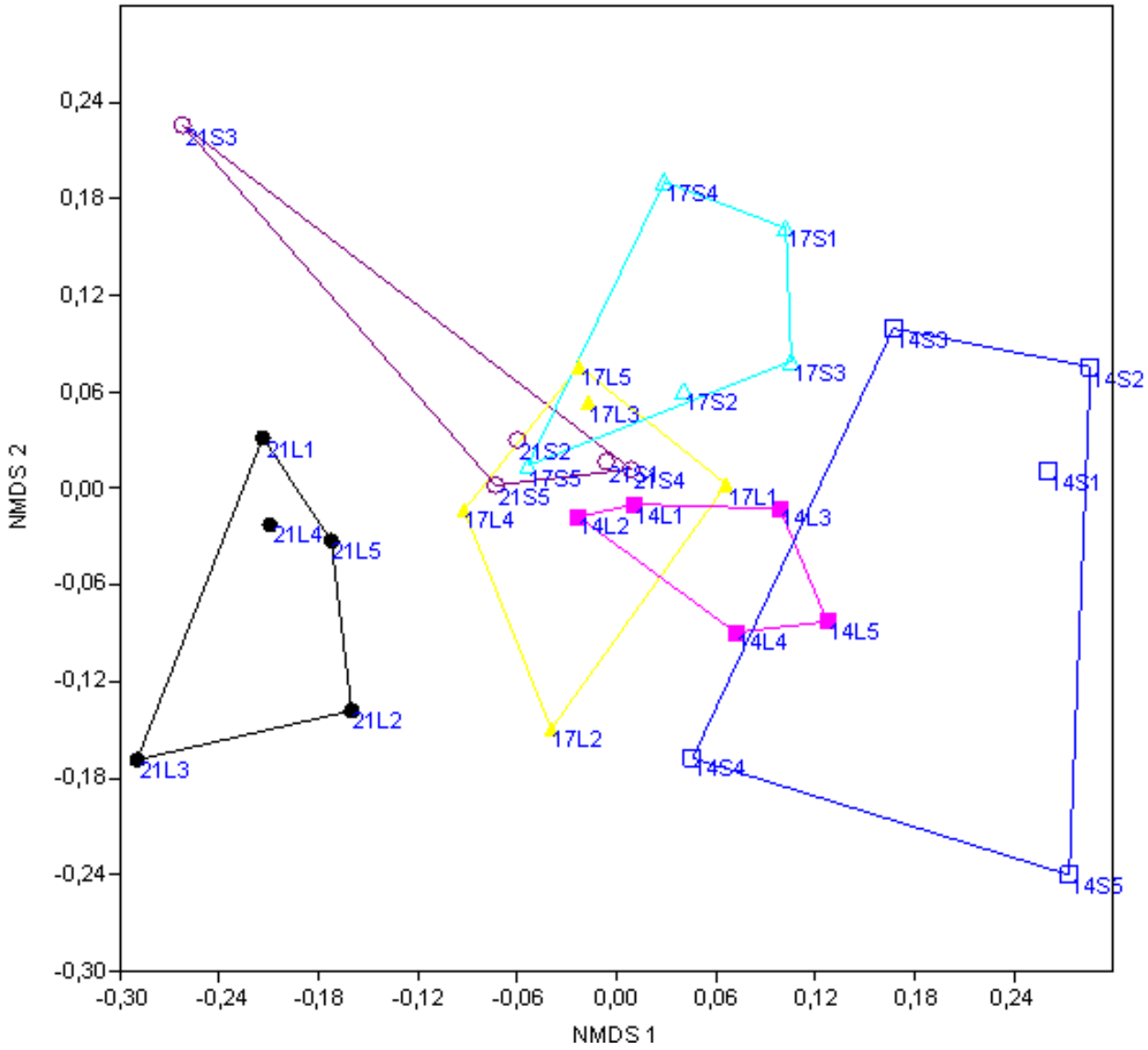

Figure 3.10: NMDS ordination based on Bray-Curtis similarities for microbiota of small and large larvae at 14 (square and filled squared), 17(triangle and filled triangle), and 21 dph (circle and filled circle); L refers to large and S refers to small larvae

3.2.3. Intestinal microbial communities in large and small larvae at 21, 24 and 28 dph

The microbial communities in the gut of large and small larvae at 21, 24 and 28 dph analyzed by PCR/DGGE are showed in Figure 3.11. Only one band marked in red frame on the gel was excised for sequencing and taxonomy assignment. Based on the observation of DGGE profiles, it can be seen that the microbial community of the larvae generally changed a little from 21 dph to 28 dph. Two strong bands in total of 54 bands were dominated at these three days in the gel, which represent to the phylum proteobacteria (*Arcobacter* genus). For the 28 dph larvae, besides these two dominating bands, there was also an appearance of another strong band in their

microbial community composition, which was determined to be the phylum proteobacteria but with genus *Sulfitobacter.* This band was also observed for some individuals at 24 and 21 dph.

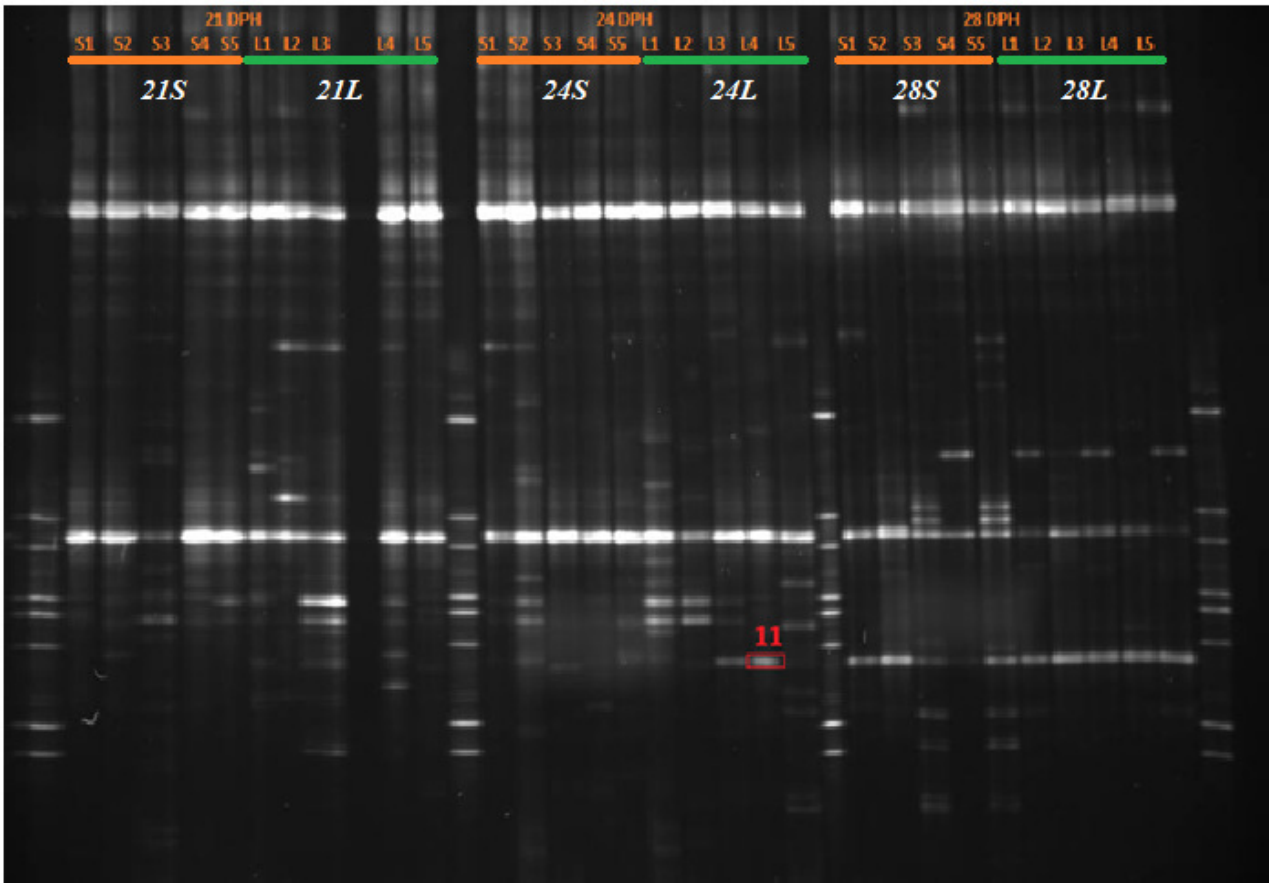

Figure 3.11: Denaturing gradient gel electrophoresis (DGGE) profiles for PCR-amplified 16S rDNA fragments from microbial communities of large (L) and small (S) cod larvae at 21, 24 and 28 dph. The marked and numbered bands are those which were excised and sequenced to assign bacterial taxa

DGGE profiles were used for the calculation of diversity indices including species richness, Shannon diversity and evenness index for gut microbiota of the larvae at 21, 24 and 28 dph (Table 3.7). A one-way ANOVA test demonstrated significant differences in richness and Shannon index between large and small individuals were recorded at 21 dph with P=0.024 and P=0.006 respectively. Further, small larvae was significant different from large larvae in evenness at 24 dph (P=0.03). For the rest, there were found no differences in diversity indices between small and large larvae ($P > 0.05$).

Table 3.7: Average band richness (S), Shannon index (H) and evenness (J) calculated from DGGE profiles of both small (S) and large (L) larvae at 21, 24 and 28 dph

DPH	21L		21S		24L		24S		28L		28S	
	Mean	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**	**Mean**	**± SD**
S	18,17	3,27	14,60	0,89	13,4	3,21	16,60	4,62	9,20	2,49	11,60	5,03
H	2,55	0,17	2,25	0,07	2,21	0,26	2,25	0,37	1,92	0,22	2,09	0,50
J	0,69	0,03	0,66	0,05	0,69	0,04	0,59	0,08	0,76	0,04	0,75	0,07

Table 3.8: ANOVA analysis for testing the differences in diversity indices between largae (L) and small (S) larvae at 21, 24 and 28 dph

		ANOVA P value		
		Band richness	**Shannon index**	**Evenness**
Between groups	**21S vs 21L**	**0.024**	**0.006**	0.78
	24S vs 24L	0.239	0.823	**0.03**
	28S vs 28L	0.367	0.491	0.90

NMDS ordination based on Bray-Curtis similarities for the intestinal microbial communities of large and small larvae at 21, 24 and 28 dph are shown in the Figure 3.12. In the plot, there is an overlap in microbial community composition between the larval groups at 21 and 24 dph, whereas the larvae at 28 dph seemed to cluster outside the larvae from other groups. Statistical analysis (ANOSIM) revealed that the intestinal microbial communities between large and small larvae at 21 dph and 24 dph are similar to each other (P=0.29 and 0.62, respectively), while a significant differences in microbial community between large and small larvae was observed at 28 dph with P=0.03.

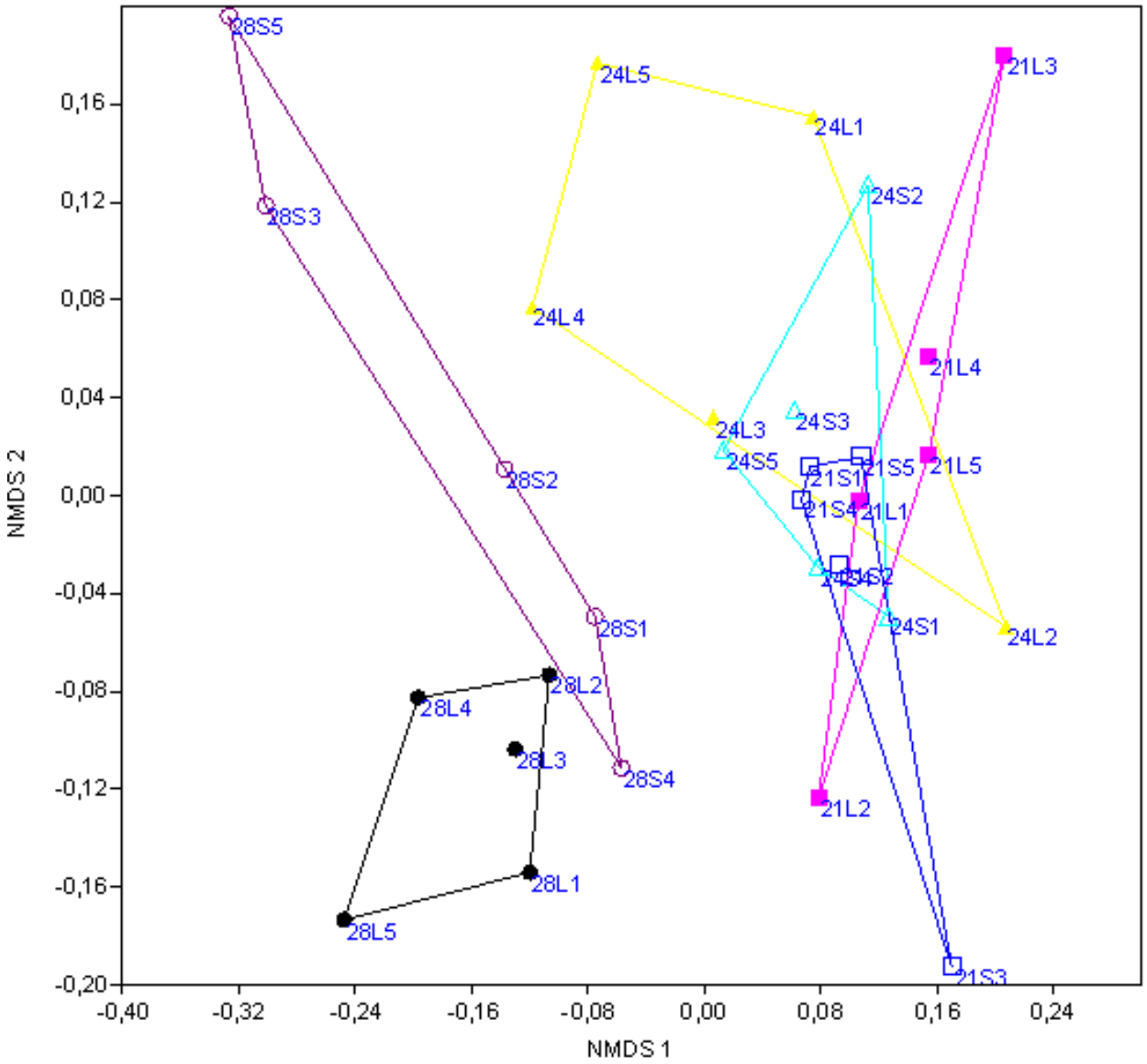

Figure 3.12: NMDS ordination based on Bray-Curtis similarities for microbiota of small (S) and largae (L) larvae at 21 (square and filled squared), 24 (triangle and filled triangle) and 28 dph (circle and filled circle); L refers to large and S refers to small larvae

The average Bray-Curtis similarities of microbial communities within and between larval groups were shown in Figure 3.13. Within groups, the similarity was highest in large larvae at 28 dph and in small larvae at 21 dph, indicating the microbiota composition among individuals within these groups is less variable. Between groups, the similarity between small and large larvae at 28 dph appeared to be lower than within groups. This indicates there are differences in microbial communities between groups.

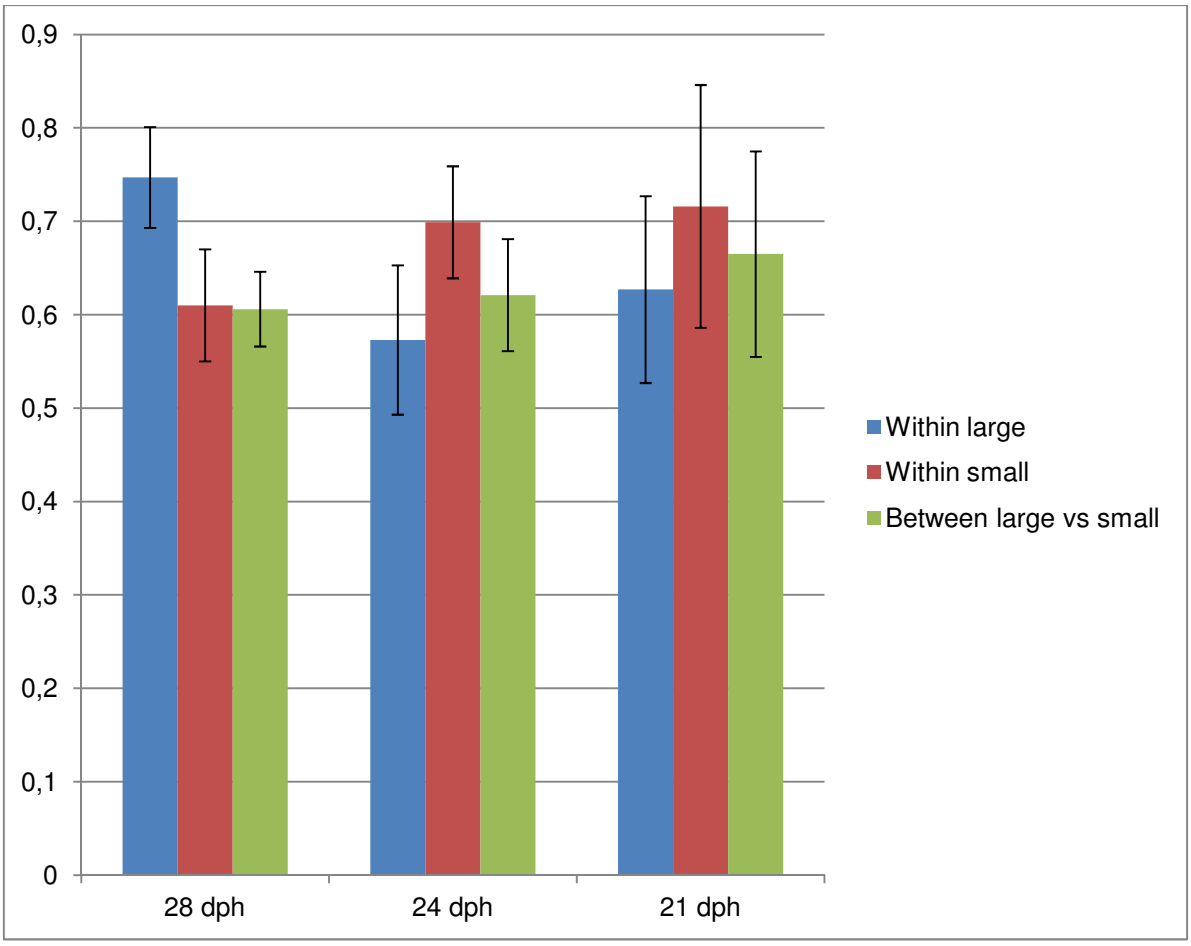

Figure 3.13: Average Bray-Curtis similarities for within and between groups of the microbial compositions of large and small larvae at 21, 24 and 28 dph

3.2.4. Intestinal microbial communities in large and small larvae at 28, 31 and 39 dph

The microbial communities in the gut samples of small and large larvae at 28, 31 and 39 dph run on another DGGE gel are presented in Figure 3.14. The bands marked in red frames and numbered as shown in the gel were excised and sequenced for taxonomy assignment. The results are reported in Table 3.9. From the DGGE profiles of the larvae at 31 and 39 dph, it can be observed that their microbiota tend to be more diverse and variable among the individuals in compared to those at 28 dph. Most of the bands of the larvae at 39 dph seem to be more evenly distributed.

The strong band appeared nearly on the top of the gel dominated in both small and large larvae at 28 and 31 dph, also in small larvae at 39 dph, but it seems to be less dominated or disappeared in the large individuals at 39 dph. This band was assigned to Epsilonproteobacteria with the genera of Arcobater (Table 3.1 in section 3.2.1).

Both band 22 and 23 strangely represent to the alpha-proteobacteria with the genus *Roseibium*. Band 9 which was assigned to the phylum alpha-proteobacteria (*Sulfitobacter*) dominating in

both large and small larvae at 28 and 31 dph, but it appeared in the larvae samples at 39 dph as a weaker band. Band 32 found in the 39 dph larvae was similar to Cyanabacteria and probably represented chloroplast DNA from algae. Other bands observed in the larvae at 39 dph were assigned to Firmicutes, Gammaproteobacteria, Alphaproteobacteria and Clostridia.

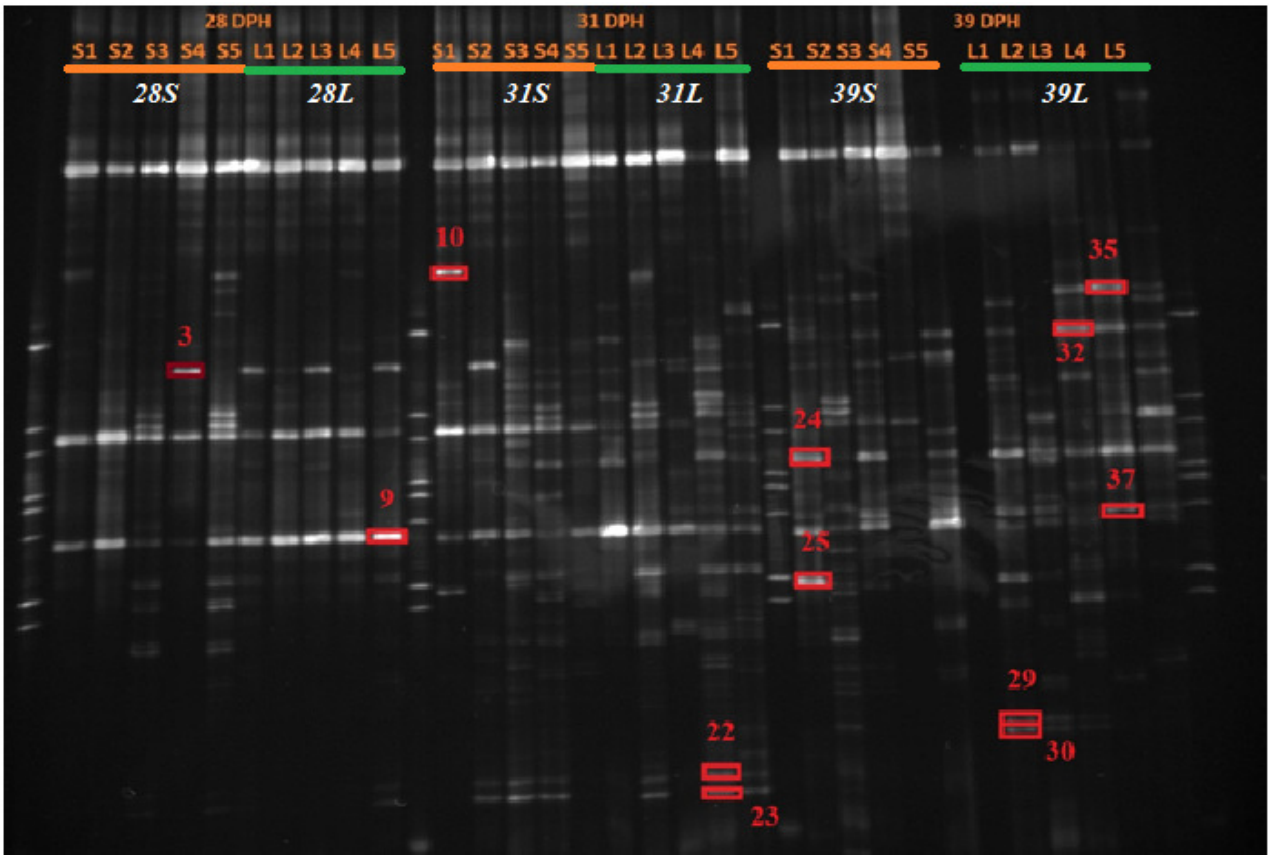

Figure 3.14: Denaturing gradient gel electrophoresis (DGGE) profiles for PCR-amplified 16S rDNA fragments from microbial communities of large (L) and small (S) cod larvae at 28, 31 and 39 dph. The marked and numbered bands are those which were excised and sequenced to assign bacterial taxa)

The diversity indices were calculated basing on peak area values resulting from Gel2k analysis of DGGE image of the larvae at 28, 31 and 39 dph. As seen in Table 3.10, the richness, diversity and evenness were similar between large and small groups at all days. One-way ANOVA analysis was confirmed this with the p value higher than 0.05 (Table 3.11).

Table 3.9: Taxonomic assignments of DGGE bands for cod larvae microbiota at 28, 31, 39 dph. Band numbers were recorded in Figure 3.14. S and L refer to small and large larvae, respectively.

Band no.	Observed in	Phylum	Class	Genus*
3	28S/28L/31S/39S	Bacteroidetes	Flavobacteria	*unclassified*
9	28S/28L/31S/31L/39S/39L	Proteobacteria	Alphaproteobacteria	*Sulfitobacter*
10	28S/28L/31S	Verrucomicrobia	Verrucomicrobiae	*Rubritalea*
22	28S/28L/31S/31L	Proteobacteria	Alphaproteobacteria	*Roseibium*
23	28S/28L/31S/31L	Proteobacteria	Alphaproteobacteria	*Roseibium*
25	31S /39S/ 39L	Proteobacteria	Gammaproteobacteria	*Aliivibrio*
24	31S/31L/39S/39L	Firmicutes		
29	39L	Proteobacteria	Alphaproteobacteria	*Unclassified*
30	39L	Proteobacteria	Alphaproteobacteria	*Unclassified*
32		Cyanobacteria	Chloroplast	*Chlorophyta*
35	39L	Firmicutes	Clostridia	*Finegoldia*
37	39L/39S	Firmicutes	Clostridia	*Unclassified*

**The lowest taxonomy level at confident threshold > 50% - As determined by Classifier - RDP*

Table 3.10: Average band richness (S), Shannon index (H) and evenness (E) calculated from DGGE profiles of both small (S) and large (L) larvae at 28, 31 and 39 dph

DPH	39L		39S		31L		31S		28L		28S	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
S	18.4	3.4	16.2	5.8	22.0	6.7	21.2	6.8	12.0	2.4	14.0	4.7
H	2.72	0.17	2.52	0.37	2.80	0.40	2.76	0.33	2.10	0.18	2.26	0.35
J	0.83	0.03	0.80	0.05	0.78	0.07	0.77	0.04	0.69	0.05	0.71	0.04

Table 3.11: ANOVA analysis for testing the differences in diversity indices between large (L) and small (S) larvae at 28, 31 and 39 dph

		ANOVA P value		
		Band richness	**Shannon index**	**Evenness**
Between groups	**28S vs 28L**	0.42	0.39	0.49
	31S vs 31L	0.85	0.85	0.92
	39S vs 39L	0.48	0.30	0.18

The average similarities of larval microbial composition within and between groups at these three days are shown in Figure 3.15. The similarities of the microbial community within the large larvae at 31 and 39 dph were similar and quite low (0.463 and 0.467, respectively), indicating large variation in microbial communities among individuals. Whereas the average similarity determined for larval microbial community within large larvae at 28 dph were much higher (around 0.7), indicating more similar microbiota between individuals within this group. Between groups, the similarity between large and small larvae at 39 dph appeared to be lower than within groups.

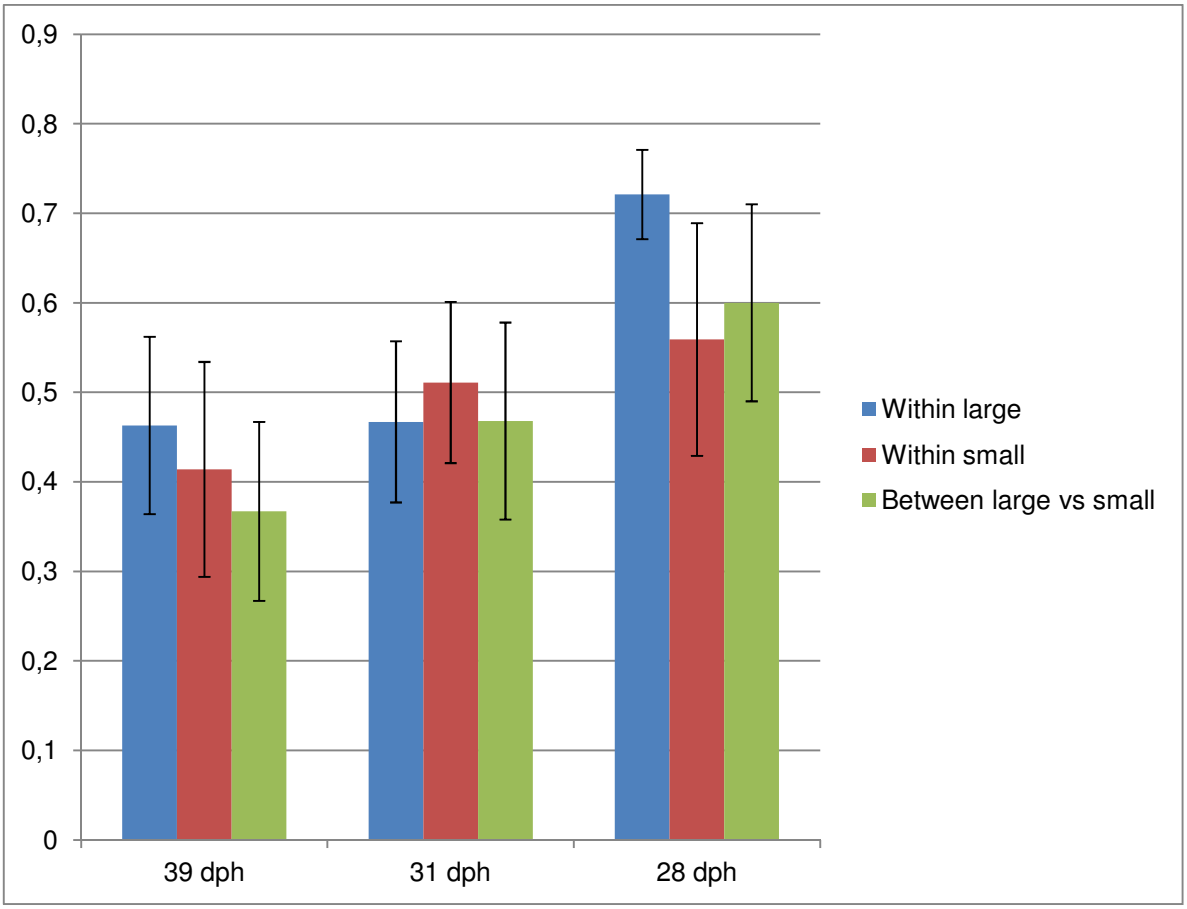

Figure 3.15: Average Bray-Curtis similarities for within and between groups of the microbial compositions of large and small larvae at 28, 31 and 39 dph

Two dimensional NMDS plot (Figure 3.16) shows that points corresponding to bacterial community of large and small larvae at 39 dph are distributed in two different areas, but the points of each group were scattered in quite large area, indicating more variable communities. ANOSIM analysis confirmed that the intestinal microbial communities of large individuals at 39 dph were significant different from those of small group at the same day (P= 0.007). Additionally, points representing microbial community composition of small larvae at 28 and 31 dph were overlapping with those of large larvae, indicating no differences in microbial

community composition between large and small individuals. Statistical analysis based on Bray-Curtis similarities indicates that the microbial communities of these groups were not significantly different (P>0.05).

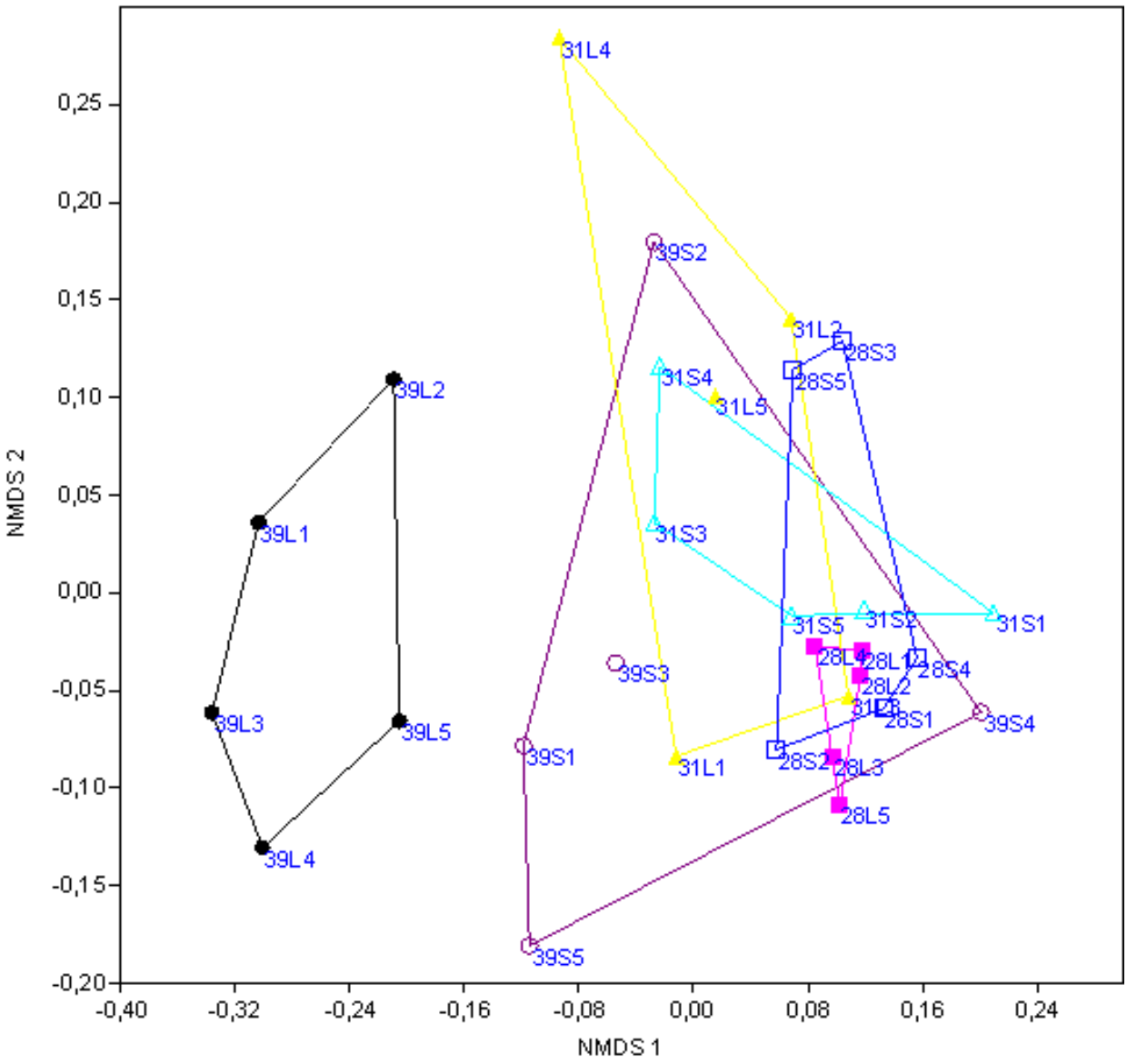

Figure 3.16: NMDS ordination based on Bray-Curtis similarities for microbiota of small (S) and largae (L) larvae at 28(square and filled squared, 31(triangle and filled triangle), and 39 dph (circle and filled circle); S and L refer to small and large larvae, respectively.

3.2.5. Intestinal microbial communities in large and small larvae at 31, 39 and 42 dph

The PCR-DGGE profiles of gut microbial community of small and large larvae at 31, 39 and 42 dph were found to be highly diverse with a high numbers of bands that are visible in the gel (Figure 3.17). Excised bands were sequenced and identified to represent gramma-, epsilon-proteobacteria and flavobacteria (Table 3.12).

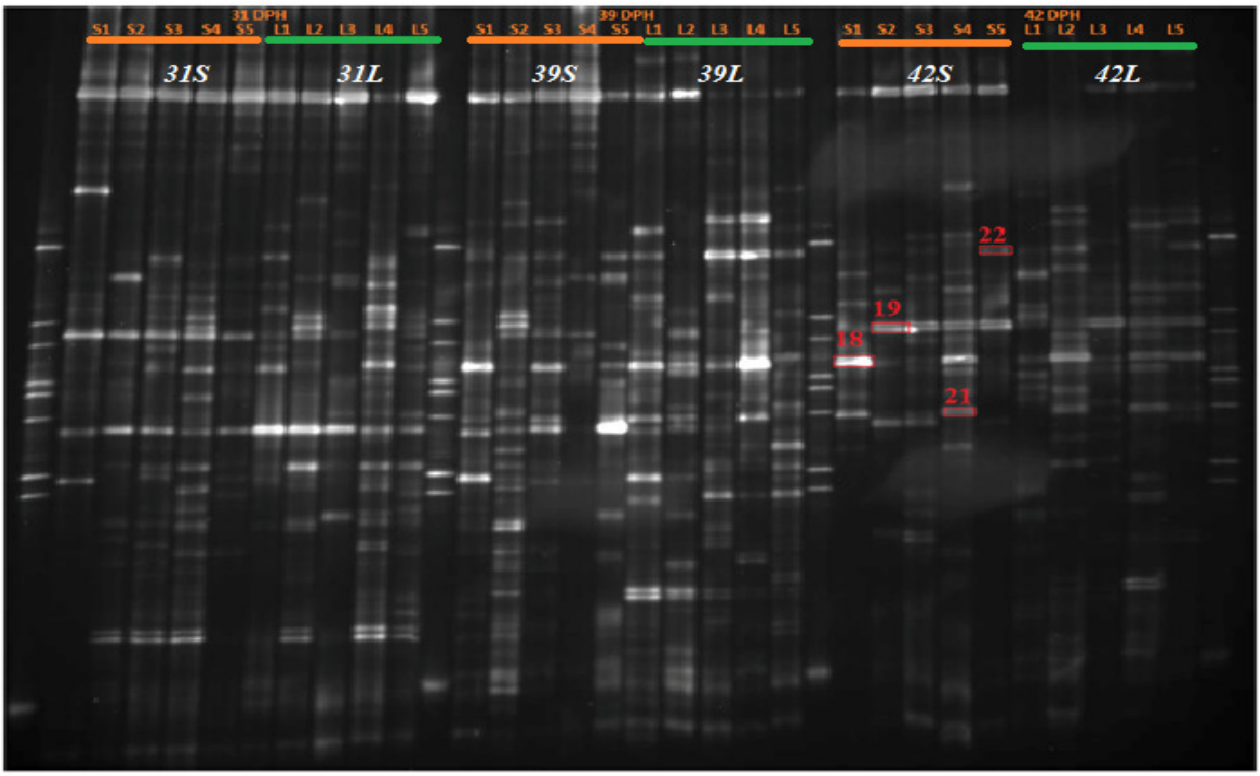

Figure 3.17: Denaturing gradient gel electrophoresis (DGGE) profiles for PCR-amplified 16S rDNA fragments from microbial communities of large (L) and small (S) cod larvae at 31, 39 and 42 dph. The marked and numbered bands are those which were excised and sequenced to assign bacterial taxa

Table 3.12: Taxonomic assignments of DGGE bands for cod larvae microbiota at 31, 39 and 42 dph. Band numbers refer to Figure 3.17. S refers small larvae and L refers to large larvae

Band no.	Observed in	Phylum	Class	Genus*
18	31S/31L/39S/39L/42S/42L	Proteobacteria	Gammaproteobacteria	*Leucothrix*
19	31S/31L/39S/39L/42S/42L	Proteobacteria	Epsilonproteobacteria	*Arcobacter*
21	39S/39L/42S/42L	Proteobacteria	Gammaproteobacteria	*Pseudoalteromonas*
22	31S/31L/39S/39L/42S/42L	Bacteroidetes	Flavobacteria	*Soonwooa*

**The lowest taxonomy level at confident threshold > 50% - as determined by Classifier - RDP*

The results of average richness, Shannon diversity and evenness were calculated for small and large larval groups for these three days (Table 3.13). The band richness in small larvae at 39 dph had very high fluctuation (SD= ± 9.6). There were no significant differences in the diversity indices between small and large larvae at all three days ($P > 0.05$, Table 3.14), except for the evenness between small and large larvae at 42 dph ($P=0.002$)

Table 3.13: Average band richness (S), Shannon index (H) and evenness (J) calculated from DGGE profiles of both small (S) and large (L) larvae at 31, 39 and 42 dph

DPH	31L		31S		39L		39S		42L		42S	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
S	24.4	4.2	23.4	6.2	28.4	3.1	23.8	9.6	15.8	2.9	14.4	4.7
H	2.90	0.20	2.86	0.30	3.11	0.12	2.86	0.40	2.62	0.19	2.37	0.37
J	0.76	0.05	0.77	0.05	0.79	0.06	0.78	0.07	0.88	0.04	0.78	0.04

Table 3.14: ANOVA analysis for testing the differences in diversity indices between large (L) and small (S) larvae at 31, 39 and 42 dph

		ANOVA P value		
		Band richness	**Shannon index**	**Evenness**
Between groups	**31S vs 31L**	0.77	0.79	0.725
	39S vs 39L	0.34	0.22	0.768
	42S vs 42L	0.59	0.22	**0.002**

The average similarities of gut microbial community within and between larval groups (Figure 3.18) revealed that larval microbial community of large individuals at 31 dph had highest average similarity (around 0.48). In addition, the similarity within small larvae at 39 dph was lowest compared to those at 31 and 42 dph, indicating more variables in microbial communities among the individuals. Further, the average Bray-Curtis similarities are highe between small and larvae than within large individuals at 31 and 42 dph. This indicates there are more differences within large individuals than between small and large larvae at 31 and 42 dph.

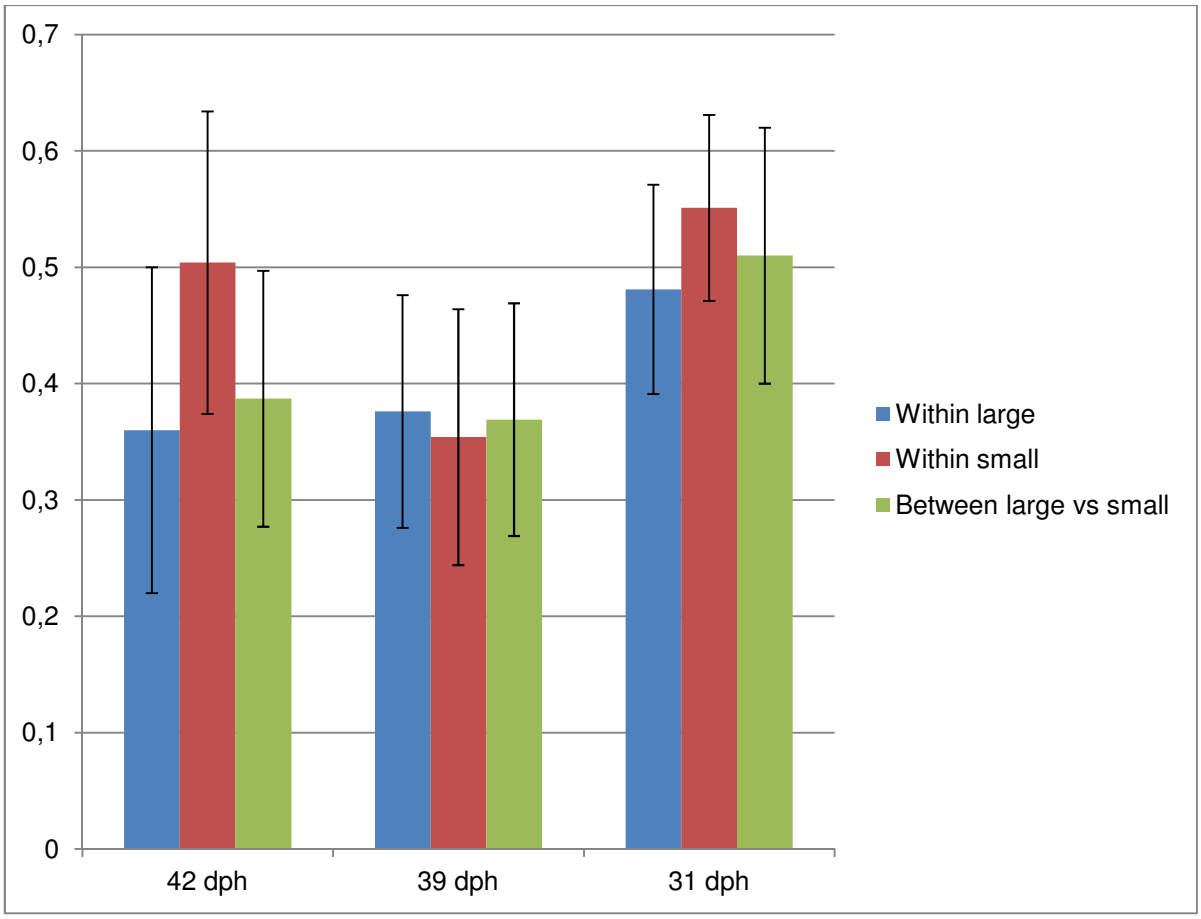

Figure 3.18: Average Bray-Curtis similarities for within and between groups of the microbial compositions of large and small larvae at 31, 39 and 42 dph

The overlapping between groups of large and small larvae at all three sampling days can be seen in both NMDS plots (Figure 3.19), and one-way ANOSIM analysis confirmed that there were no significant differences in microbial community composition between them ($P>0.05$)

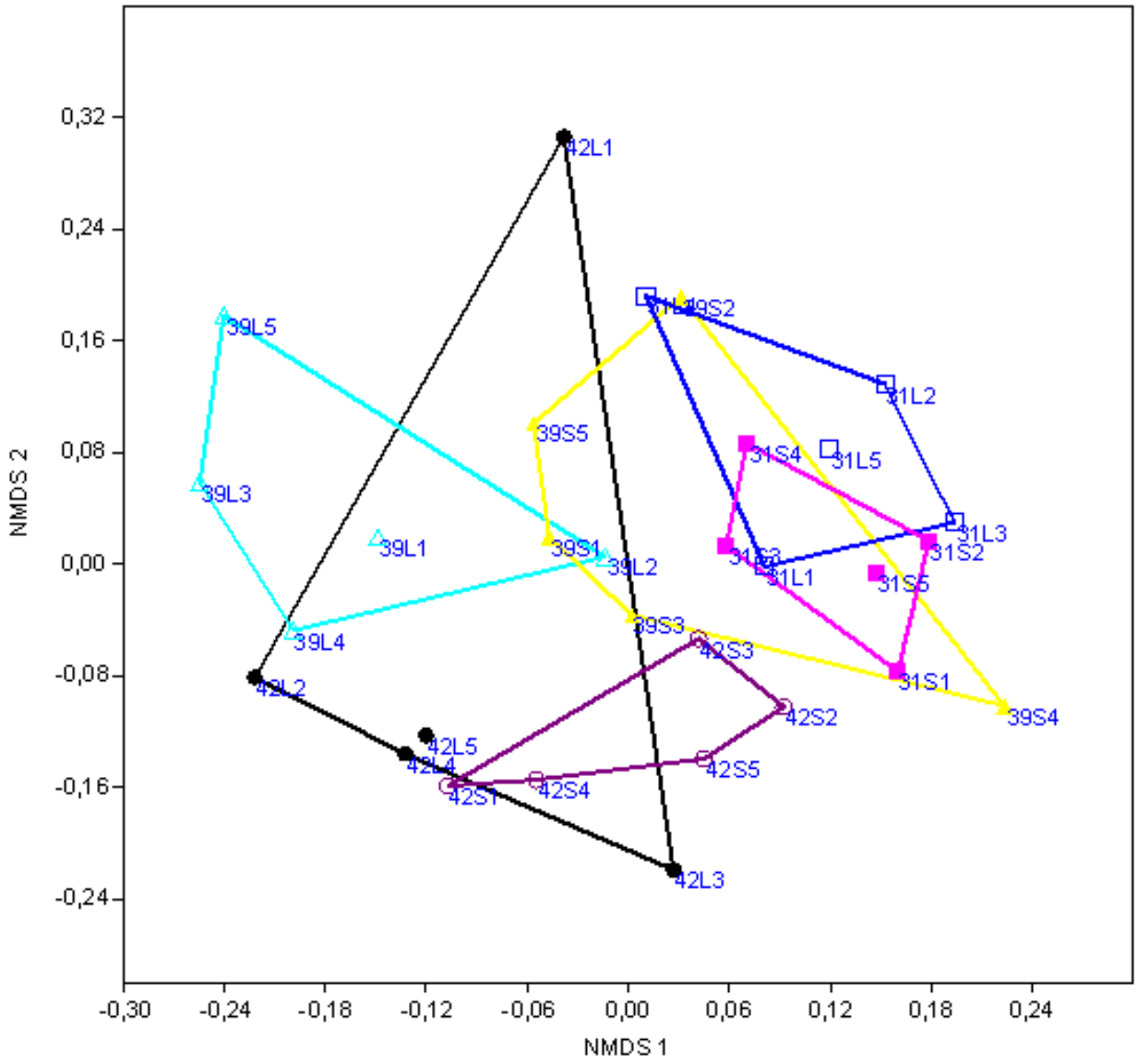

Figure 3.19: NMDS ordination based on Bray-Curtis similarities for microbiota of small (S) and largae (L) larvae at 31 (square and filled squared), 39(triangle and filled triangle) and 42 dph (circle and filled circle)

3.3. The correlations of the gastrointestinal microbial community composition of cod larvae with their growth and age

According to the Figure 3.1; 3.2 and 3.3, there were a clear separations in weight, length and height of small compared to large larvae at the same age, which was contributed to the differences in larval growth rate. As mentioned in section 3.1, the microbiota of 10 individuals for each sampling time (7, 10, 14, 17, 21, 24, 28, 31, 39, and 42 dph) was analyzed. The larval microbiota between each day was compared. The ordinations by NMDS based on Bray-Curtis similarity indicated that larval microbiota in the gut of cod larvae differed between sampling

days (Figure 3.20). This was confirmed by one-way ANOSIM analysis that revealed significant differences in the microbiota between the 10 groups ($P < 0.05$, Table 3.15).

Table 3.15: One-way ANOSIM analysis of the microbial community between the 10 larval groups; dph refers to days post hatching

		R value	***P value***
Between groups	7 dph vs 10 dph	0.8182	0.0002*
	10 dph vs 14 dph	0.5739	0.0003*
	14 dph vs 17 dph	0.5842	0.0001*
	17 dph vs 21 dph	0.2996	0.0014*
	21 dph vs 24 dph	0.5511	0.0002*
	24 dph vs 28 dph	0.6244	0.0001*
	28 dph vs 31 dph	0.1891	0.0151*
	31 dph vs 39 dph	0.3813	0.0002*
	39 dph vs 42 dph	0.2353	0.0034*

***: p<0.05**

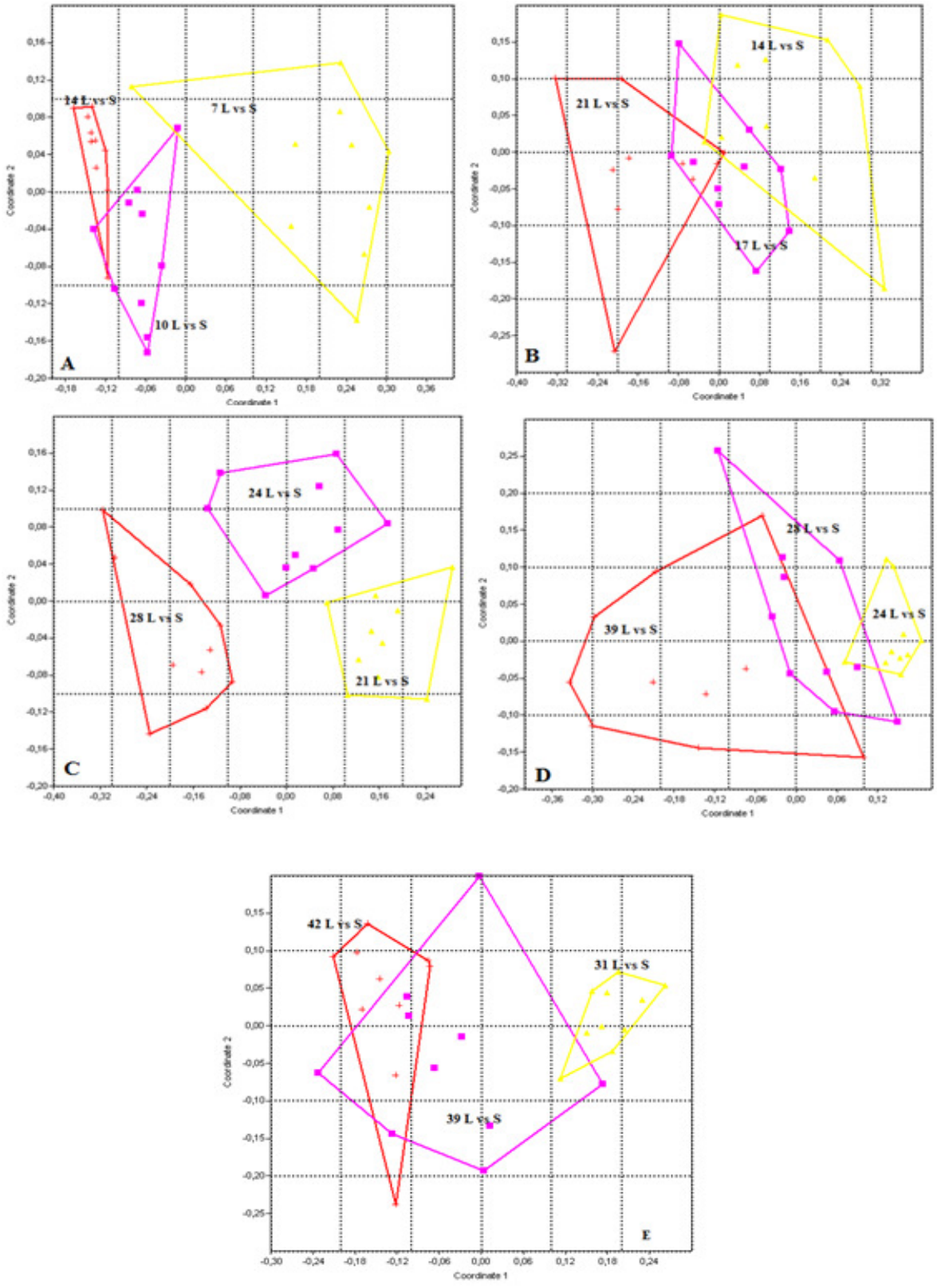

Figure 3.20: Non-metric MDS of Bray-Curtis similarities for comparison of gut microbiota of cod larvae sampled at ten different sampling days (A); 14-17-21 dph (B); 21-24-28 dph (C); 28-31-39 dph (D); 31-39-42 dph (E). S and L refer to small and large larvae, respectively**.**

The size (in terms of weight of the same old individuals) and the age of the larvae (larvae at different developmental stages) were reconstructed based on microbial community composition using factor analysis and environmental regression (CABFAC). The model revealed that both size and age were correlated to intestinal microbial composition of individual larvae. However, the variation in gut microbiota was less impacted by the size of the larvae than by host age (Figure 3.21). This is indicated by the lower value of coefficient of determination (R^2) in the reconstruction found for weight versus reconstruction based on age. For weight, it is observed that $0.65 < R^2 < 0.79$ (Figure 3.21 A, B, C), while the age of the larvae explained at least 82% of the variations in microbiota of the larvae ($0.82 < R^2 < 0.89$, Figure 3.21 F, G, H). However, this is not reflected in the last two gels, where the weight and age of the larvae at 28, 31, 39 and 42 dph have similar impacts on bacterial composition with the similar determination coefficient of 0.85 (Figure 3.21 D, E and I, J). The significance of weight versus age on the composition of the gut microbiota was also analyzed by plotting Bray-Curtis similarities of two-and-two larvae versus the difference in age and weight, respectively (Figure 3.22). A negative correlation is showed between Bray-Curtis similarity of two-and-two larvae and difference in age and weight of the larvae, but with a low correlation coefficient ($R^2 < 0.34$). However it is still possible to say that the similarity of microbial community in gut larvae decreased with the increase of their age.

Two-way NPMANOVA confirmed that the age (DPH) has more strongly effects on the gut microbiota of larvae than their size (in term of weight).

Table 3.16: Two-way NPMANOVA test of the effect of age (DPH) and size of same old larvae (L vs. S) on the composition of the gut microbiota

D7-10-14	**F**	**P-value**
DPH	11,803	**0,0001**
L vs. S	1,0225	**0,3617**

D14-17-21	**F**	**P**
DPH	4,4779	**0,0001**
L vs. S	3,2771	0,0018

D21-24-28	**F**	**P**
DPH	6,8038	**0,0001**
L vs. S	0,95572	**0,455**

D28-31-39	**F**	**P**
DPH	5,2661	**0,0001**
L vs. S	1,4367	**0,1683**

D31-39-42	**F**	**P**
DPH	4,2476	**0,0001**
L vs. S	2,5893	**0,0076**

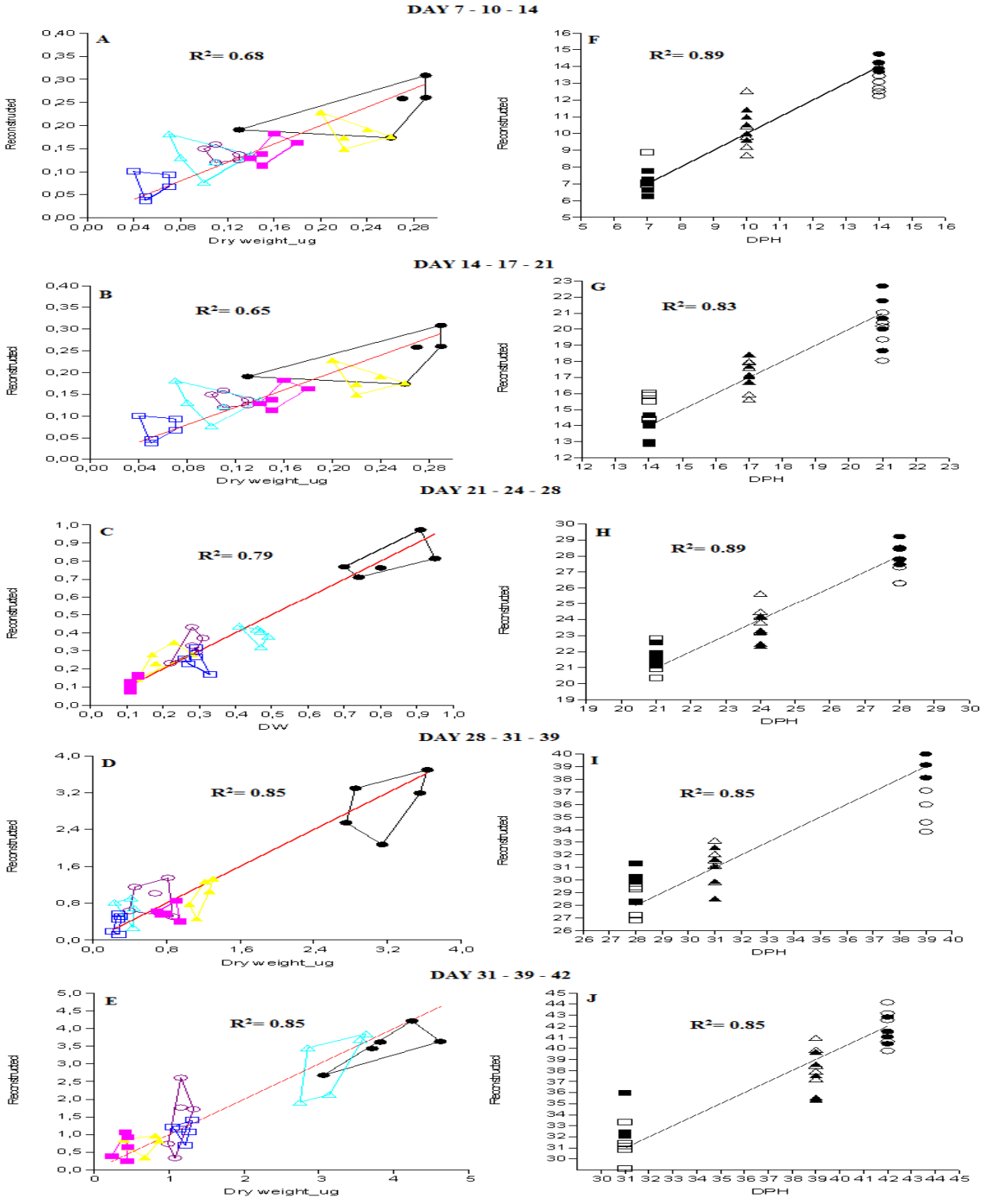

Figure 3.21: Reconstruction of weight and age data of cod larvae based on intestinal microbial community composition using factor analysis and environmental regression (CABFAC). DPH refers to days post hatching

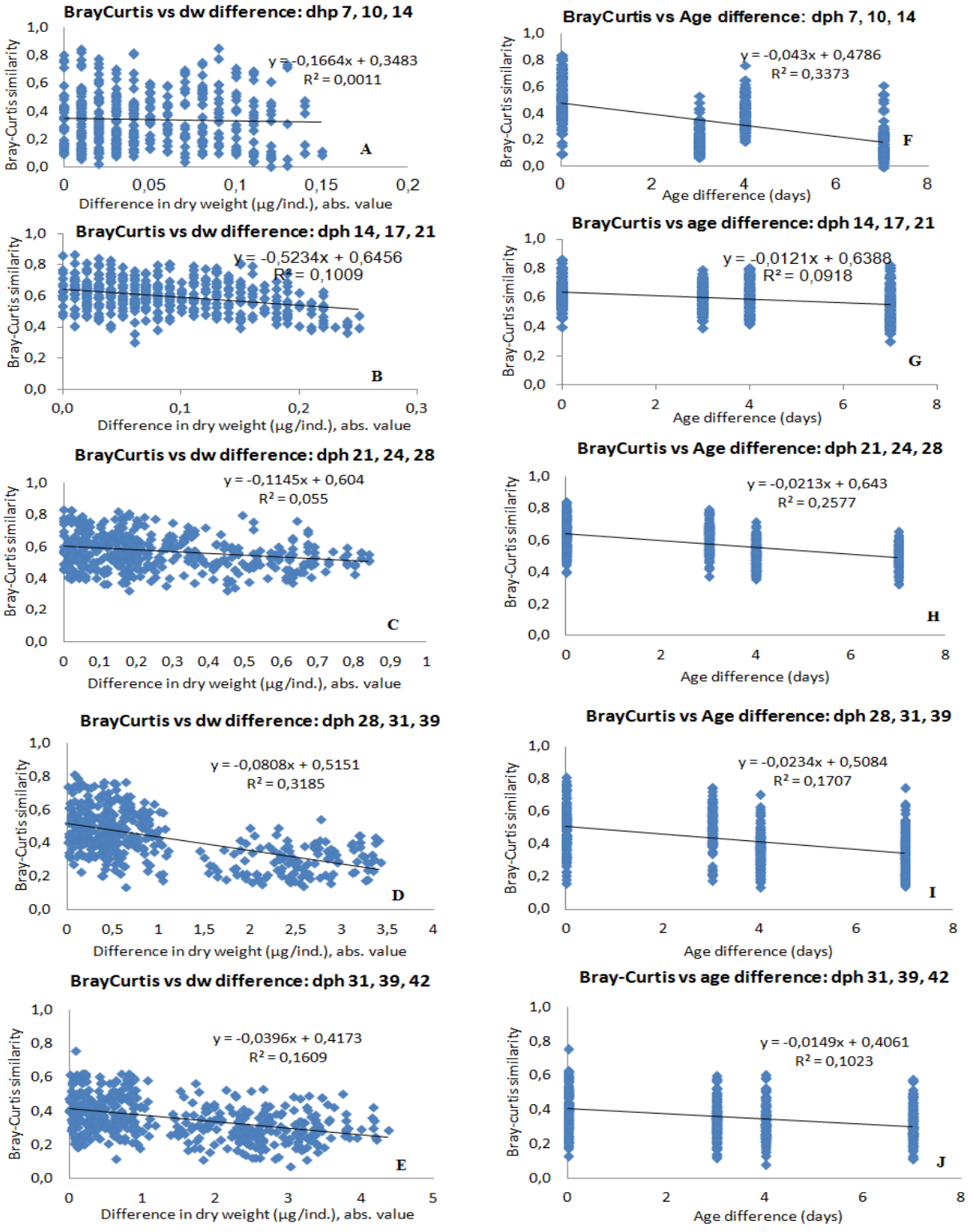

Figure 3.22: The correlations between individual Bray-Curtis similarities calculated from microbial composition of the larvae and the difference in both age and size

4. Discussion

4.1. Evaluation of the methods for characterization of microbial community

The intestinal microbial communities of cod larvae were examined using the PCR-DGGE molecular method. Nested PCR was applied in this study to avoid co-amplification of eukaryotic DNA (Bakke, De Schryver et al. 2011). The sequencing results of excised DGGE bands revealed that the nested PCR was successful because most of the bands were assigned to bacterial taxa. In general, good quality gels were obtained with clear separation of bands**.** However, DGGE which is mainly used in this experiment is only a semi-quantitative method. Muyzer (1998) mentioned that DNA sequence heterogeneity of one bacterium is one of the main problems associated with DGGE method.

In the present study, a total of 100 cod larvae were sampled at different days post hatching and analyzed on 5 DGGE gels. Thus the larval sampled at day 14, 21, 28, 31 and 39 were analyzed twice in two different gels. The comparison of the intestinal microbial community of small and large individuals at day 21 on two gels (Figure 3.7 and 3.9), differences in diversity was observed due to the appearance of some more weak bands in Figure 3.9 not being visible in Figure 3.7. This could be explained by better staining and more PCR products on the gel in Figure 3.9, leading to a little higher band richness compared to the larvae samples in Figure 3.7. Thus, between- gels comparisons should be avoided.

In this study, the sequences obtained from isolated bacteria and the DGGE bands were analyzed with the Classifier tool in the Ribosomal Database project for comparison with nucleotide sequences in the database. Theoretically, the different bands in the DGGE gel represent different sequences because they have different melting behavior. However, one problem occurred in the DGGE gel of day 7-10-14 (Figure 3.5 and Table 3.1). Surprisingly two different bands (numbered 9 and 10) far down in the gel were assigned to represent the same class of Actinobacteria with the genus *Propionibacterium.* Sequence alignment showed that the two sequences are identical (Figure 4.1). The exact cause behind this is unknown, but potential reasons may be anomalous melting behavior or secondary structure formation of DNA single strands (Thornhill, Kemp et al. 2010).

```
38  Gel1   9 TGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGG 60
39  Gel1  10 TGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGG 60
             ************************************************************
38           CCTTCGGGTTGTAAACCGCTTTCGCCTGTGACGAAGCGTGAGTGACGGTAATGGGTAAAG 120
39           CCTTCGGGTTGTAAACCGCTTTCGCCTGTGACGAAGCGTGAGTGACGGTAATGGGTAAAG 120
             ************************************************************
38           AAGCACCGGCTAACTACGTG 140
39           AAGCACCGGCTAACTACGTG 140
             ********************
```

Figure 4.1: Aligned sequences of 16S rDNA amplicons excised from band number 9 and 10 in Gel 1: Day 7, 10, 14 dph larvae

Another problem was observed for bands number 11 and 12 on the gel of day 7-10-14 (Figure 3.5). These are the most dominating bands and both assigned to the class of Epsilonproteobacteria and the genus *Arcobacter.* However, Sequence alignment of these 'upper" and 'lower" bands in the gel represent different sequences (Figure 4.2). Further, comparison of the 16S rDNA sequences with the nucleotide sequence available in Ribosomal Database Project revealed high similarity of the sequence from band 11 with the value of 91 %, while only 74% of similarity observed in band 12. Furthermore, our results show that sequenced bands were only assigned to the level of genus. Therefore, one more explanation for this case might be that these sequences represent to the same genus, but with different species.

```
41 Gel1 12  TGGGGAATATTGCACAATGGACGAAAGTCTGATGCAGCAACGCCGCGTGGAGGATGACAC 60
40 Gel1 11  TGGGGAATATTGCACAATGGAGGAAACTCTGATGCAGCAACGCCGCGTGGAGGATGACAC 60
            ******************** **** ********************************
41 Gel1 12  ATTTCGGTGCGTAAACTCCTTTTATATGGGAAGATAATGACGGTACCATATGAATAAGCA 120
40 Gel1 11  ATTTCGGTGCGTAAACTCCTTTTATATAAGAAGATAATGACGGTATTATATGAATAAGCA 120
            *************************  ***************  *************
41 Gel1 12  CCGGCTAACTCCGTG 135
40 Gel1 11  CCGGCTAACTCCGTG 135
            ***************
```

Figure 4.2: Aligned sequences of 16S rDNA amplicons excised from band number 11 and 12 in Gel 1: Day 7, 10, 14 dph larvae

The amplified 16S rDNA fragments of two different band 22 and 23 on the gel of day 28-31-39 (Figure 3.11) were sequenced and assigned to the same genus *Roseibium.* This could be explained by a little "messy" on the sequencing result of the band 23 as shown in the Figure 4.3, which revealed a very low similarity percentage of only 51%.

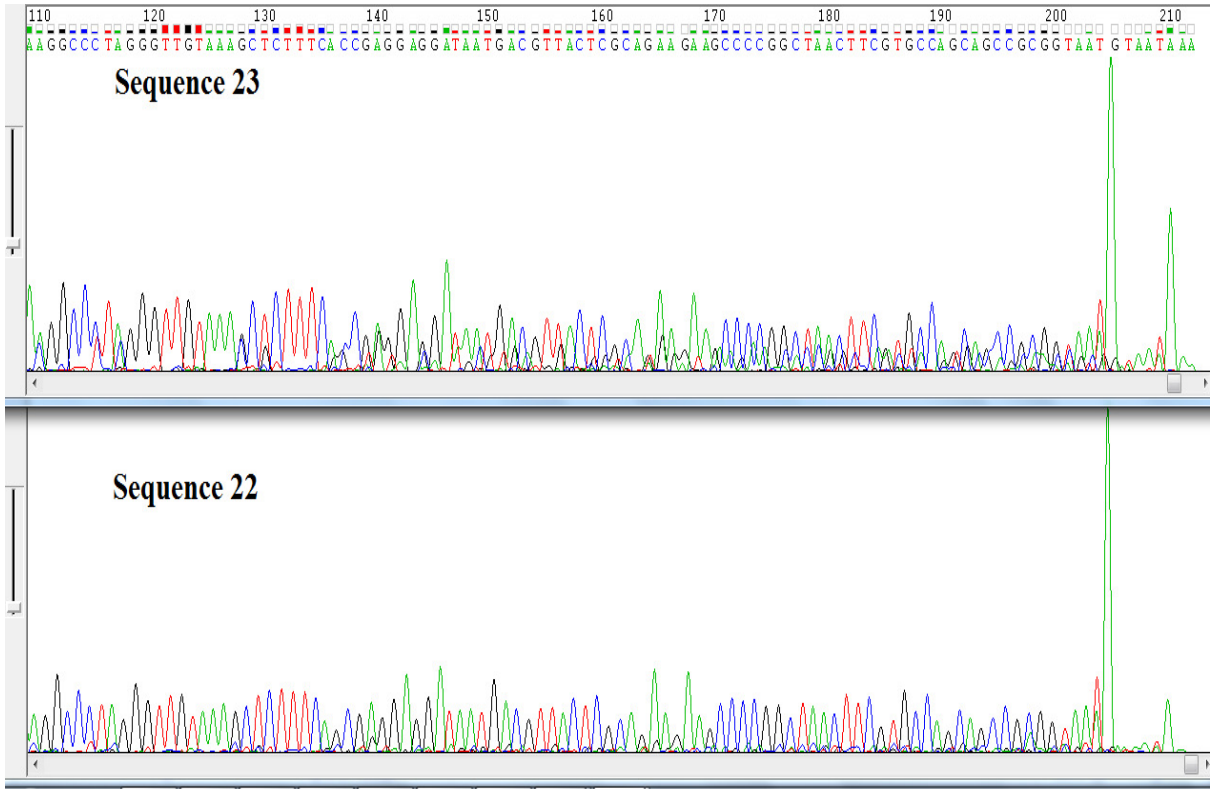

Figure 4.3: The sequences of the 16S rDNA gene of a Roseibium strain isolated from the band number 22 and 23

According to these problems, it can clearly to say that although 16S rDNA gene sequencing is highly useful and convenient in regards to bacterial classification, sometimes it fails to identify some bacteria and for the excised bands, the taxonomy could only be identify to the genus level.

4.2. Comparisons of the intestinal microbial community of small and large cod larvae at the same age

This study was dependent upon growing fish individuals with a sufficient difference in size. Significant differences in larval length, weight and height were obtained between the two groups of small and large larvae at all ten different sampling days (Figure 3.1; 3.2; 3.3). Therefore, these larval samples were applicable for this study.

By characterizing the microbial communities associated with small and large larvae, we were able to statistically test for possible differences in the microbiota of small and large individuals at

the same age. In general, our results indicate significant differences in the larval gut microbiota between small and large individuals of the same ages were detected for 4 of 10 ages (14, 21, 28 and 39 dph). Thus, the results presented in this study do not support the hypothesis of cod larval intestinal microbiota as a factor contributing to the difference in their growth rate (i.e. differences in size for larvae at the same age). The experiment of Sjulstad (2011) showed that size of Atlantic cod larvae was strongly correlated to their intestinal microbiota. However, this might due to the fact that only larvae at 43 days after hatching were used in her study. Further, in humans (Bajzer, 2006) and mice (Ley *et al*., 2006; Turnbaugh *et* al., 2006) important differences in the intestinal microbial communities were recorded between lean and obese individuals. There are some factors which may contribute to this inconsistency. The potential reason could be that these studies were performed for adult stages, whereas our experiment studied the early developmental stages of cod larvae. Further, fish in contrast to terrestrial animals have closer contact with the microbiota in the environments due to their aqueous habitat. The bacteria in the ambient environment are continually ingested with water by the fish gut, but some of them probably inhabit in fish intestine temporarily, and then are ejected by the fish (Hansen and Olafsen 1999). Therefore, these bacteria may not contribute to the microbial differences between small and large larvae at the same age.

When inspecting the DGGE band patterns of small and large larvae at day 7 (Figure 3.5), a gastrointestinal microbiota without dominating band was revealed by high evenness indices (0.84 and 0.86, Table 3.1) and a high number of bands. Based on the taxonomy assignment (Table 3.1), it is clearly seen that Firmicutes and Actinobacteria appeared with similar abundance in both small and large individuals on this day. Further, the presence of Proteobacteria with the genera *Arcobacter* in both groups of small and large individual at most sampling days, except day 7, 39 and 42, is consistent with the previous study by Sjulstad (2011).

For the taxonomic assignment of DGGE bands, two most predominant bands in both small and large individuals at day 10, 14, 17, 21 and 24 are represented by the same phylum of Epsilon-proteobacteria (*Arcobacter*) and with similar abundances. However, there is a change in the difference between small and large larvae from day 28 to the end of the experiment, which is related to the lower abundance of *Arcobater* and appearance of new species (e.g. *Sulfitobacter*). Microbial communities for both small and large larvae at day 28 changed from the previous sampling times with the appearance of a new genus of Alpha-proteobacteria (*Sulfitobacter*), but it seemed to be in higher abundance in large individuals.

The DGGE patterns at day 31, 39, 42 revealed larval intestinal microbial communities with more variations among individuals and higher number of bands. Still some differences were observed at the species level between small and large larvae at day 31 and day 39. Example of this is Firmicutes (band number 24, 35 and 37, Figure 3.11) which seems to be appeared at higher abundances in the large larvae. Ley *et al.* (2006b) showed that the differences in GI microbiota between lean and obese mammals were related to the relative abundance of the bacterial divisions Firmicutes. Lean individuals have lower abundance of Firmicutes compared to obese individuals. Furthermore, *Aliivibrio* sp. (Band 2, Table 3.6) was only observed in DGGE profiles of small cod larvae at day 14 even at lower abundance. This species is a pathogenic bacteria causing disease in Atlantic cod (*Gadus morhua*) (Schrøder et al. 1992), and could have contributed to the lower growth rate of these larvae in comparison to the large larvae at the same age. However, the number of bands sequenced in this study is low; therefore, the obtained sequencing results just give an idea about the composition of the microbiota in larval intestine rather than conclusion about whether specific taxa were associated with small and large growth rate.

4.3. The effect of growth rate and age on the gastrointestinal microbial community composition of cod larvae

It was demonstrated in this study that there was a negative correlation between the Bray-Curtis similarity in microbial composition of two-and-two larvae and difference in their age and size, but with low value of coefficient determination (R^2<0.34) (Figure 3.22). The intestinal bacterial communities of the larvae changed with increasing of size (in term of weight) and age of the larvae. In concordance with these findings, Yan *et al.* (2012) concluded that the developmental stage of Zebrafish was a significant predictor of intestinal microbial turnover. The difference in age and size between larval groups implies that the gastrointestinal system could be at different stages for larvae at different age and size (Dabrowski 1984). The increasing physiological complexity of the developing larval gastrointestinal tract might also contribute to a change in microbial environment in the intestine (Cahill 1990; Bergh 1995; Olafsen 2001), possibly allowing the development of some more niches in the larval intestine. Ringø (1999) and Hansen *et al* (1999) also reported that microbial communities of marine fish changes during their developmental stages, and probably is affected by the structure of intestinal tract. Besides, the more developed larvae will have higher ability to fight opportunistic pathogens as a consequence of the development of their immune system, which could influence on their microbial composition.

The results from Figure 3.21 shows that the variation in gut microbiota was less impacted by the size than by host age of the larvae at 7, 10, 14, 17, 21, 24 and 28 dph. However, the size and age of the larvae after 28 dph were found to have a similar impact on bacterial composition with high determination coefficients of 0.85 (Figure 3.16 D, E and I, J). It might be explained that the differences in size between small and large larvae was generally smaller for the younger larvae (up to 28 dph) than for the older larvae (from 31 to 42 dph). A higher difference in size found for the larvae from 28 to 42 dph implies that the larvae were more developed.

NMDS plots and statistical analysis showed that the intestinal microbial community was significantly different between each sampling day during the 42 days of the experiment (Figure 3.20 and Table 3.15). A decrease in complexity of the larval microbiota with age was indicated by DGGE banding patterns up to 28dph. A high number of bands were observed at onset of first feeding stage (larvae at 7 dph; Figure 3.5) then a reduction in band richness and diversity was observed up to 28 dph. After 28 dph, an increase in the richness and diversity of the larval microbiota with increasing age (Figure 3.14 and 3.17). Reitan et al. (1998) mentioned that marine larvae drink water before yolk-sac is consumed, thus the intestinal bacterial flora is influenced by bacteria in the surrounding water at early stage. However, the number of total intestinal bacteria increases substantially as the larvae start eating (Eddy and Jones, 2002). The DGGE profiles of the larvae at day 10 to day 24 post hatch (Figure 3.5, 3.8, and 3.11) show that the intestinal microbiota of the larvae during these days has not changed much and was strongly dominated by a few species, e.g., two most strong bands appeared in DGGE gels represented *Arcobacter*. The changes in number of bands and banding positions indicate that new bacterial strains were established in the samples taken after day 24, leading to the increase in complexity of microbial community, further some bands became more abundant, while others disappeared. This change in bacterial compositions could be explained by a more developed gut, possibly offering more niches to the intestinal microbiota. This change could also be a consequence of starting to feed the larvae with *Artemia*. (Table 2.1), so the intestinal microbial community might be affected by the influx of bacteria, associated with live feed organisms (Bergh, Naas et al. 1994). However, it is difficult to interpret whether the changes in intestinal microbiota of cod larvae were caused by live feed or other factors because we did not analyse the microbiota from feed samples due to limited time of doing the experiment.

5. Conclusions

a. This experiment revealed that significant differences in the intestinal microbial communities between small and large cod larvae of the same age was found only for 4 of the 10 ages examined from 7 to 42 dph (14, 21, 28 and 39 dph). Thus our study indicates that the composition of gut microbiota is not a major contributor to the differences in the growth rate of equally old larvae.

b. Some of the DGGE bands were sequenced to assign their taxonomy. However, the number of bands sequenced in this study is relatively low. Therefore, it was not possible to conclude whether specific taxa were associated with low and high growth rate.

c. It seems to be a general conclusion that the intestinal microbial composition of individual larvae were impacted by both growth rate and age of the larvae, which is revealed by the reconstructions of larval age and weight based on intestinal microbial community composition, and also by a negative correlation between Bray-Curtis similarity of two-and-two larvae and difference in age and size of the larvae. It means that the decrease in the similarity of two-and-two larvae was found with the increase of larval age or size. However, the variation in gut microbiota was less impacted by the size of the larvae than by host age up to 28 dph, whereas it was similarly affected by both age and size after 28 dph.

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Appendices

Appendix 1: Modified protocol for DNA extraction following the Powersoil® DNA Isolation Kit

1. Add 60 µl of Solution C1 to Power Bead tube and then vortex briefly
2. Add the fish larvae to the Power Bead tube
3. Vortex at maximum speed for 10 minutes
4. Incubate tubes for 10 minutes at 65°C, and then for other 10 minutes at 95°C
5. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature
6. Transfer approximately 400-500 µl of the supernatant to a clean 2 ml Collection Tube
7. Add 250 µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes
8. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g
9. Avoiding the pellet, transfer around 600 µl of supernatant to a clean 2 ml Collection
10. Add 200 µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes
11. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g
12. Transfer around 700 µl of supernatant into a clean 2 ml Collection Tube
13. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds
14. Load approximately 675 µl onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature
15. Add 500 µl of Solution C5 and centrifuge at room temperature for 30 sec at 10,000 x g
16. Discard the flow through.
17. Centrifuge again at room temperature for 1 minute at 10,000 x g
18. Carefully place spin filter in a clean 2 ml Collection Tube
19. Add 100 µl of Solution C6 to the center of the white filter membrane
20. Centrifuge at room temperature for 30 seconds at 10,000 x g
21. Discard the Spin Filter. The DNA is now ready in the tube
22. Quantify DNA concentration with Nanodrop

Appendix 2: Recipes for solutions used in DGGE

1. **Denaturing solution 0%**

   8% acryalmide in 0.5 x TAE (per 250ml):

40% acrylamide solution	50ml
50 x TAE	2.5ml

   Store the solution at 4^{o}C, protect from light

2. **Denaturing solution 80%**

   8% acryalmide in 0.5 x TAE, 5.6 M urea, and 32% formamide 0.5 x TAE (per 250ml):

40% acrylamide solution	50ml
50 x TAE	2.5ml
Deionized formamide	84g

   Store the solution at 4^{o}C, protect from light. This solution must be filtered before pouring the gel

3. **50 x TAE buffer**

Tris-HCl	242g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

   dH_2O up to 1000 ml. The buffer was then sterilized by autoclaving.

4. **SYBR Gold staining solution**

SYBR Gold	4 µl
TAE (50 x)	600 µl
MiliQ water	30 ml

5. **APS 10%**

   Dissolve 10g of ammonium persulfate in 100 ml miliQ water.

   Then sterile filter the solution, distribute in eppendorf tubes (250 µl in each), and then keep frozen.

Appendix 3: Protocol for reamplification and purification of PCR products

1. Re-amplification of DGGE bands

10 x reaction buffer: 2.5µl

dNTP (10mM each): 0.5µl

MgCl2 (25mM): 0.5µl

BAS: 0.75µl

Primer fwd (10µM): 0.75µl

Primer revs (10µM): 0.75µl

Taq pol.: 0.125µl

H_2O: 19µl

- ✓ Template (from gel elute): 1µl
- ✓ Vortex and spin down tubes with band material prior to addition to the new PCR reaction.
- ✓ Primers: 338F-GC-M13+518R

PCR program:

95^{o}C 3 min

90^{o}C 30 sec

5^{o}C 30 sec 40 cycles

72^{o}C 1min

72°C 10min

10°C hold

- ✓ Check amounts of product on 1% agarose gel
- ✓ A total volume of 15 µl should be sent for sequencing at Eurofins MWG.
- ✓ For bands with good yield, use 5µl PCR product + 10µl water

2. Purification PCR product

- ✓ Add 5µl buffer PN to 50µl PCR product and mix
- ✓ Place a QIA quick spin column in a provided 2ml collection tube
- ✓ Apply the sample to the QIA quick column and centrifuge for 1min at 13.000 rpm
- ✓ Discard the flow-through and put QIA quick column back into the same tube
- ✓ To wash QIA quick column, add 750µl of buffer PE and centrifuge for 1 min at
- ✓ 13.000 rpm
- ✓ Discard the flow-through and place the QIA quick column back in the same tube and centrifuge foe additional 1 min at 13.000rpm
- ✓ Place QIA quick column in a clean 1,5ml micro-centrifuge tube
- ✓ To elute DNA add 30µl sterile water to the center of the QIA quick membrane and centrifuge the column for 1 min at 13.000 rpm