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# Effect of water treatment systems on gut microbial community in reared larvae of Atlantic Cod (Gadus morhua)

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*Master's Thesis in Marine Coastal Development*

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**Trondheim, May 2012**

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**Thi My Hanh Truong**

## Abstract

Interactions between bacteria and fishes affect health and survival, especially during the first fish growth stage. The establishment of the gut microbial in fishes is believed to be influenced by the microbial composition of the rearing water.

The purpose of this study was to examine whether different water treatment systems can be used as a tool to obtain microbial control in the rearing of cod larvae. More specifically the aim was to investigate to what extent the water treatment systems influence on the rearing water microbiota and the cod larval microbiota. This was tested by comparing the microbial community structure of the rearing water and the larval microbiota from three water treatment regimes: a flow-through system (FTS), a microbial maturation system (MMS) and a recirculation aquaculture system (RAS). The microbial communities of MMS and RAS systems are typically dominated by K-selected species or non-opportunity bacteria, whereas microbial communities of FTS systems are probably predominated by r-selected or opportunistic bacteria. For each system cod larvae were reared in three replicate tanks.

A PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) methodology was applied to characterize the microbial communities in this study. Total DNA of water, feed and larvae samples were extracted, and used as template in PCR to amplify a fragment encompassing the variable region 3 of the highly conversed bacterial 16S rRNA gene. Multivariate statistics based on the DGGE profiles were used to compare microbial communities.

The results indicated that there were significant differences between microbial community (MC) of water as well as of larval MC from the three different water treatment systems. The rearing water MC in RAS were mainly influenced by the incoming water MC, while the rearing water MC in MMS and FTS were more similarities with the MC of the incoming water and the feed. Moreover, the water and the larval MC in RAS and MMS were more stable over time compared to FTS. The larval MC was a major determined by the rearing water MC. Therefore, the water treatment systems can control the rearing water MC, and systems with K-selection give more stable and reproducible MCs. These treatment systems can be also used for controlling larval MC.

## Table of Contents

## Abbreviations

| | |
|---|---|
| ANOSIM: | analysis of similarity |
| APS: | ammonium persulphate |
| DGGE: | denaturing gradient gel electrophoresis |
| DNA: | deoxyribonucleic acid |
| dph: | day post hatching |
| FTS: | flow-through system |
| H': | Shannon diversity index |
| J': | evenness index |
| MC: | microbial community |
| MMS: | microbial maturation system |
| MS22: | tricaine methane sulphonate |
| NMS: | non-metric multidimensional scaling |
| PCR: | polymerase chain reaction |
| RAS: | recirculation aquaculture system |
| RDP: | ribosomal database project |
| S: | band richness |
| SE: | Standard error |
| TAE: | tris base, acetic acid and EDTA |
| TEMED: | tetramethylethylenediamine |

## List of figure

## List of table

# 1. Introduction

Atlantic cod (*Gadus morhua*) is one of the well-known cold-water species and an important commercial fish species that has been traded for a millennium on 4 continents (Kurlansky 1999). It is forecasted that Atlantic cod will become the second most economically important marine finfish species, after Atlantic salmon farming in Europe (Jørstad *et al*). It has been known as a good candidate for aquaculture (Svåsand *et al*., 2004). Atlantic cod on the market is supplied by both fisheries and farming. However, the wild stocks of Atlantic cod have been reduced steadily throughout the past few decades (Figure 1.1). Parallel with the declining of wild stocks, considerable efforts have been put into developing Atlantic cod farming (Svåsand *et al*., 2004; it was demonstrated in Figure 1.2).

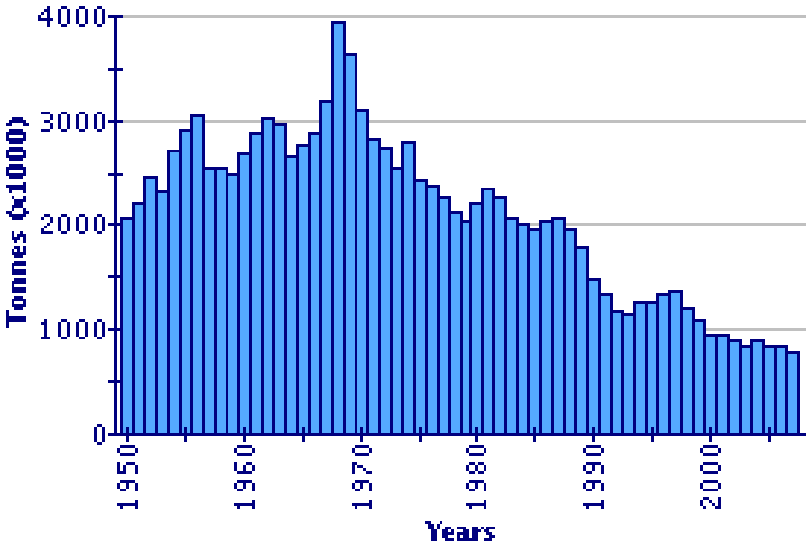



**Figure 1.1: Global Capture production for G*adus morhua* from 1950 to 2009**
*(FAO Fishery Statistic)*

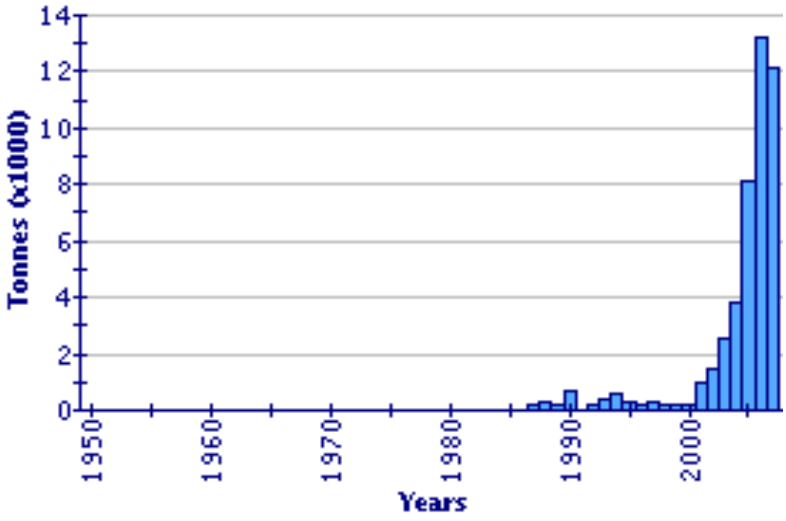



**Figure 1.2: Global Aquaculture production for *Gadus morhua* from 1950 to 2009**
*(FAO Fishery Statistic)*

The cod products on the market come mostly from European water. For example in 2008 the European countries supplied 95% in the total cod product of the world, and Norway was the largest supplier (38%) followed by Russia (32%), Iceland (25%), Canada (4%) and US (1%) (Figure 1.3) (www.tradexfoods.com/reports).

In 2008, Norwegian aquaculture supplied the market with 18.000 tones of cod, which was higher than other species such as e.g. halibut (2000 tones) and char (500 tones) (Figure 1.4). The cod production was continuously improved in 2009 and 2010 correspond with 21.000 tones, whereas the halibut and chart provided for stable use during 3 years (2008-2010).

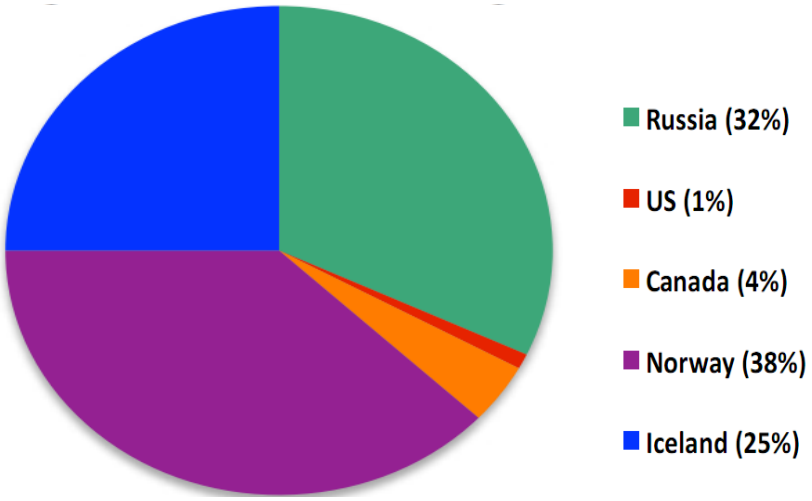



**Figure 1.3**: **Atlantic Cod global supply in 2008**
*(Food and Drug Organization of the United Nation)*

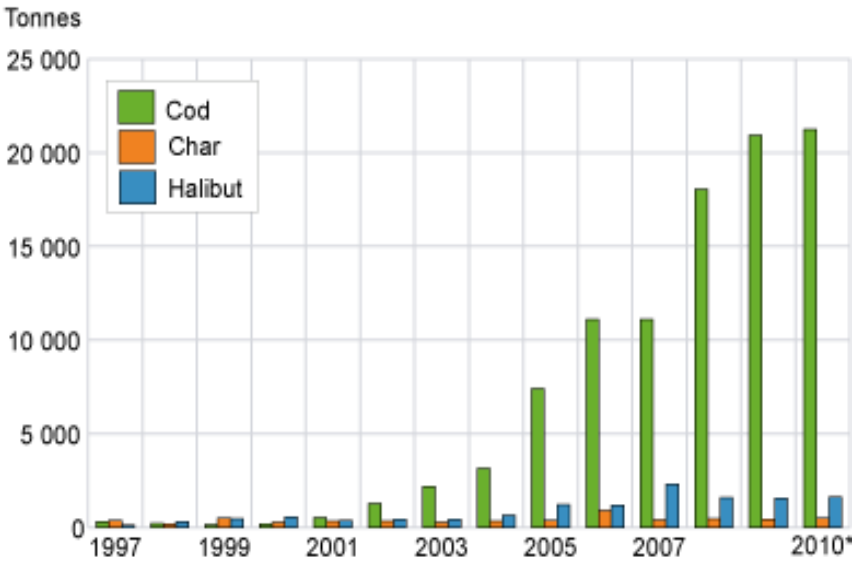



**Figure 1.4**: **Production of some fish farming fish in Norway.**
*(2011- Statistics Norway /http://www.ssb.no/akvakultur_en/)*

Generally, cod aquaculture in Norway has relatively large product quantity and quality. However, it still has some challenges, for example in terms of juvenile quality as well as large-scale fry production. One of the main challenges in fry production is high mortality

during the larval stage, especially at first-feeding (live feed) and weaning. Hence it is difficult to predict product of cod fry, and this causes adverse impacts on economic outcomes (Bergh *et al*., 2005; Lee *et al*., 2001; Mikkelsen *et al*., 2004 and Samuelsen and Bergh, 2004). Many reports showed that low quality juveniles significantly reduce the profitability for the farmers (Svåsand *et al*., 2004).

The huge mortality of larvae is related to several factors including feeding and nutrition, poor quality broodstock, culture condition (light, green water, larvae density) and disease control (gastrointestinal bacteria, probiotics, control of bacteria in rearing water) (Gimenez *et al*., 2008; Szkudlarek *et al*., 2007; Vine *et al*., 2006) and negative interactions between microbes in water and the larvae (Gatesoupe, 1999; Munro *et al*., 1995). Microbiology of the rearing water was of particular interest. The microbial communities of the rearing system may affect the survival, growth rate and quality of larvae. The characteristics of the microbial community of the rearing system will depend on several factors including the water treatment system. It is possible that the water treatment regimes influence microbial composition directly by killing or selection of potential opportunistic fish pathogens, or by providing condition for non-opportunistic and harmless bacteria (Vadstein *et al*., 2004). Opportunistic bacteria are typically r-strategists, with high growth rates, and are favored when there is excess of substrates and low level of competition. K-strategists are often non-pathogenic. They typically have lower growth rates and are favored when these is competition for nutrients. Since most pathogenic bacteria are r-strategist, the water treatment in aquaculture should always aim to favor the K-strategistic, non-opportunistic species, and avoid pathogenic opportunists (r-strategy). Two different water treatment systems used in marine fish rearing to select for non-opportunistic bacteria are microbial maturation of inlet water and recirculation system. In contrast to those, the flow-through system uses UV radiation or ozonation for disinfection, resulting in selection for r-strategists (Salvesen *et al*., 1999).

### 1.1. The water treatment systems used for rearing of fish larvae

- Flow - through system (FTS)

According to the traditional flow-through (FT) Aquaculture Systems, water is introduced into rearing tanks of culture system only once and is then discharged back to the aquatic environment (Rethink Inc and Canadian aquaculture system Inc, 2010), and filter method for

inlet water is simply UV radiation or sand filters. Almost all bacteria of the inlet water from the FTS system are removed, and then there will be a problem with recolonization and selection for r-strategists. Hence, high densities of fish and the live feed cause increased and oscillated organic load in fish tanks, at that time with the decline in competition among bacteria will promote growth of r-strategies (opportunities bacteria) (Hess-Erga *et al*., 2010). The flow of water through the culture system supplies oxygen to the fish and carries dissolved and suspended wastes out of the system. Maintaining water quality in culture system is obtained by replacing all the system water before the dissolved oxygen concentrations drop below minimum acceptable limits and concentration of contaminants (i.e. ammonia, solids, and carbon dioxide) accumulates to above maximum acceptable limits.

The FTS is applied commonly to aquatic animal culture with market size and broodstock, whereas it is not applied universally in rearing of larvae. In salmon culture it is common to use flow-through systems (Pennell *et al*., 2001) and it is the second most popular aquaculture production systems in the US, in terms of number of facilities (USDA 1998). For a unit production capacity of 1kg fish/year, FT systems released 0.16 L/min of effluence (MacMillan 1992) that compared to 0.0034 L/min of pond systems and 0.0002 L/min of recirculation systems (Losordo, 1991; Losordo *et al*., 1994). Effluent pollutants, especially phosphorus, have caused environmental concerns as excessive discharge of P to receiving waters that may lead to water quality degradation through eutrophication (Stickney 1994). In addition, a predicted but not well documented difference between FT system and recirculation system is a microbial community composition where the FT is selected dominant r-strategy while K-strategists are selected dominant by recirculation systems.

- Microbial maturation system (MMS)

Microbial maturation of intake water from the MMS was introduced into the fish tanks after disinfecting the intake water by biofilter. Thus, MMS can control recolonization with K-selection, and has a stable bacteria community over time. Those are the reason why the MMS was encouraged to be applied for rearing tanks achieving microbial maturation (Salvesen et al., 1999). Microbial community in the MMS occupied by K-strategists, and result in improved performance of the fish larvae in the early stages of first feeding (Vadstein *et al*., 1993; Skjermo *et al*., 1997).

Theoretically, non-opportunistic bacteria or K-strategists were beneficial to the larvae. The researchers therefore believed that primary colonization of the skin and gut surfaces of fish larvae by non-opportunistic may establish a commensally microflora, which can protect the larvae and avoid infection by opportunistic and pathogenic bacteria. The process of stabilizing the microbial community of the water in the biofilter is termed microbial maturation and previous experiments indicate that its results in enhance larval growth and survival (Skjermo *et al*., 1997; Vadstein *et al*., 1993).

Microbial matured water has been tested and applied in several experiments as well as in hatching industry with marine fish larvae during the last decades. According to Skjermo et al (1997) when used in incubation of Atlantic halibut (*Hippoglossus hippoglossus*) yolk sac larvae, MMS resulted in 76% or higher survival and improved feeding incidence as well as increased reproducibility between replicates. Correspondingly, turbot (*Scophthalmus maximus* L) larvae maintained in microbial matured water showed faster growth than larvae maintained in membrane filtered water, and reached 51% higher weights during the experimental period (14-16 days) (Skjermo *et al*., 1997). Similarly, Munro et al (1995) has reported that the survival rate of turbot larvae could be increased from 4.6% to 32.4% in microbial matured system. Currently, the Center of Aquaculture (NTNU and SINTEF, Trondheim) is using microbial matured water as a standard condition in the first feeding experiments with marine larvae.

- Recirculation aquaculture system (RAS)

Recirculation aquaculture systems are systems in which the rearing water is re-used after undergoing treatments (Rosenthal *et al*., 1986). These systems provide opportunities to reduce water consumption (Martins *et al*., 2010; Verdegem *et al*., 2006) and to improve waste water management and nutrient recycling (Martins *et al*., 2010 and Piedrahita, 2003). Bio-filters including heterotrophic and nitrification are necessary in RAS, where the heterotrophic bacteria consumes organic matter. Moreover, it results in better hygiene and disease management than in FT systems (Summerfelt *et al*., 2009 and Tal *et al*., 2009) because the K-strategists (non-opportunistic bacteria) were shown to dominate in the system (Konneke *et al*., 2005) and to control the microbial communities (Zohar *et al*., 2005). Also related to microbiota in RAS, Attramadal et al. (2012a) showed that the microbial community composition in RAS developed a more diverse and stable microbiota over time compared to

the FTS. The low ammonium and nitrite concentrations in these systems are a result of nitrification and denitrification. Denitrification is carried out by facultative anaerobic bacteria that utilize either organic (heterotrophic denitrification) or inorganic (autotrophic denitrification) compounds as electron source to reduce nitrate to form nitrogen gas ($N_2$). Thus the RAS is known as an environmentally friendly aquaculture system.

The RAS was applied in aquaculture in the late 1980's and fish production in RAS has increased significantly in volume and species diversity (Martins *et al*., 2005; Rosenthal, 1980 and Verreth *et al*., 1993). So far, more than 10 species are produced in RAS (African catfish, eel and trout as major freshwater species and turbot, sea bass and sole as major marine species). Recently, new facilities of RAS were established in the UK (sea bass), France (salmon), and Germany (different marine species) (Martins *et al*., 2010).

The efficiency of RAS is clear when compared with some other systems. For example Joensen (2008) reported an increase of smolt size from 50 - 70g in flow-through farms to 140-170g in RAS. In addition, Terjesen et al. (2008) suggested an increased smolt quality (growth and survival) after sea transfer of RAS cultured smolts. In Norway a production of 85 million smolts in RAS is foreseen (Campo *et al*., 2010). Similarly, Verner-Jeffreys et al. (2004) reported improved growth and survival of halibut larvae in a recirculation system. Not only fish but also shrimp has been cultured in RAS. Two trials were performed with cultured shrimp from market size (20g) to broodstock (40-60g) in recirculation aquaculture system versus a flow-through (FT) pond. These results shown that growth rate of shrimp in RAS were lower than in FT (Clete *et al*., 2003). However, broodstock in RAS maintained good growth and high survival.

### 1.2. Establishment of gut microbial community in fish larvae

During the first days after hatching there is an intimate relationship between fish and the water bacteria that eventually may affect establishment of a normal mucosal microflora or result in epidemic disease. The primary colonization by bacteria on the skin and mucosal surfaces of fish larvae gut were known to be non-opportunistic, which can protect larvae from pathogenic or opportunistic bacteria (Hansen and Olfsen, 1999). In addition, bacteria in intestinal system may play a role as a contribution to the nutrient uptake by the metabolizing nutritional compounds or synthesizes factors needed by the host at an early life stage. Early

exposure to high bacterial densities may be important for immune tolerance. E.g. for the zebra fish, it has been shown that the gut microbiota is necessary for the development of the immune system (Kanther and Rawls, 2010). Thus the establishment of a protective intestinal microflora will increase survival and growth of the fish larvae. Hence, the quality as well as quantity of early phase of several marine fish species highly depends on knowledge and possibility to control the complex interactions between the cultured organisms and the bacterial communities which develop at the mucosal surfaces, in the surrounding water, and the rearing systems (Hansen and Olfsen, 1999; Skjermo *et al*., 1997).

Bacterial adhesion and colonization of the egg surface occur within several hours after fertilization, and both non-pathogenic and pathogenic bacteria can be found on the surface of fish eggs both in culture and natural condition. The flora which ultimately develops on the egg appears to reflect the bacterial composition and load of the ambient water, but species specific adhesion at the surface of eggs may also play a role in development of the egg epiflora (Hansen and Olfsen, 1999). Even though, the bacteria colonize only the outside of the egg, these bacteria influenced the gastrointestinal microbial communities of larvae (Romero and Navarrete, 2006; Verner-Jeffreys *et al*., 2006; Verner-Jeffreys *et al*., 2003).

Hansen et al. (1999) reported that the primary intestinal microflora was established at the yolk sac stage. It was demonstrated that the gastrointestinal microbe seem independent of first feeding. This was explained by the fact that larvae need to consume seawater to osmoregulate and this is a way for bacteria to infiltrate into the gastrointestinal tract. In addition establishment of a gut microflora is likely to go through several stages. Microbial community composition has changed in each stage and depends on the structure of the intestinal tract. For example in Halibut (*Hippoglossus hippoglossus*) larvae the number of mucous cells increases during development from pelagic larvae to bottom-dwelling, and as a result the chemical composition of the mucus changes and this may affect microbial adhesion and colonization in gastrointestinal (Ottesen and Olafsen, 1997).

During the larvae stage, ingestion of bacteria may present antigens and be an important basis for the formation and development the immune system (Davina *et al*., 1982; Rombout and Berg, 1985; Kanther and Rawls, 2010). This may result either in antigen priming or in development of immune tolerance to specific bacterial strains. These bacterial strains consist

of aerobic, facultative anaerobic and obligate anaerobic forms and they are the principal colonizers in the GI tract of fish (Nayak 2010).

In juvenile and adult stages of fish, local mucosal and secretory immune responses play an important role in protection against bacterial pathogens (Hart *et al*., 1987; Trust, 1986). However, the mechanisms of defense function are not yet clear. It has been suggested that absorptive enterocytes in the intestinal epithelium may function as an antigen-sampling device, thereby presenting antigenic determinants to intraperitoneal lymphoid cells (Davina *et al*., 1982; Rombout and Berg, 1989). It has been demonstrated that endocytosis of bacterial antigens in intestinal enterocytes of cod (*Gadus morhua*) and herring (*Clupea harengus*) larvae (Olafsen and Hansen, 1992) are involved in stimulation of the developing immune system.

In conclusion, it is obvious that a gastrointestinal microbiota will become established actively take up bacteria from the water soon after hatching in marine larvae. The reason may be that the marine larvae need to drink on order to osmoregulate before they start eating. This is good opportunity for bacteria infiltration into intestine of larvae. Additionally, it has been shown that turbot larvae had an active uptake of both bacteria and algae at rates 100 times higher than the drinking rate (Reitan et al., 1998). The composition as well as the development of the microbial community in the gut of fish larvae will also depend on the water is already mentioned, together with external environmental factors. A prediction is that pioneer bacterial strains may be adapted to the ecological niche formed in the larval gut, and will persist and develop into components of the "adult" microflora.

### 1.3 Interaction between microbial community and fish at first feeding stage.

Both in natural seawater as well as in aquaculture, setting bacterial densities are often significantly high, that is illustrated by level of $10^6$ cells per mL (Maeda 2002) and these bacteria move easily in the aquatic environment and between habitats and hosts.

Bacterial colonization may have adverse effects on egg (Hansen *et al*., 1992) and on the developing embryo (Bergh *et al*., 1992; Bergh *et al*., 1997), and may result in delayed hatching and even halt egg hatching because bacterial overgrowth may result in hypoxia in the developing embryo (Helvik 1991). In addition, Kjørsvik et al. (1991) showed that there is a negative correlation between bacterial colonization and the physical characteristic of fish

eggs. Some adherent pathogens on fish eggs may damage the chorion; dissolve the egg shell by releasing exoproteolytic enzymes (Hansen and Olafsen, 1989). Further, some bacteria produce exotoxins or toxic metabolites that may harm the developing embryo, such as *Flexibacter ovolyticus*, and induce high larval mortalities after hatching (Hansen *et al*. 1992).

Parallel with the harmful of bacteria given above have a positive effect to the host as well. For example, those were believed that had significance in the formation of gastrointestinal microbial for later stage of fish (larvae, juvenile). However, knowledge still lacks about which specific microbes may benefit or harm the larvae, and these activity mechanism (Olafsen, 2001).

There are two mechanisms by which the pathogens interact with the host at the larvae stage, which are uptake of antigens and immune stimulation. The marine cultured or natural food organisms for fish larvae may serve as vectors for transfection of fish pathogens, and this is the main base for uptake of antigens in the larvae (Olafsen, 2001; Tamplin and Capers, 1992; Tamplin and Fisher, 1989). Further, this is the first stimulation of the immune system of fish.

When bacteria have colonized the gastrointestinal tract of larvae, they set up interaction between bacteria and larvae, where the interaction may be implemented in three forms (specificity, establishment of normal microbiota or building first line defense). At that time, the larvae also interact with opportunistic pathogens in water or in feed. A schematic presentation of the interactions between bacteria and fish egg and fish larvae is shown in Figure 1.5.

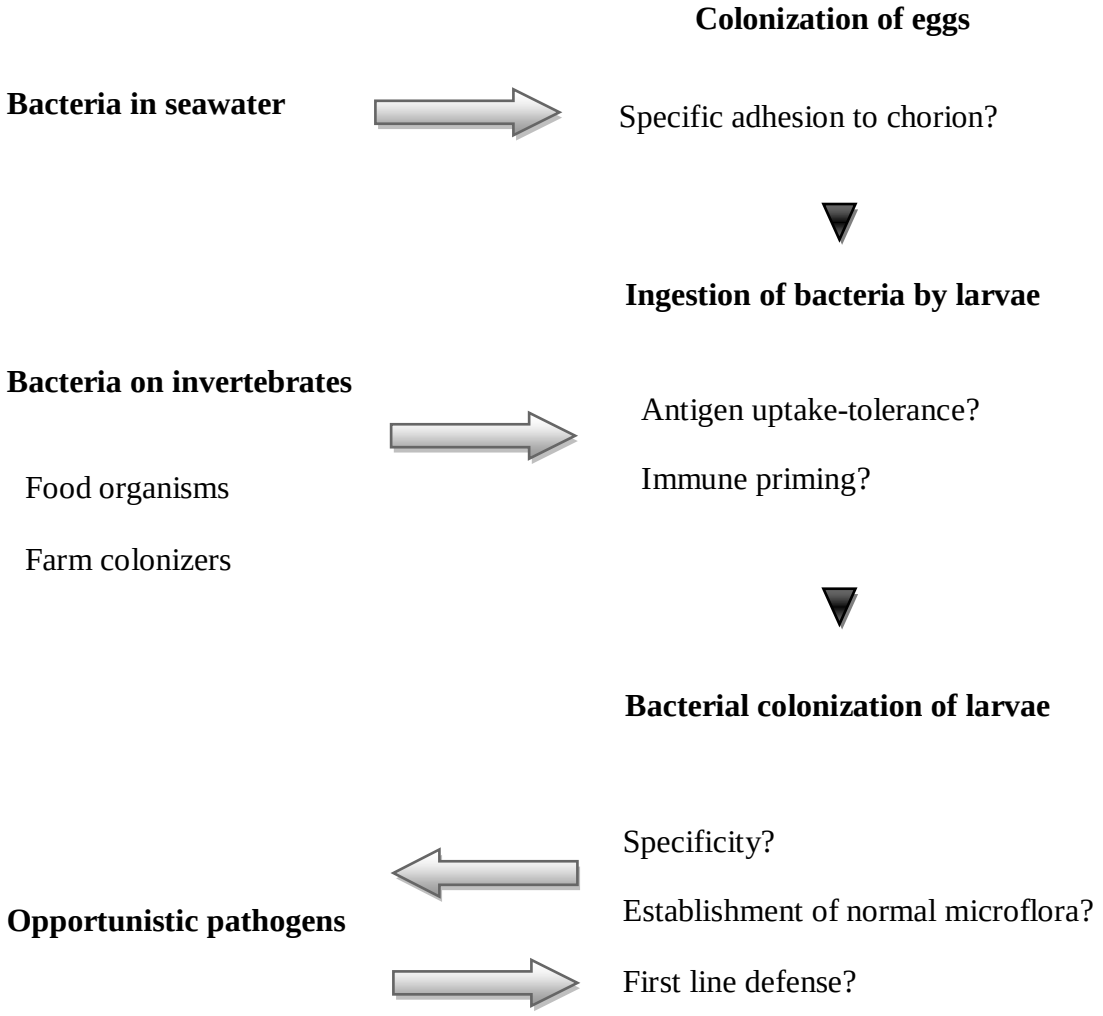



**Figure 1.5: Interactions in bacterial colonization of fish egg and larvae** (*Olafsen, 2001*)

## 1.4 Molecular methods for describing microbial community structure

The microbial diversity and its role in nature is poorly understood. Classification based on morphological traits is difficult due to small size and analog morphology. Moreover 99% of all microorganisms in nature can't be cultured with nutrient medium, but these microorganisms reproduce quickly in their nature environment, and therefore classification of microorganisms based on cultivable dependent physiological and biochemical features is nearly impossible (Amann *et al*., 1995).

To understand the microbial diversity at a different level such as the genetic, the molecular biological techniques were developed. These are culture-independent methods, where microbes are grouped according to similarities in their genes (Woese 1987).

In exploration of microbial diversity in all kind of natural samples with culture independent methods, the conserved regions of 16S rRNA gene have been used for development of universal primers (Hugenholtz *et al.,* 1998). One reason for this is that 16S rRNA is ubiquitous in all microorganisms (Watanabe 2001). Furthermore, the regions of 16S rRNA gene are conserved enough to allow the design of PCR primers that target various classifiable groups, while other regions of the gene are variable enough to provide phylogenetic comparisons of microbial community member (Woese 1987).

Microbial community composition can be analyzed based on profiles generated from the physical separation of rRNA or DNA sequences on a gel, thanks to the PCR amplification and subsequent comparisons of the sequences of the PCR amplicons (Muyzer 1999). Four methods that allow us to determine the bacterial communities' structure in samples include amplified ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE) and terminal-restriction length polymorphism (T-RFLP).

PCR-DGGE is a commonly used method in identification of the bacterial flora in environment microbial ecology (Øvreas 2000; Schafer and Muyzer, 2001). In addition, these methods have been used to study the bacterial flora of Atlantic halibut larvae (Jensen *et al*., 2004) as well as early life stage of salmon (Romero and Navarrete, 2006). Yang et al. (2007) described the microbial community composition of the skin, gastrointestinal, liver and ovary of puffer fish (*Takifugu obscurus*) by PCR-DGGE analysis.

The theoretical aspects of DGGE method was first described by Fischer and Lerman. (1983) and DGGE is known as a powerful method (Bernard *et al*., 2001 and Kawai *et al*., 2002) with sensitivity near to 100% with respect to resolve different DNA fragments by as little as one single nucleotide (Dolinsky *et al.,* 2002). DGGE can determine the dominant member of microbial communities with medium phylogenetic resolution (Sanz 2007; Sanz and Kochling, 2007). The main strong point of DGGE is that it allows us to monitor the spatial/temporal changes in microbial community structure, and provides a simple view of the

dominant microbial species within a sample. In addition this method enables analysis of many samples at the same time during a short time. DGGE has some limitations in microbial community studies such as sequence information is limited to 500bp fragments of 16S rRNA sequences. Moreover, it may lack the specificity required for the phylogenetic identification of some organisms (Gilbride *et al.*, 2006). Also multiple copies of the rRNA gen exist in some organisms and multiple bands may then occur for a single species on the gel (Nubel *et al.*, 1997).

## 1.5 Hypothesis of the study

The hypothesis of this thesis was:

It is possible to manipulate the microbiota of the water by the help of water treatment systems, and use this as a tool to modulate the microbiota associated with larvae.

More specifically we wanted to test the different stages of this hypothesis:

1. Water treatment systems selects for different microbial communities.
2. The microbial community of the water in the rearing tank is mainly determined by the microbial community of in-flowing water.
3. The microbial community of the water in the tank strongly influence the microbial community associated with the larvae.

We tested this hypothesis in a first feeding experiment with cod by comparing three different water treatment systems, and the microbial communities were investigated by nested PCR/ DGGE analysis. Nested-PCR with two rounds of amplification was used to avoid co-amplification of eukaryote DNA (Bakke et al., 2011). Multivariate statistics was also used to compare microbial community structures.

# 2. Materials and method

## 2.1 Experimental setup and sampling schedule:

The experiment was carried out for 60 days from 23rd January to 24th March 2011 at Sealab. Atlantic cod larvae were reared with water from three different systems: flow-though system (FTS), microbial maturation system (MMS) and recirculation aquaculture system (RAS). Each of the water system was with triplicate tanks, resulting in a total of 9 tanks. The tanks of FTS received inlet water that had passed through filter (UV irradiation). In the MMS, inlet water passed through a filter with UV irradiation and then passed bio-filter to obtain controlled recolonization of the water under K-selected before it was introduced to tanks. In the RAS, inlet water passed three filters (first a sand filter, second a protein skimmer filter and third a bio-filter) before it was presented in tanks, tanks outlet also reused after it passed through a bio-filter (heterotrophic) and bio-filter (nitrify). The separation in two designated bio-filters (heterotrophic and nitrification) may increase efficiency of heterotrophic maturation and nitrification by securing optimal selection pressure for each process. The flow scheme of the experiment is show in Figure 2.1

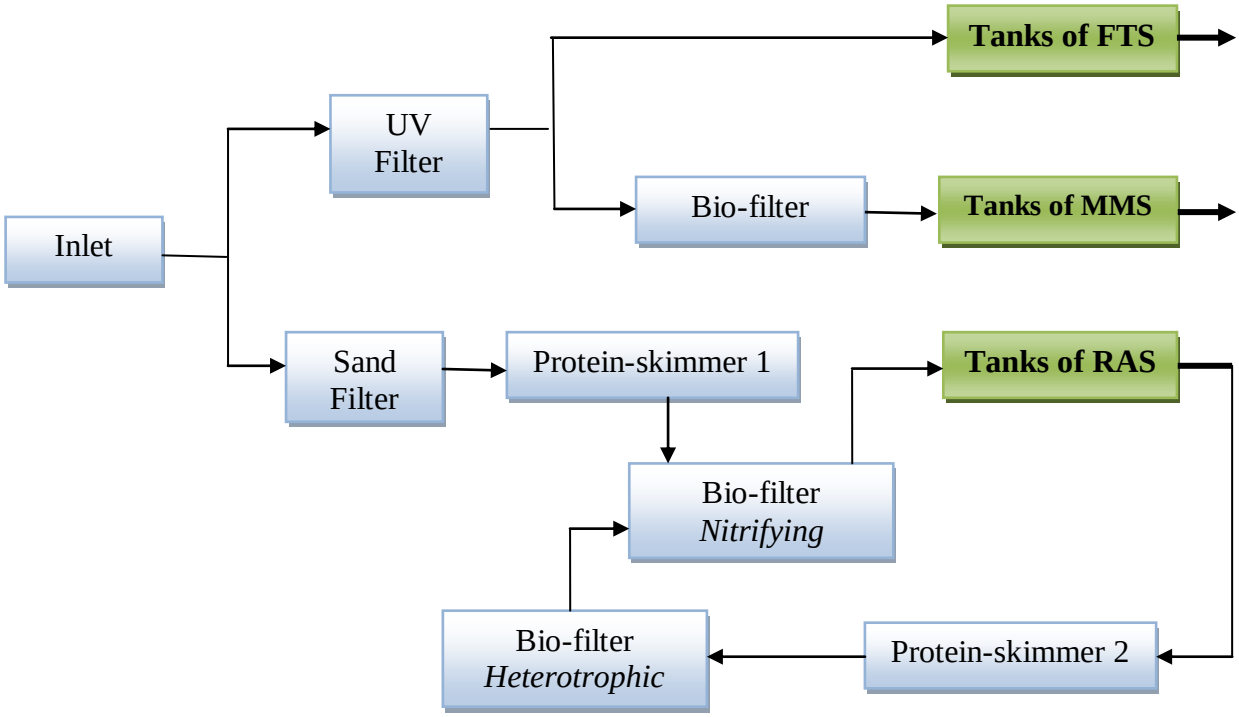
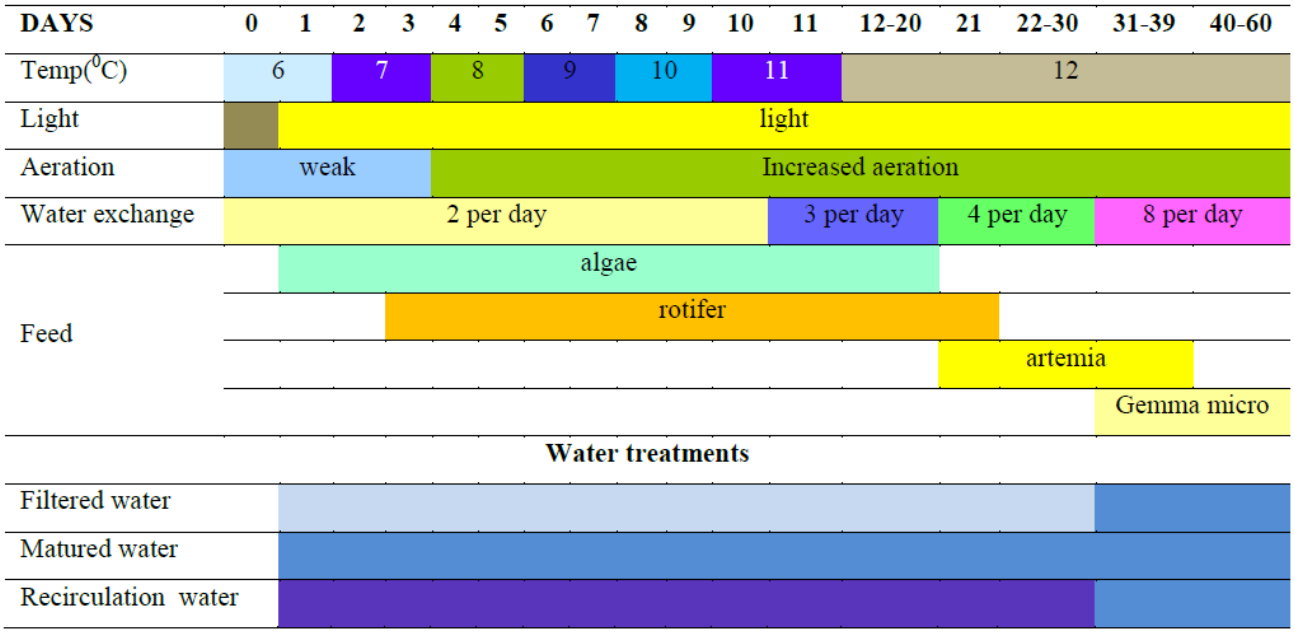



**Figure 2.1: The flow scheme of three water treatment systems in experiment.**

The rearing conditions in the experiment, such as temperature, light regime, aeration, feed and water exchange, were similar for all three water treatments. Feed was added to the tanks with an automatic robot. During the first 30 days, these nine tanks received water from three different water treatment systems (3 tanks for each treatment system). However from day 31 to day 60, all the tanks were introduced the same water, which was microbial maturation system. The rearing conditions are shown in Table 2.1.

**Table 2.1: The rearing condition of the experiment during 60 days**




***Sampling***

The sampling schedule for larvae, feed and water is described in Table 2.2

**Table 2.2: Sampling schedule in the experiment**

The number of samples collected the same day

| Samples \ Day | | 0 | 01 | 03 | 04 | 08 | 12 | 16 | 17 | 19 | 23 | 26 | 30 | 38 | 46 | 54 | 60 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Water | input | 3 | 3 | | 3 | 3 | 3 | 3 | | 3 | 3 | 3 | 3 | 1 | 1 | 1 | 1 |
| | tanks | | 3 | | 9 | 9 | 9 | 9 | | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| Larvae | | | | | | 27 | | | 27 | | | | 27 | | 27 | | 27 |
| Feed | input | | | 1 | | 1 | | | 1 | | 1 | 2 | | | | | |
| | tanks | | | | | 9 | | | 9 | | | | 3 | | | | |

*Water samples*:

In each of the experimental tank (9 tanks), 40ml of water sample were collected at the middle depth of the center by sterile plastic pipette (25 ml) inside a tube with net to prevent larvae from getting into pipette. Then the water sample was transferred to sterile syringe (50ml) and water was filtered through a sterile filter tip (0.2µm size) (Dynagard, Microgon InC). Afterward, the filter tips were stored at $-20^0$C.

In each system, inlet water was also sampled: three samples were collected in each system at the site before running into the tank.

*Live feed samples*:

From each of the tank, 100ml of water in the tank were collected with a beaker. The live feed samples (rotifer/artemia) were rinsed with sterile water in a sterile sieve. Then a microscope was used for collecting 200 rotifers or 100 artemias in a plastic Petri dish. And afterward a sterile syringe was used to collect the rotifer/artemia and filter them through sterile filter tip. The filter tips were stored at $-20^0$C.

*Cod larvae samples*:

From each treatment tank, a plastic tube was placed at middle depth in the center of the tank, and 12 larvae were sampled (a total 108 individual in one sampling). The larvae were transferred to a beaker where they were anaesthetized by MS 222 and measured. Thereafter, the larvae were rinsed twice in sterile seawater before transferring individually to Eppendof tubes (1.5ml) and immediately preserved in liquid nitrogen. These tubes were stored at $-20^0$C.

## 2.2 Methodology

DNA was extracted from water, feed and larvae samples and used as template in the polymerase chain reaction (PCR) to amplify a fragment of the bacterial 16S rRNA genes. Subsequently, the PCR products were analyzed by DGGE. The taxonomic affiliations of the predominant community members, as represented in the DGGE band pattern, were determined by re-amplification and DNA sequencing. An overview of the microbial community determination process is described in figure 2.2.

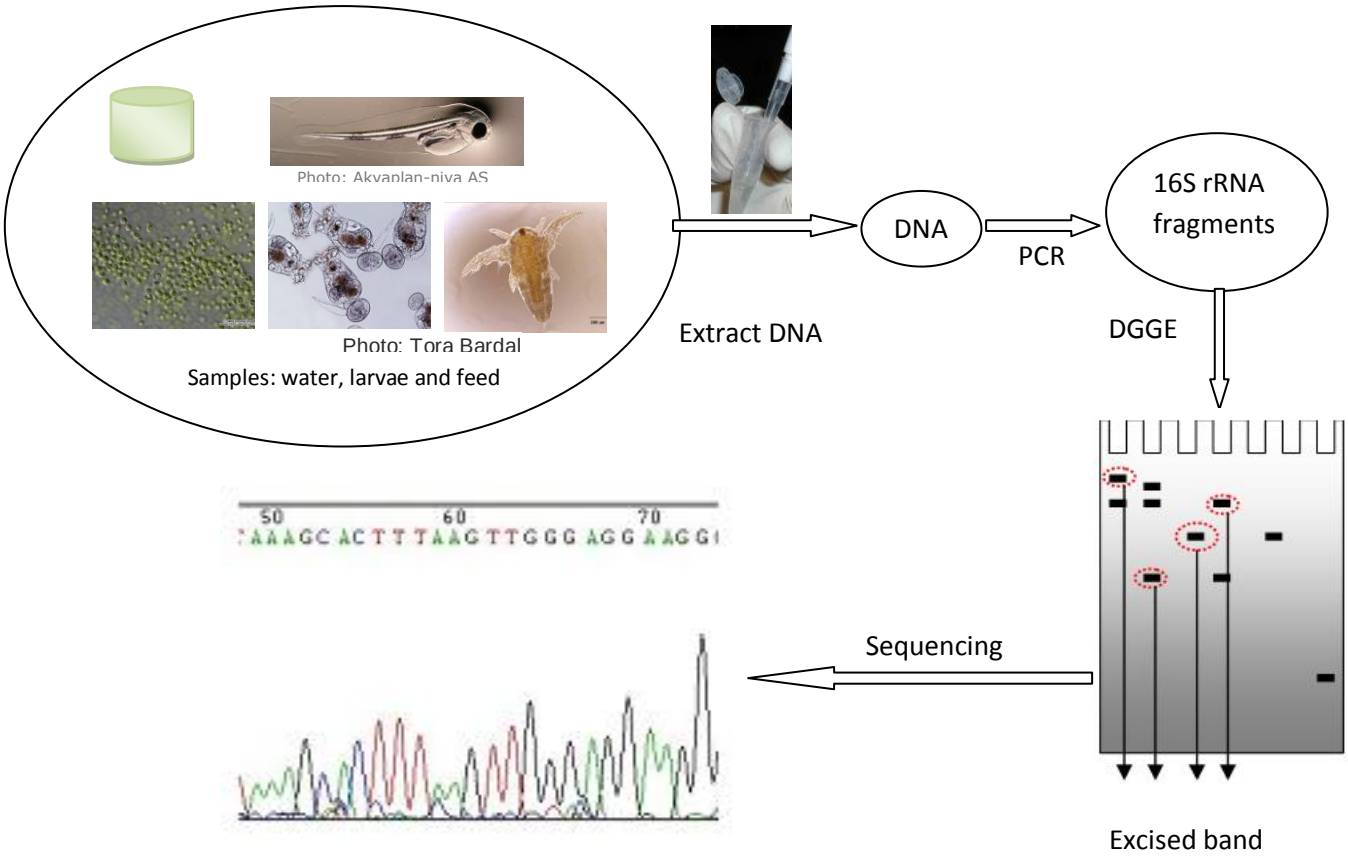



**Figure 2.2: Flow diagram of the different steps in the analysis of microbial community structure by PCR-DGGE.**

### *2.2.1 DNA extract*

DNA was extracted from water samples, feed samples and cod larvae samples by using the commercially available kit (DNeasy blood and tissue kit Qiagen).

*DNA extraction from cod larvae*:

Extraction began with the addition of 180µl enzymatic lysis buffer to the Eppendoft tube with the sample and broken the larval by using the pipette before incubation these mixture at 37°C for one hour. Proteinase K (40µl) and 180µl ATL buffer were then added, and mixed by vortexing before incubated at 55°C for approximately 2 hours. AL buffer (200µl) was added, and the tube was incubated at $70^0$C for 10 minutes. Then 96% ethanol (300µl) was added, the tube was vortexed and the lysate was thereafter transferred to the Dneasy column. For the rest, the manufacturer's protocol was followed. DNA concentration was determined using a NanoDrop spectrophotometer.

*DNA extraction from water and live feed samples:*

The generally principle was similar to DNA extract from cod larvae; however there were small differences during the lysis steps. The details are given in Appendix 1.

### *2.2.2 PCR*

DNA extraction products were identified concentration by NanoDrop. If the DNA concentrations of sample was higher than 10ng/µl, it was diluted to 10ng/µl or 20ng/µl before used as a template in the PCR reaction.

A fragments of the 16S rRNA gene was amplified using a nested PCR approach with the two different primer pairs EUB8F/984YR and 338F GC/518R, with two rounds of amplification; external and internal respectively. Two primers pair are shown detail in Table 2.3.

**Table 2.3: Primer sequences used in this study**

| Primer | Sequence (5'-3') |
|---|---|
| **EUB8F** | AGA GTT TGA TCM TGG CTC AG |
| **984YR** | GTA AGG TTC YTC GCG T |
| **338F-GC** | cgcccgccgcgcgcggcgggcggggcgggggcacgggggg<br>ACT CCT ACG GGA GGC AGC AG |
| **518R** | ATT ACC GCG GCT GCT GG |

External PCR: The primer pair EUB8F and 984YR was used with standard PCR condition 10mM dNTP, 25mM $MgCl_2$, Tag DNA polymerase (0.125µl Qiagen), the accompanying reaction buffer, and approximately 10ng DNA template, (for water samples also BSA was added) in a total reaction volume of 25µl. The PCR cycling conditions were as follows: denaturation at $95^0$C for 3 min, 20 cycles for $95^0$C for 30s, $50^0$C for 30s and $72^0$C for 60s, and the reaction was terminated with an extension step of $72^0$C for 10 min.

Internal PCR: The primer pair 338F GC and 518R was used with 2µl product from the external PCR as template, in a total reaction volume of 50µl. For the rest, the reaction conditions were identical to those used for the external PCR. The following temperature cycling was used: 22 cycles for $95^0$C for 30s, $53^0$C for 30s and $72^0$C for 60s.

PCR products were examined and verified by agarose gel electrophoresis with 1% agarose gel with GelRed$^{TM}$, and 1x TAE as electrophoresis buffer. PCR product (5µl) with loading buffer (1µl) was applied to the wells. The gels were run approximately 45 minutes at 140 Volts and then photographed under UV light. PCR products were only accepted for further analysis when a simultaneous negative control (non template control) showed no amplification.

### *2.2.3 DGGE*

DGGE analysis of PCR products was performed with the INGENY phorU system, using 8% acrylamide gels with a denaturing gradient ranging from 30% to 55%.

The glass plates, spacers and comb were cleaned well with water prior to assemble. Further, glass plates were cleaned with 95% ethanol and Kimwipe paper to remove electrostatic charges and ensure that the gel would pour uniformly. The glass plates were assembled in the gel cassette together with spacer and the comb.

The DGGE solutions were made with a mix of 0% and 80% denaturing acrylamide solution to a total volume of 48mL (100% denaturing conditions are defined as 7 M Ure and 40% formamide). These solutions were prepared according to the protocol described in Table 2.4. A gradient maker was used to cast the gradient gel. The gradient maker was rinsed with MilliQ water before casting 8 mL of 0% denaturing acrylamide solution, on top of the gel. Ultimately, the gel was left at room temperature at least 120 minutes to polymerization.

**Table 2.4: The recipe for the DGGE solution**

| Denaturating % | 0% | 80% | TEMED + 10% APS | Total volume |
|---|---|---|---|---|
| 30 | 15 ml | 9 ml | 16µl + 87µl | 24ml |
| 55 | 7,5ml | 16,5ml | 16µl + 87µl | 24ml |

*TEMED (Tetramethylenediamine); APS (Ammonium persulphate)*

TAE (0.5x) was prepared in buffer tank and heated to 60$^0$C. The gel apparatus was placed into the buffer tank, tilting the entire assembly and lowering slowly to avoid air bubbles beneath the gel. All the wells were rinsed with TAE buffer using a syringe before loading

samples (5-9 µl PCR products mixed with 4 µl loading dye). The electrophoresis was run at 100V for approximately 17 hours.

The gel was stained within 1 hour with SYBR Gold dye (Invitrogen) solution and incubated in the dark. Recipe for dye solution see at Appendix 3.

The gel was washed with tap water, and gently transferred to the UV plate. The gel was photographed and the bands were visualized under UV light (G: BOX, Syngene).

### *2.2.4 Sequencing of bands*

The DGGE bands of interest were excised from the gel with sterile pipette tips and transferred to 20 µL of sterile water, for re-amplification and DNA sequencing. The DNA was allowed to diffuse into the water at $4^0$C overnight. From the elute 1µL was used as a template and re-amplified using PCR with a pair of primer (338F-GC-M13 and 518R). The PCR products were purified using the Qiaquick PCR purification kit (Qiagen), and finally sent to Eurofins MWG for sequencing. The protocols for re-amplification as well as the purification are described in detail in Appendix 4.

### *2.2.5 Gel image and statistical analysis*

The DGGE gel images were analyzed with the software program Gel2K (developed by Svein Norland at Dept. of Biology, University of Bergen), to facilitate the analysis of gel images. This program converts band profiles to histograms, where the peaks correspond to DGGE bands. Peak areas, whose values reflect the intensities of the bands, were exported to Excel spread sheets and used for statistical analysis.

The peak areas for each band were normalization by dividing on the total peak area for all bands in the lane, and then took the logarithm of fractional peak areas to calculate band richness, diversity and evenness index. The peak areas also converted to percent for each sample, took the square root of the percentages, and then used to compare samples with a non-metric multidimensional scaling (NMS) based on Bray-Curtis dissimilarities.

*Sample description:*

The band richness (S), the Shannon weaver index (H) of general diversity and the Pielou's evenness index (J') within the microbial populations as well as the similarities between the

microbial community composition of different water sources and rearing larvae were calculated from DGGE profiles.

Parameters such as S, H' and J' were calculated using the peak areas area data

**(i)** Species richness is number of different species in a given lane.

**(ii)** The Shannon diversity index was calculated using the following function:

$$H' = -\sum_{i=1}^{S}(p_i \ln p_i)$$

*S*: the number of bands. Also it called species richness.

*Pi:* the relative abundance of each band, calculated as the proportion of individuals of a given species to the total number of individuals in the community:

$$\mathbf{P_i = n_i/N}$$

*N*: the total number of all individuals

$n_i$: the number of individuals in species i; the abundance of species i.

It can be shown that for any given number of species, there is a maximum possible$H'$, $H_{max} = \ln S$ which occurs when all species are present in equal numbers.

**(iii)** The evenness index of species was calculated base on formula

$$J' = H'/H_{max} = H'/\ln(S)$$

A one-way ANOVA, Tukey multiple comparison in the Past software package was used to determine if S, H' or J' were significantly different between samples

*Sample comparisons*:

Non-metric Multidimensional Scaling (NMS) with Bray-Curtis dissimilarity was applied to give an overview of the similarities or dissimilarities between the different bacteria community composition of the samples.

The Bray-Curtis measurement of dissimilarity could not be applied to earlier data standardization as it does not accept negative values (Quinn and Keough, 2002; Bray and Curtis, 1957) which are generated when the data are in scaled. The Bray-Curtis co-efficient compares two species in terms of their minimum abundance at each site. So to have data are in scaled, it needs to take the exported DGGE data, converts it to percent, then takes the square root of the percentage and subsequently run NMS using Bray-Curtis.

NMS works in a space with an ordination (scaling) of the different bacteria composition of the samples in full-dimensional space and then the bacteria composition of interest are points in ordination space with a stress value (goodness-of-fit), those are result of NMS, the stress values considered good if the stress value is less than 0.2 (Clark, 1999). The main objective of NMS is to seek an ordination in which the distances between all of samples are, as far as possible, in rank-order agreement with samples' dissimilarities in bacteria composition.
A statistical analysis ANOSIM was used to test of bacteria community composition was similar between groups by comparing within and between group dissimilarities.

*Taxonomy assignment*

The DNA sequences determined for DGGE bands were quality checked and trimmed for primer sequences using Clone Manager (Sci-Ed). The classification tool of the RDP (Ribosomal Database Project; http://rdp.cme.msu.edu) was used for assigning taxonomy to the sequences.

# 3. Result

## 3.1 Microbial community composition of water in water treatment systems

A total of 27 samples of inlet water representing the three water treatment systems were used to investigate the bacteria communities by PCR-DGGE. These samples were collected during the live feed period (day 1, 4, 8, 12, 16, 19, 23, 26 and 30). The results are shown in Figure 3.1, where each lane on the DGGE gel image corresponds to one sample from the inlet water qualities FTS, MMS and RAS. A total of 66 unique bands were identified in the DGGE gel by the Gel2k software.

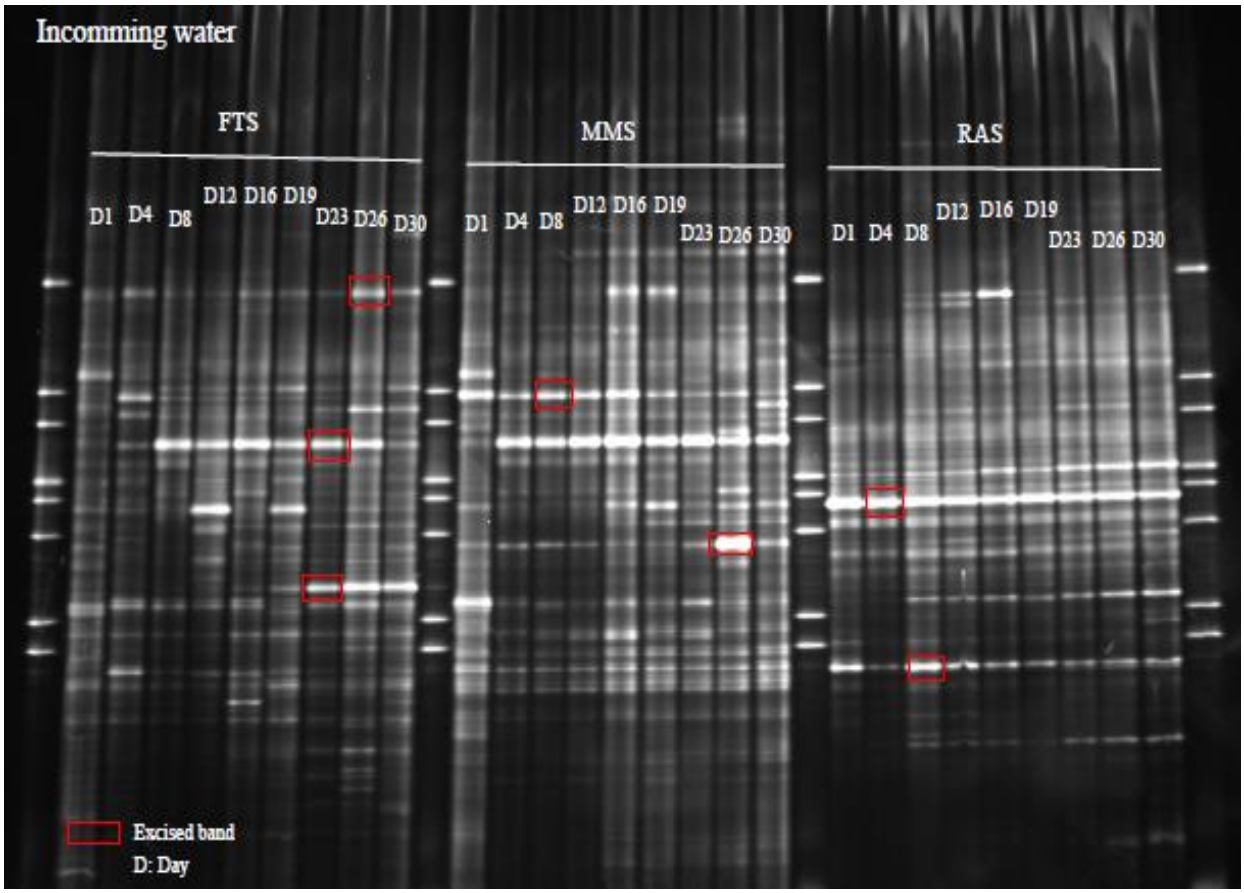



**Figure 3.1: DGGE analyses of PCR-amplified 16S rRNA fragments of bacteria in water from the three water treatment systems** (D: sampling day; red frame: sequenced bands)

In the DGGE gel, each band is likely to derive from one distinct bacteria population, so the number of dominating population of bacteria can be estimated based on the total number of bands in the DGGE gel profile. For all the DGGE profiles, the richness index (S) the diversity index (H') and the evenness index (J') were determined. In the RAS, the band richness and

diversity was highest at day 8 in the experiment, whereas for MMS and FTS there was a tendency that the richness and diversity was highest at the end of the experiment (Table 3.1). Based on one-way ANOVA and Tukey, there were no significant differences between the groups, except that the richness index of MMS was significantly higher than the index of FTS ($p=0.035$).

**Table 3.1: Specific richness (S), diversity index (H') and evenness index (J') of microbial community from incoming water systems**

| **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| RAS-D30 | 24 | 2.65 | 0.83 | MMS-D30 | 35 | 3.16 | 0.89 | FTS-D30 | 26 | 2.86 | 0.88 |
| RAS-D26 | 29 | 2.85 | 0.85 | MMS-D26 | 31 | 3.00 | 0.87 | FTS-D26 | 24 | 2.64 | 0.83 |
| RAS-D23 | 27 | 2.83 | 0.86 | MMS-D23 | 31 | 2.83 | 0.82 | FTS-D23 | 23 | 2.42 | 0.77 |
| RAS-D19 | 24 | 2.62 | 0.82 | MMS-D19 | 29 | 2.87 | 0.85 | FTS-D9 | 24 | 2.76 | 0.87 |
| RAS-D16 | 24 | 2.66 | 0.84 | MMS-D16 | 27 | 2.79 | 0.85 | FTS-D16 | 24 | 2.59 | 0.82 |
| RAS-D12 | 28 | 2.81 | 0.84 | MMS-D12 | 23 | 2.32 | 0.74 | FTS-D12 | 19 | 2.23 | 0.76 |
| RAS-D8 | 30 | 2.84 | 0.83 | MMS-D8 | 24 | 2.30 | 0.72 | FTS-D8 | 15 | 1.83 | 0.67 |
| RAS-D4 | 19 | 2.17 | 0.74 | MMS-D4 | 21 | 2.34 | 0.77 | FTS-D4 | 21 | 2.62 | 0.86 |
| RAS-D1 | 18 | 2.39 | 0.83 | MMS-D1 | 26 | 2.84 | 0.87 | FTS-D1 | 25 | 2.90 | 0.90 |
| **Average** | **24.7** | **2.6** | **0.8** | **Average** | **27.4** | **2.7** | **0.8** | **Average** | **22.3** | **2.5** | **0.8** |
| **SE** | **1.4** | **0.0** | **0.0** | **SE** | **1.4** | **0.1** | **0.0** | **SE** | **1.1** | **0.1** | **0.0** |

Non-metric multidimensional scaling (NMS) plot based on Bray-Curtis similarities for the MC of the water from the three water treatment systems are shown in Figure 3.2. Each of point on the figure represents one individual sample and corresponds to a lane in Figure 3.1 or a sample in Table 3.1. The closer points are, the more similar the samples are in microbial community composition. Figure 3.2 indicates that the points representing the samples of the three treatment systems are distributed in three different areas with a clear clustering indicating that the water of the three different systems has different microbial composition. ANOSIM analysis confirmed that the differences in the microbial communities of the water in all three systems were significantly different ($R=0.8941$, $p<0.0001$). Figure 3.2 further shows that points corresponding to bacteria communities of RAS and MMS are distributed in two different areas and the points are relatively close together. For FTS points are more scattered, indicating less stable MC.

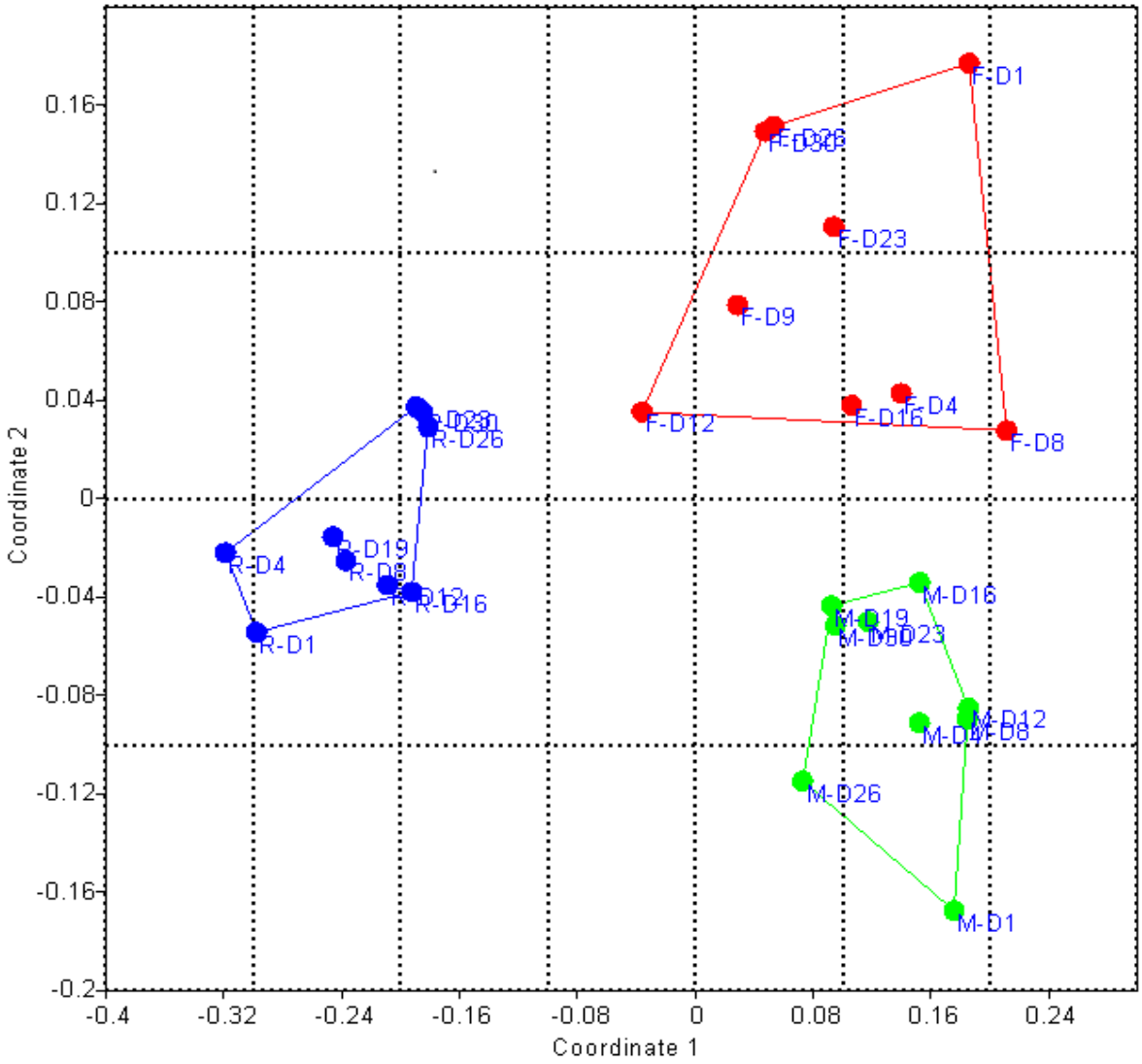



**Figure 3.2: Ordination of microbial community composition of water from three water treatment systems analyzed by NMS using Bray-Curtis similarities** (M: MMS, R: RAS, F: F TS and D_number: sampling day)

The average Bray-Curtis similarities of microbial communities within and between groups of water samples from different treatments were calculated (Figure 3.3). Within groups, RAS and MMS had higher similarity than FTS. Between groups, the microbial composition of MMS and FTS had higher similarity than that of RAS and FTS and the lowest similarity is microbial composition was between RAS and MMS (Figure 3.3)

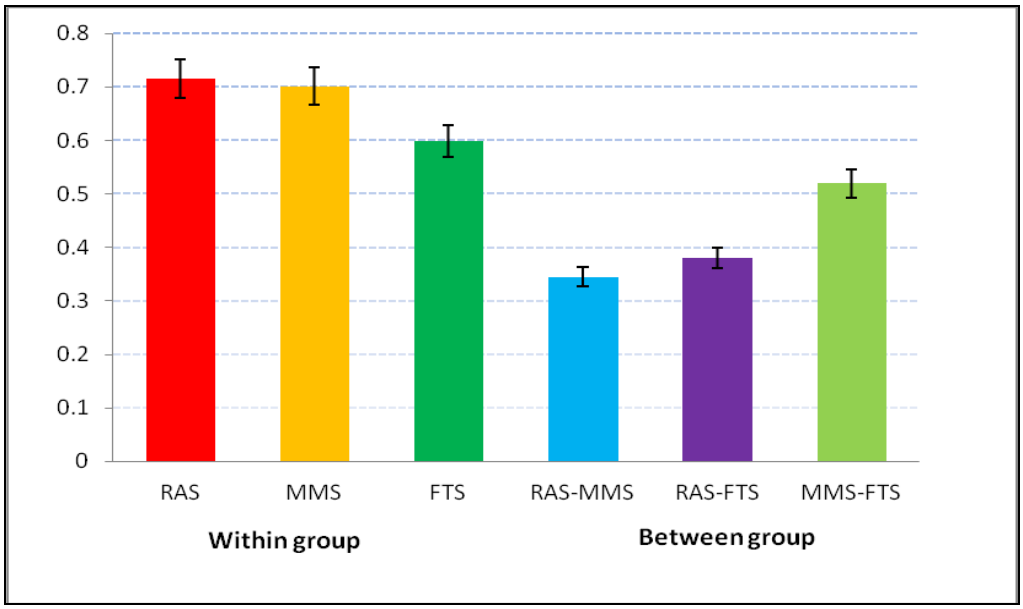



**Figure 3.3: Average Bray-Curtis similarities of MC composition of incoming water between and within the three different water treatment systems**

The results presented above (Figure 3.2 and 3.3) indicated that the microbial community composition in RAS and MMS apparently were more stable than in FTS over time.

## 3.2 Factors affecting microbial communities in rearing water

### *3.2.1 Flow-though system (FTS)*

The MC composition of water and feed samples from the FTS were analyzed by PCR-DGGE, and the resulting DGGE gel is shown in Figure 3.4. Incoming water and feed was sampled throughout the live feed period (day 3, 4, 8, 12, 17, 19, 23, 26 and day 30), while water in the three rearing tanks were sampled at day 8, day 16 and day 30.

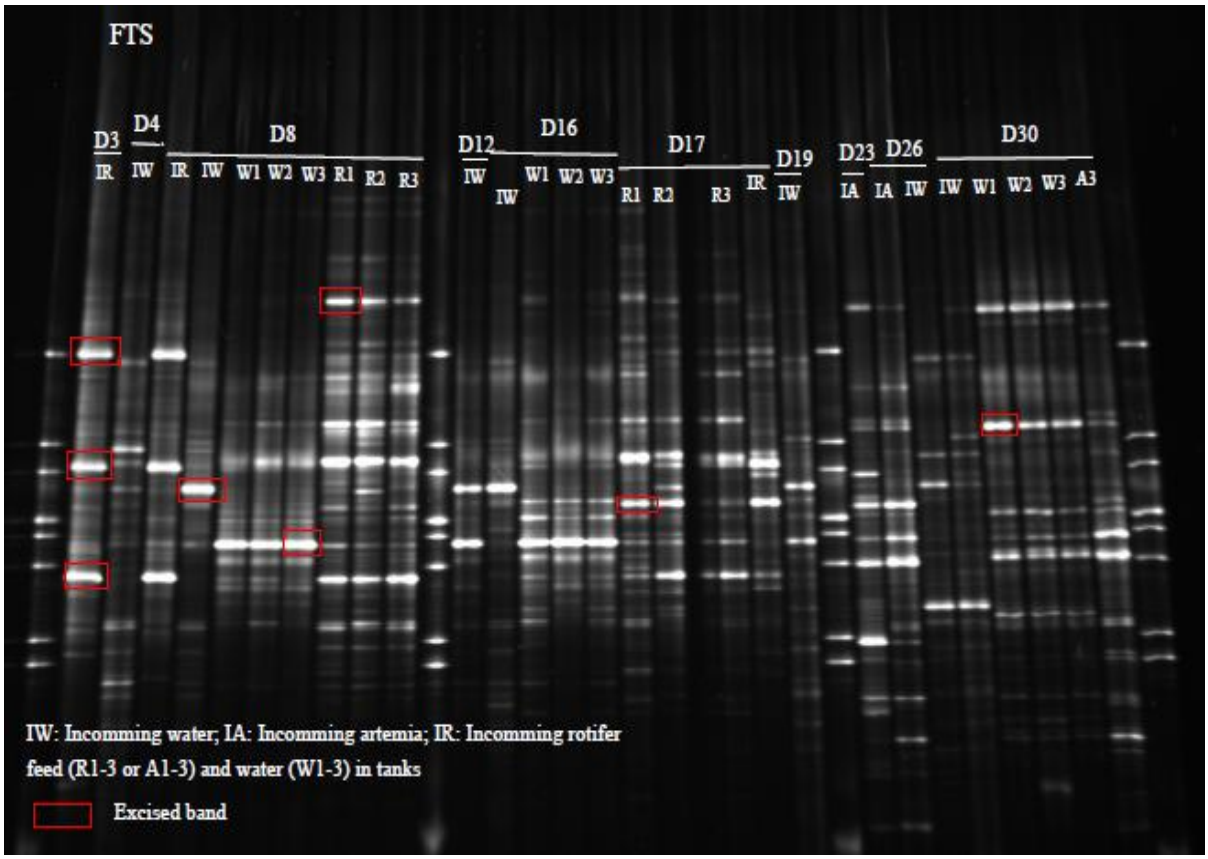



**Figure 3.4: DGGE analysis for PCR-amplified 16S rRNA fragments from the microbial communities of feed and rearing water in flow-through system** (IW: intake water, IA: incoming arterima, IR: incoming rotifer, R1-3: rotifer in tanks, W1-3: water samples in tanks, red frame: sequencing bands)

Richness, diversity and evenness were calculated for all the DGGE profiles (Table 3.2). The band richness (S) and the diversity index (H') for the incoming water samples were a bit lower than for the water samples in the tanks. Incoming live feed samples had lower band richness, diversity and evenness indexes than live feed samples from the tank. Generally, the band richness and the diversity in water samples seem to be lower than in live feed samples. ANOVA and Tukey analysis confirmed that the band richness and diversity index of live feed samples from the tanks was significantly higher than for the tank water and the inlet water samples ($p<0.05$). For evenness index of feed samples (incoming and tanks feed) in comparison with water samples (inlet and rearing water) was no significant difference. Moreover the band richness, the diversity of incoming feed samples in comparison with rearing water and inlet water samples these were no significant difference ($p>0.05$).

**Table 3.2: Band richness (S), diversity index (H') and evenness index (J') of the microbial communities of feed and rearing water samples in the flow-through system** (IA: incoming artermia; IR: incoming rotifer; IW: incoming water; R: rotifer in tank; A: artermia in tank; W: water in tank; D: sampling day)

| **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** |
|---|---|---|---|---|---|---|---|
| W3D30 | 13 | 1.88 | 0.73 | IWD30 | 13 | 2.26 | 0.88 |
| W2D30 | 17 | 2.33 | 0.82 | IWD26 | 9 | 1.65 | 0.75 |
| W1D30 | 16 | 2.18 | 0.79 | IWD19 | 19 | 2.18 | 0.74 |
| W3D16 | 15 | 2.00 | 0.74 | IWD16 | 17 | 1.86 | 0.66 |
| W2D16 | 12 | 1.79 | 0.72 | IWD12 | 12 | 1.47 | 0.59 |
| W1D16 | 18 | 2.35 | 0.81 | IWD8 | 15 | 1.98 | 0.73 |
| W3D8 | 15 | 1.94 | 0.71 | IWD4 | 15 | 2.38 | 0.88 |
| W2D8 | 14 | 1.87 | 0.71 | **Average** | **14.2** | **1.9** | **0.7** |
| W1D8 | 13 | 1.78 | 0.69 | **SE** | **1.2** | **0.1** | **0.0** |
| **Average** | **14.7** | **2.0** | **0.7** | A3D30 | 18 | 2.42 | 0.84 |
| **SE** | **0.6** | **0.0** | **0.0** | R3D17 | 14 | 2.20 | 0.83 |
| IAD26 | 18 | 2.46 | 0.85 | R2D17 | 15 | 2.13 | 0.79 |
| IAD23 | 19 | 2.48 | 0.84 | R1D17 | 28 | 2.72 | 0.82 |
| IRD17 | 18 | 2.16 | 0.75 | R3D8 | 28 | 2.58 | 0.77 |
| IRD8 | 18 | 1.81 | 0.63 | R2D8 | 29 | 2.69 | 0.80 |
| IRD3 | 19 | 2.08 | 0.71 | R1D8 | 29 | 2.71 | 0.80 |
| **Average** | **18.4** | **2.1** | **0.7** | **Average** | **23.0** | **2.4** | **0.8** |
| **SE** | **0.2** | **0.1** | **0.0** | **SE** | **2.6** | **0.0** | **0.0** |

NMS analysis based on the Bray-Curtis similarities (Fig. 3.5) indicated that the MCs composition representing incoming water, rearing water, incoming rotifers and rotifers in tank separates into four separate groups. Points representing MCs composition of the artermia in tanks and incoming artermia samples are positioned part from the points representing rotifer samples in the plot (Fig. 3.5), these feed organisms represent different MC.

ANOSIM analysis based on average Bray-Curtis similarities indicated that the MC composition of the four sample types (incoming water, incoming feed, water and feed in tank) of FTS system were significantly different from each other, with p values equal to or lower than 0.001 (Table 3.3).

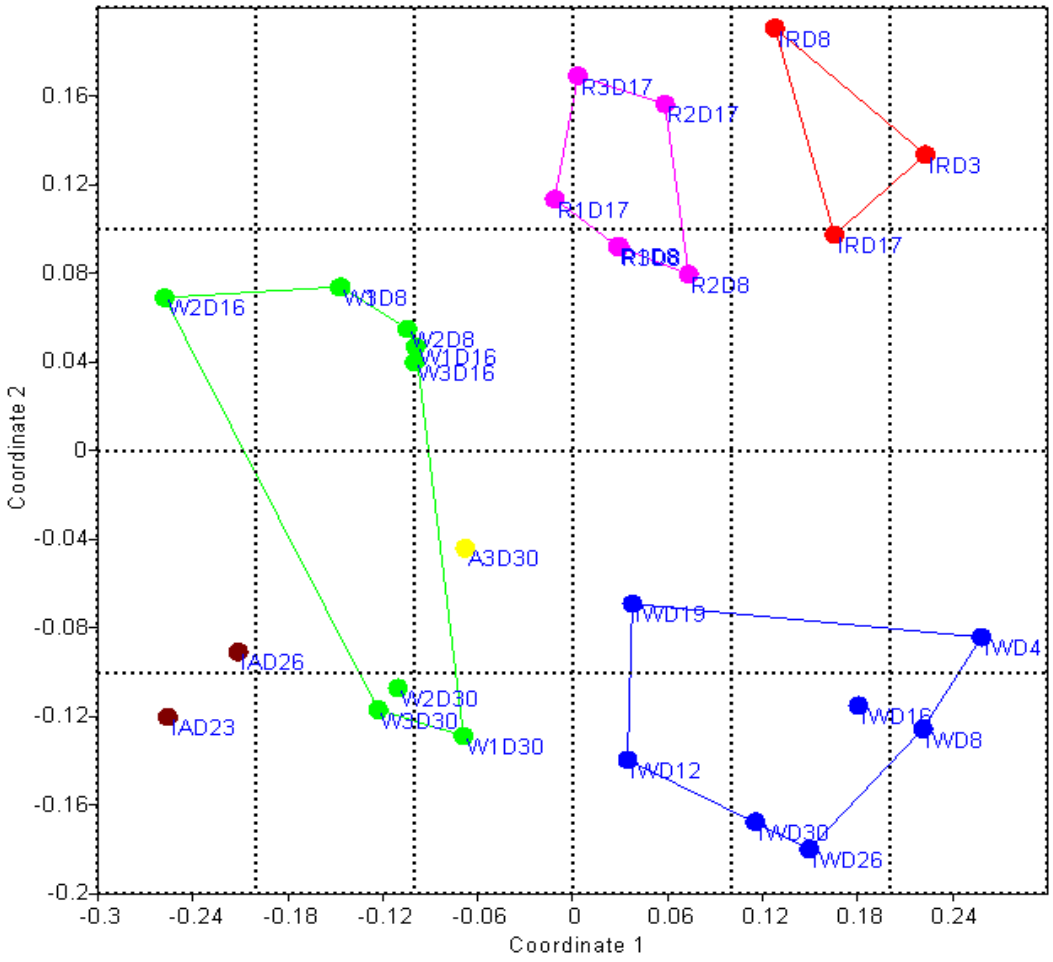



**Figure 3.5: Ordination of the microbial composition of water and feed samples of the FTS system by NMS using Bray-Curtis similarity** (the lines and points green: rearing water samples, blue: incoming water samples, red: incoming rotifer samples, pink: rotifer in tanks samples, brown: incoming artermia samples and yellow: artermia in tank).

**Table 3.3: ANOSIM analysis of the microbial community from water and live feed samples in flow-through system (FTS)**

| | Overall analysis | R value | p value |
|---|---|---|---|
| | | **0.6539** | **0.0001** |
| **Between groups** | Incoming feed - Feed in tank | 0.4396 | 0.0081 |
| | Incoming feed - Incoming water | 0.6793 | 0.0018 |
| | Incoming feed - Water in tank | 0.5729 | 0.0019 |
| | Feed in tank - Incoming water | 0.9427 | 0.0010 |
| | Water in tank - Feed in tank | 0.5299 | 0.0002 |
| | Water in tank - Incoming water | 0.7655 | 0.0002 |

### *3.2.2 Microbial maturation system (MMS)*

The MCs of four sample types from the MMS system were investigated by PCR-DGGE, including incoming feed, inlet water, and feed, water in tanks. These samples were collected during the first day 30 of experiment. The resulting DGGE gel is shown in Figure 3.6.

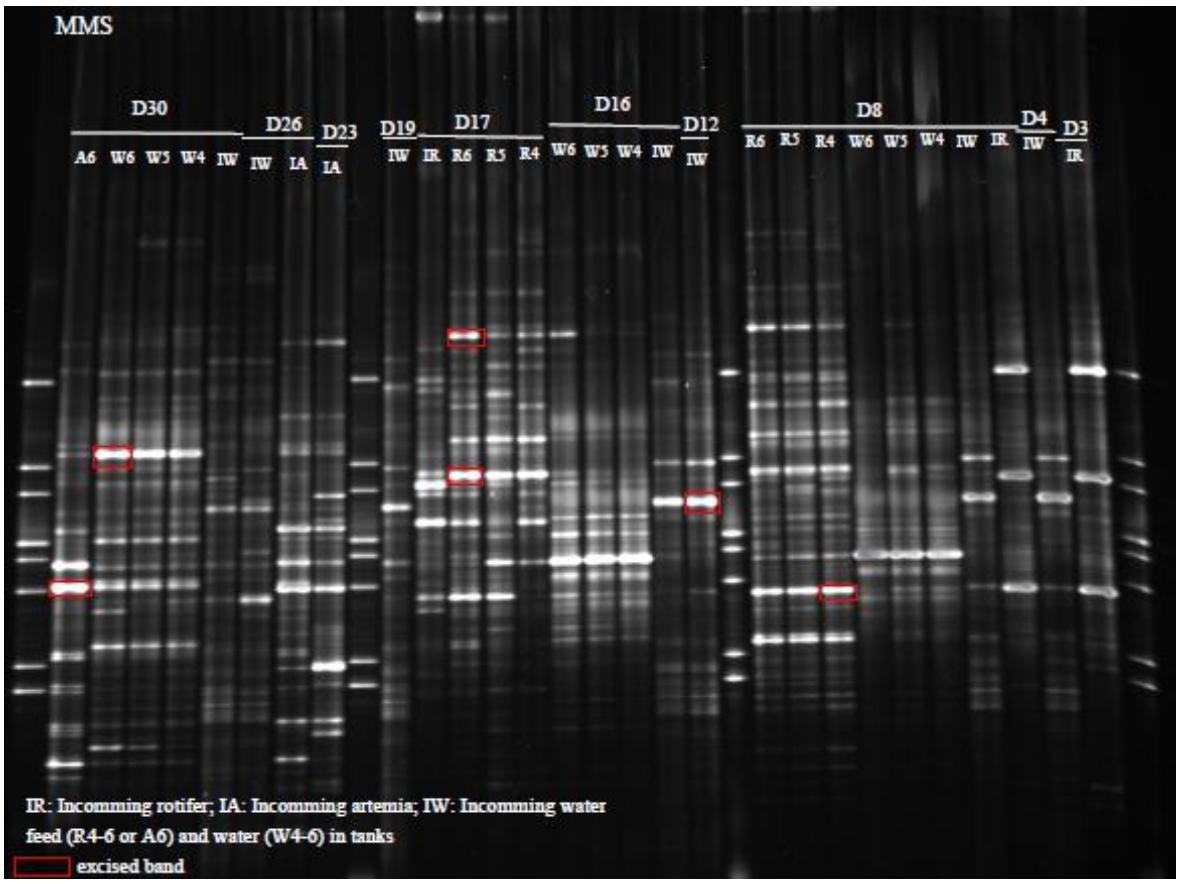



**Figure 3.6**: **DGGE analyses for PCR-amplified 16S rRNA fragments from the bacterial communities of feed and rearing water in the MMS system** (IW: inlet water, IR: incoming rotifer, IA: incoming artemia, W4-6: water in tank, R4-6: rotifer in tank, A6: artemia of tank 6, red frame: sequenced bands)

Band richness, diversity and evenness were calculated for each DGGE profile (Fig. 3.6). The results are shown in Table 3.4. The average band richness is highest for feed samples in tank (25.7±2.2), followed by rearing water samples (18.4±1.3), then incoming feed (16.6±1.5) and incoming water (15.5±1.1). ANOVA and Tukey analysis showed that the richness and the diversity index between groups (feed in tanks vs inlet water and incoming feed) were significantly difference ($p<0.05$). The evenness index was not significantly different between the four sample types. The band richness from feed samples representing different replicate

tanks was less variable at day 8 than at day 17. For rearing water, the band richness was more stable for samples representing different rearing tanks, and had a tendency to increase over time, from day 8 to day 30.

**Table 3.4: Band richness, diversity index and evenness index of MCs in the MMS system** (W4-6: water in tank 4-6, IW: inlet water, IA: incoming artemia, IR: incoming rotifer, R4-6: rotifer in tank 4-6, A6: artemia of tank 6, D_number: sampling day).

| Samples | S | H' | J' | Samples | S | H' | J' |
|---|---|---|---|---|---|---|---|
| W4D8 | 14 | 2.31 | 0.88 | R4D8 | 32 | 2.99 | 0.86 |
| W5D8 | 16 | 2.40 | 0.87 | R5D8 | 28 | 2.82 | 0.85 |
| W6D8 | 12 | 1.99 | 0.80 | R6D8 | 32 | 2.65 | 0.77 |
| W4D16 | 17 | 2.25 | 0.79 | R4D17 | 18 | 2.36 | 0.82 |
| W5D16 | 18 | 2.44 | 0.84 | R5D17 | 27 | 2.77 | 0.84 |
| W6D16 | 21 | 2.53 | 0.83 | R6D17 | 25 | 2.53 | 0.78 |
| W4D30 | 22 | 2.67 | 0.86 | A6D30 | 18 | 2.30 | 0.79 |
| W5D30 | 23 | 2.54 | 0.81 | **Average** | **25.7** | **2.6** | **0.8** |
| W6D30 | 23 | 2.70 | 0.86 | **SE** | **2.2** | **0.0** | **0.0** |
| **Average** | **18.4** | **2.4** | **0.8** | IWD4 | 11 | 1.83 | 0.76 |
| **SE** | **1.3** | **0.0** | **0.0** | IWD8 | 14 | 1.71 | 0.65 |
| IRD3 | 14 | 1.66 | 0.63 | IWD12 | 16 | 2.00 | 0.72 |
| IRD8 | 14 | 1.72 | 0.65 | IWD16 | 13 | 1.84 | 0.72 |
| IRD17 | 15 | 2.17 | 0.80 | IWD19 | 17 | 2.27 | 0.80 |
| IAD23 | 18 | 2.39 | 0.83 | IWD26 | 18 | 2.25 | 0.78 |
| IAD26 | 22 | 2.70 | 0.87 | IWD30 | 20 | 2.67 | 0.89 |
| **Average** | **16.6** | **2.1** | **0.7** | **Average** | **15.5** | **2.0** | **0.7** |
| **SE** | **1.5** | **0.1** | **0.0** | **SE** | **1.1** | **0.1** | **0.0** |

The MCs of the four sample groups (incoming feed, inlet water, feed and water in tanks) of the MMS system were analyzed by NMS based on the Bray-Curtis similarities (Figure 3.7). The NMS plot showed a clear clustering of the water samples (inlet and rearing water) and of the rotifer feed samples (incoming and in tank). Three points referring to incoming artemia and artemia in tank samples clustered relatively close to the points representing the rearing water samples. The MCs of the rotifers in tank samples were also more similar to the water in tank samples. ANOSIM analysis based on Bray-Curtis similarities confirmed that the MCs of these four samples types were significant difference ($p<0.05$; Table 3.5).

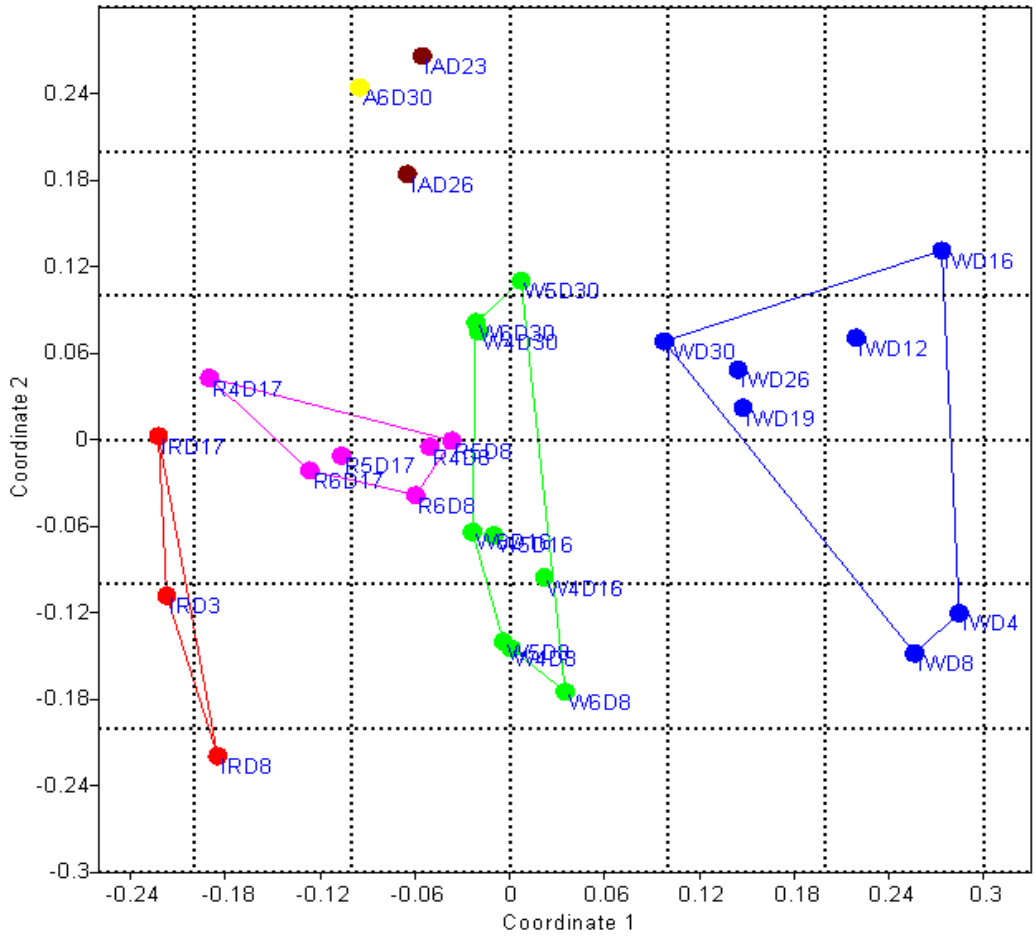



**Figure 3.7**: **Ordination of the microbial composition of water and feed samples of the MMS system by NMS based on Bray-Curtis similarity** (the points and lines blue: incoming water samples, green: rearing water samples, red: incoming rotifer samples, pink: rotifer in tanks samples, brown: incoming artermia samples, yellow: artermia in tank). The stress value in analysis is 0.228

**Table 3.5**: **ANOSIM analysis of the microbial community from feed and water samples in microbial maturation system**.

| | | R value | p value |
|---|---|---|---|
| **Overall analysis** | | **0.5899** | **0.0001** |
| **Between groups** | Incoming feed - Feed in tank | 0.3401 | 0.0275 |
| | Incoming feed - Incoming water | 0.6959 | 0.0012 |
| | Incoming feed - Water in tank | 0.6502 | 0.0005 |
| | Feed in tank - Incoming water | 0.6900 | 0.0007 |
| | Water in tank - Feed in tank | 0.4687 | 0.0006 |
| | Water in tank - Incoming water | 0.7555 | 0.0040 |

### *3.2.3 Recirculation aquaculture system (RAS).*

The MC compositions of a total of 28 samples (including water and feed) from the RAS were analyzed by PCR-DGGE, and the resulting DGGE gel is shown in Figure 3.8. Generally, the MCs of water in RAS system appeared to be stable between replicate tanks taken at the same time, and band patterns of inlet and rearing water seemed to be similar.

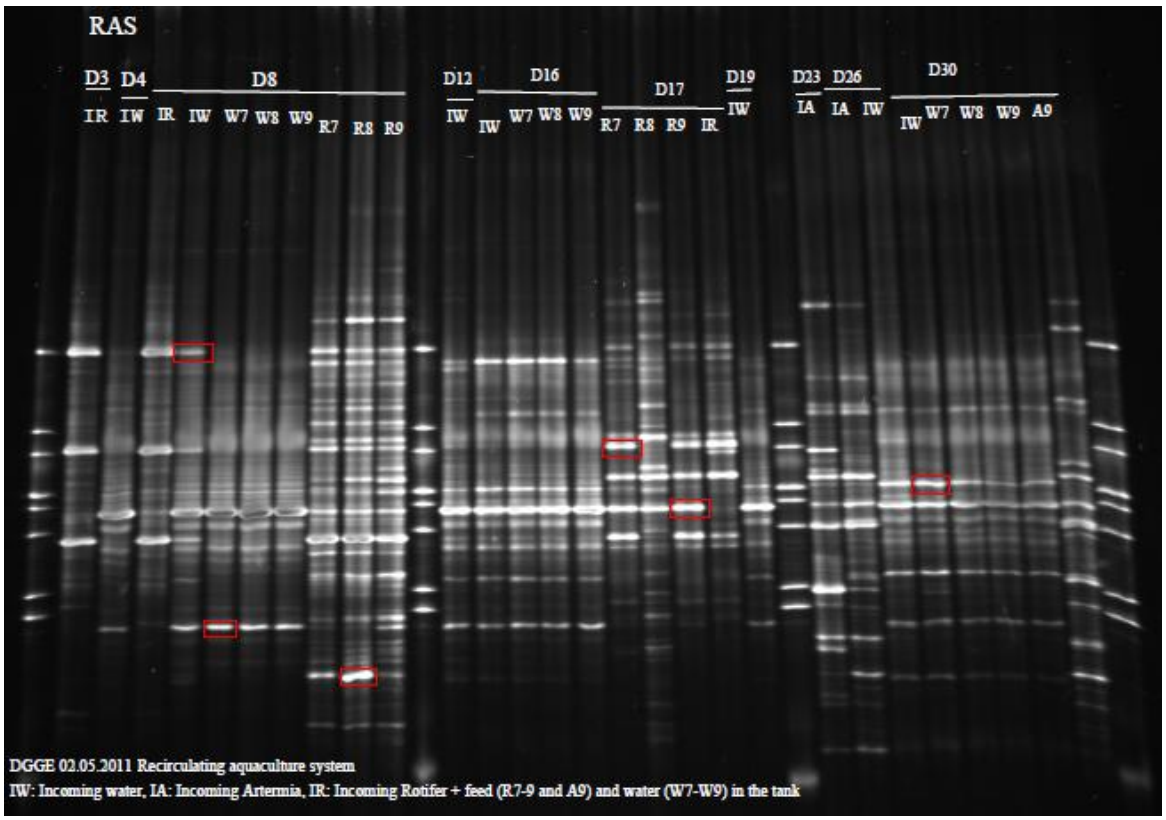



**Figure 3.8: DGGE gel for PCR-amplified 16S rRNA fragments from microbial communities in the RAS** (IW: inlet water, IR: incoming rotifer, IA: incoming artemia, W7-9: water in tank, R7-9: rotifer in tank, A9: artemia in tank and red frame: sequencing bands).

The band richness, diversity and evenness index of the MCs in RAS (Table 3.6) showed that average band richness was highest in live feed in tanks (23.5±3.3) followed by incoming water (17.4±1.5), water in tanks (16.8±1.0) and incoming live feed (16.4±1.6). It also seemed to be a tendency that band richness of water samples decreased with time, was stable between replicate tanks. The diversity index was nearly equal and varied on the range from 2.2 to 2.6. The highest evenness index was 0.8±0.0 in live feed in tank and the lowest was 0.7±0.0 in incoming live feed. ANOVA and Tukey analysis revealed no significantly differences between groups ($p>0.05$).

**Table 3.6: Band richness, diversity index and evenness index of microbial composition in the RAS** (W7-9: water in tank 7-9, IW: inlet water, IA: incoming artemia, IR: incoming rotifer, R7-9: rotifer in tank 7-9, A9: artemia of tank 9, D_number: sampling day)

| Samples | S | H' | J' | Samples | S | H' | J' |
|---|---|---|---|---|---|---|---|
| W9D30 | 11 | 2.29 | 0.95 | IWD30 | 13 | 2.19 | 0.85 |
| W8D30 | 11 | 2.19 | 0.91 | IWD26 | 15 | 2.31 | 0.85 |
| W7D30 | 10 | 1.98 | 0.86 | IWD19 | 13 | 1.91 | 0.74 |
| W9D16 | 17 | 2.42 | 0.85 | IWD16 | 16 | 2.26 | 0.81 |
| W8D16 | 17 | 2.36 | 0.83 | IWD12 | 20 | 2.38 | 0.79 |
| W7D16 | 17 | 2.43 | 0.86 | IWD8 | 23 | 2.68 | 0.85 |
| W9D8 | 20 | 2.25 | 0.75 | IWD4 | 22 | 2.54 | 0.82 |
| W8D8 | 22 | 2.24 | 0.73 | **Average** | **17.4** | **2.3** | **0.8** |
| W7D8 | 23 | 2.47 | 0.79 | **SE** | **1.5** | **0.0** | **0.0** |
| **Average** | **16.4** | **2.2** | **0.8** | A9D30 | 15 | 2.45 | 0.91 |
| **SE** | **1.6** | **0.0** | **0.0** | R9D17 | 14 | 2.04 | 0.77 |
| IAD26 | 17 | 2.37 | 0.84 | R8D17 | 26 | 2.79 | 0.86 |
| IAD23 | 19 | 2.57 | 0.87 | R7D17 | 14 | 2.19 | 0.83 |
| IRD17 | 15 | 2.21 | 0.82 | R9D8 | 33 | 3.22 | 0.92 |
| IRD8 | 19 | 2.28 | 0.77 | R8D8 | 33 | 3.11 | 0.89 |
| IRD3 | 14 | 1.75 | 0.66 | R7D8 | 30 | 2.99 | 0.88 |
| **Average** | **16.8** | **2.2** | **0.7** | **Average** | **23.5** | **2.6** | **0.8** |
| **SE** | **1.0** | **0.1** | **0.0** | **SE** | **3.3** | **0.1** | **0.0** |

NMS analysis base on the Bray-Curtis similarities (Figure 3.9) revealed partly overlap of microbial community similarity in the inlet and the rearing water. This illustrate that the MC of the rearing water was similar to the MC of the inlet water. The microbial communities of tank and incoming feed samples were separated.

The ANOSIM test based on Bray-Curtis similarities showed that the microbial communities composition between incoming feed and feed in tank and between incoming and rearing water were not significantly different ($p>0.2$). Thus, the ANOSIM test supported the observation that the inlet water and the rearing water of the RAS tanks were similar. However, the MC of the rest of the groups was significantly different (Table 3.7).

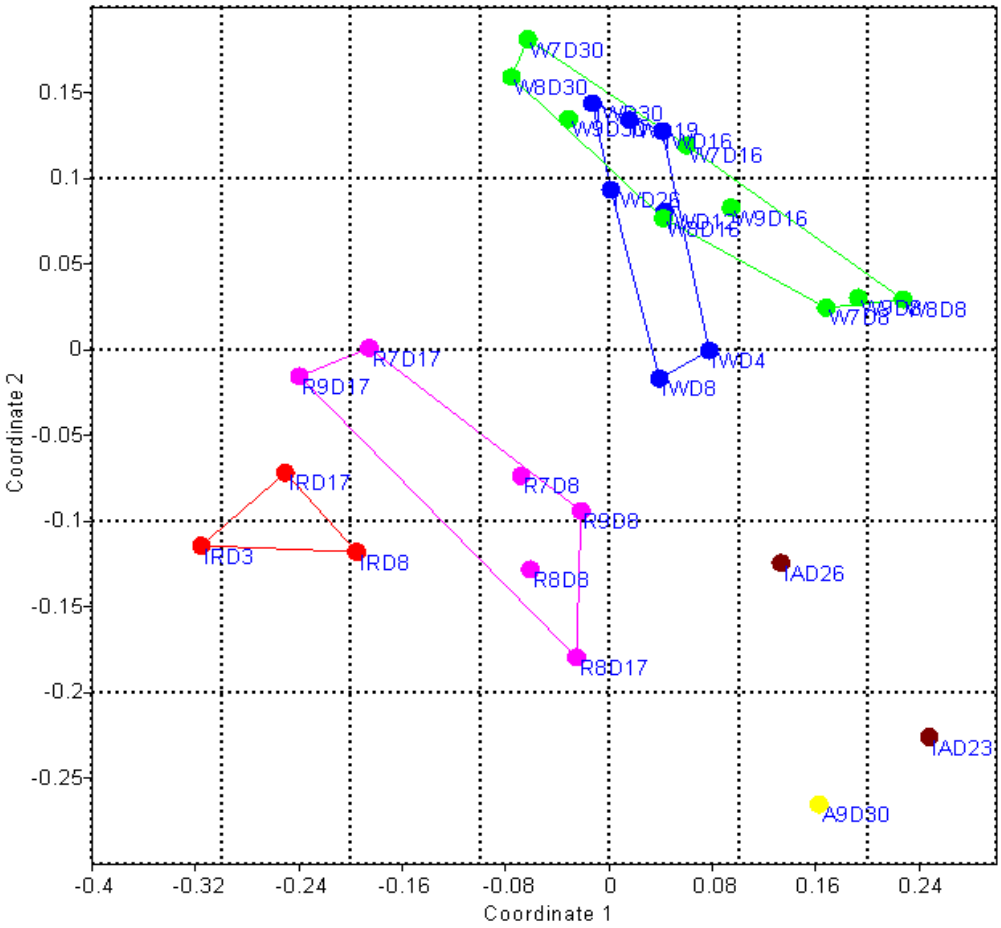



**Figure 3.9**: **Ordination of the microbial composition of water and feed samples of the RAS system by NMS based on Bray-Curtis similarity** (the lines and points blue: incoming water samples, green: rearing water samples, red: incoming rotifer samples, pink: rotifer in tanks samples, brown: incoming artermia samples and yellow: artermia in tank). The stress value in analysis is 0.187

**Table 3.7**: **ANOSIM analysis of the microbial community between four sample types** (IF-incoming feed, FT-feed in tanks, IW-incoming water, WT-water in tanks) collected from the recirculation aquaculture system

| **Overall analysis** | | **R value** | **p value** |
|---|---|---|---|
| | | **0.4176** | **0.0001** |
| **Between groups** | IF- FT | 0.0654 | 0.2258* |
| | IF-IW | 0.5797 | 0.0043 |
| | IF -WT | 0.6058 | 0.0012 |
| | FT - IW | 0.6103 | 0.0006 |
| | WT-FT | 0.5934 | 0.0001 |
| | WT -IW | 0.0209 | 0.2922* |

** p>0.05 (no significant difference)*

### *3.2.4 Similarity of MCs between replicate tanks, and between inlet water, feed and rearing water*

The average Bray-Curtis similarities of microbial communities between replicate tanks of each water treatment were calculated (Figure 3.10). The replicate tanks of the RAS had highest similarity, and those were higher than FTS by 11.6% and MMS by 1.5%. Thus these results support the findings described above (Fig. 3.3 and 3.9) indicating that the water MC is more stable in the RAS than in the other systems. However, the average similarities in MC of incoming water were lower (RAS, MMS and FTS reached 0.71; 0.7 and 0.58 respectively (Figure 3.3). ANOVA analysis showed that the between-tank similarity for the RAS was significant higher than for the FTS (p=0.0132).

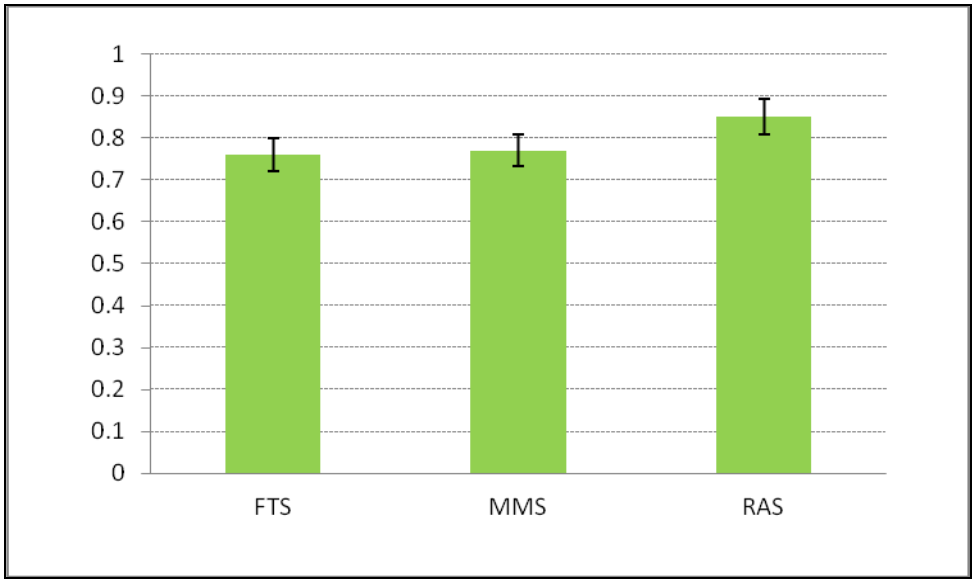



**Figure 3.10**: **Average Bray-Curtis similarities of MC composition between replicate tanks from the three water treatment systems.**

To determine whether the MC of tank water is mainly influenced by the MC of in-flowing water or by the MC of the feed, the MC of incoming water and feed was compared to the MC of the rearing water for all three treatment systems by calculating average Bray-Curtis similarities. As shown in Figure 3.11, the average similarity between the intake water MC and rearing water MC from MMS and FTS were similar (0.26), while in RAS, the similarity between intake water and rearing water MC was considerably higher (0.74). The average Bray-Curtis similarities between the incoming feed MC and the rearing water MC were 0.22, 0.37 and 0.44 in FTS, MMS and RAS respectively (Figure 3.11). T-test analysis indicated

that the similarities between intake feed and rearing water MC, and between inlet water and rearing water MC in the FTS and MMS were not significantly different ($p>0.05$), while RAS had contrary result (Table 3.8).

In conclusion the MC of rearing water of RAS was more strongly determined by the MC of in-flowing water, while for MMS and FTS, the rearing water MC seemed to be similarly influenced by inlet water and feed MC.

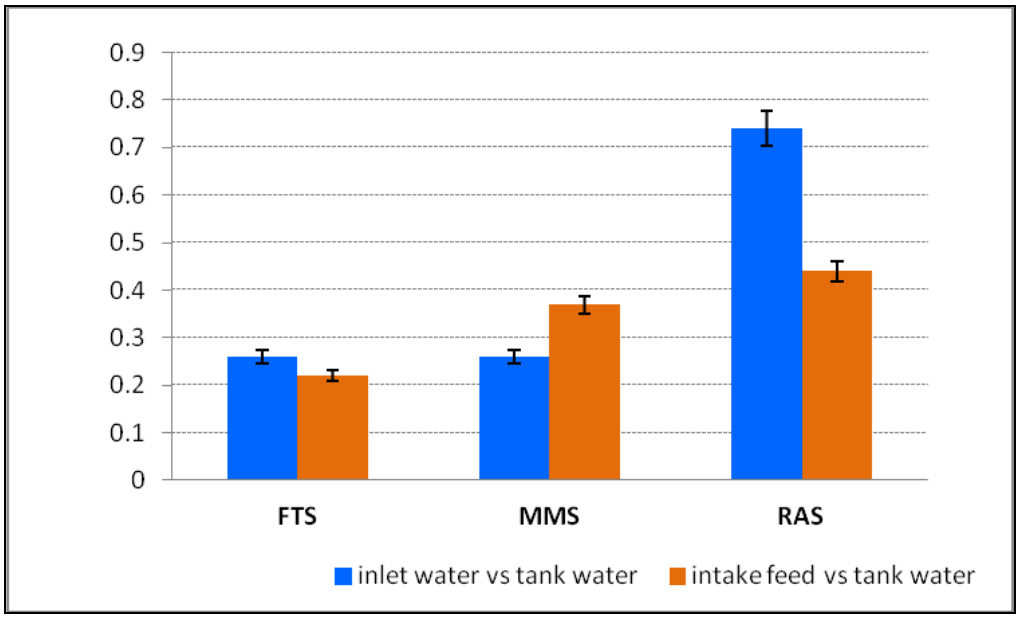



**Figure 3.11: Average Bray-Curtis similarities between rearing water MC and inlet water MC, and between rearing water MC and feed MC for the three water treatment systems**

**Table 3.8**: **T-test analysis of similarities between rearing water MC and intake water MC, and between rearing water MC and intake feed MC from the RAS, MMS and FTS**

| System | Between groups | P-value |
|---|---|---|
| **RAS** | Intake feed, rearing water vs Inlet water, rearing water | 0.002* |
| **MMS** | Intake feed, rearing water vs Inlet water, rearing water | 0.377 |
| **FTS** | Intake feed, rearing water vs Inlet water, rearing water | 0.971 |

**$p<0.05$, significant difference*

## 3.3 Microbial community composition of the gut in cod larvae from the three water treatment systems

### *3.3.1. Larval microbiota at day 8 post hatching*

A total of 9 individuals of average size from each rearing system were selected to investigate the MC composition by PCR-DGGE. There were in total 41 unique bands visible in the DGGE gel with the software program Gel2K. One band was dominating in the DGGE profile of all larvae. Among the 41 bands, eight bands were excised for DNA sequencing and taxonomic assignment.

Six bands from the larval samples were determined to be Proteobacteria, Bacteroidetes and Firmicutes, of which Proteobacteria represented the dominating band of all larval. Additionally, two bands representing the water samples were identified as Proteobacteria.

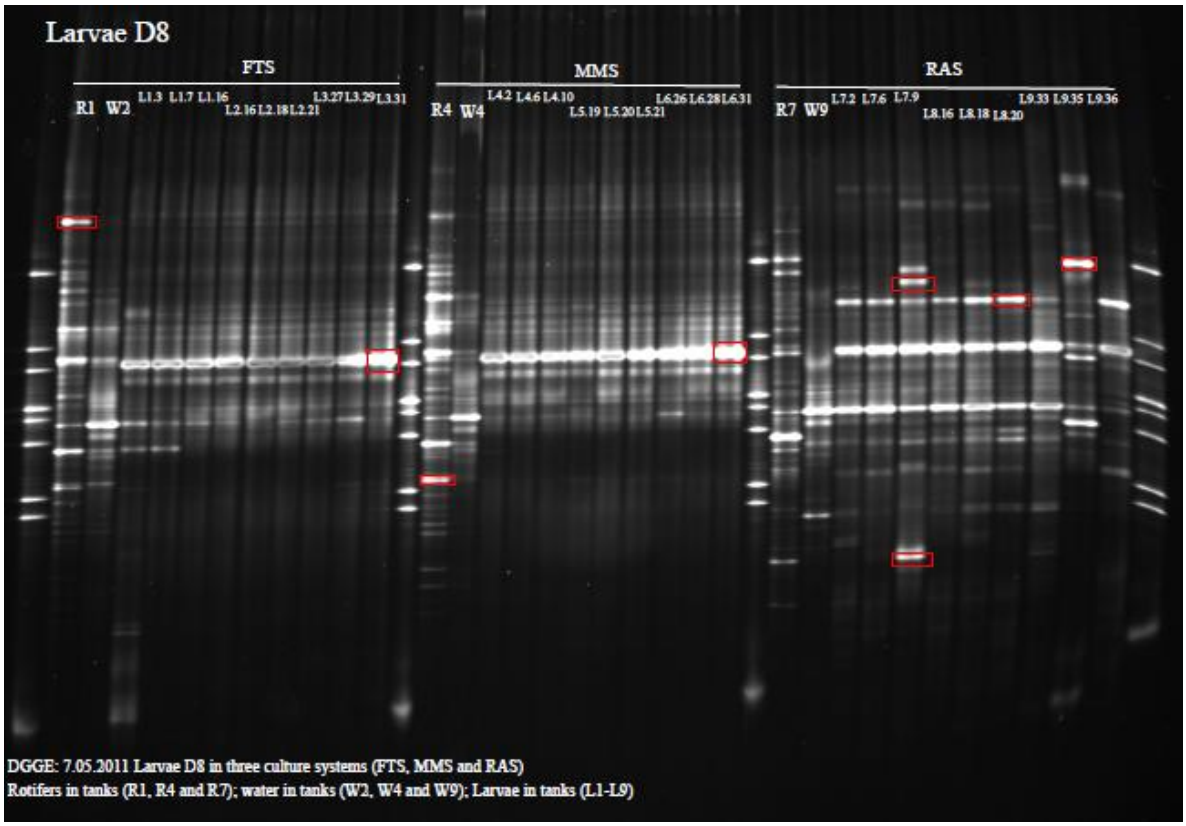



**Figure 3.12: DGGE gel image of 16S rDNA fragments representing the microbial community composition of feed, water and larvae samples at 8 day post-hatching in RAS, MMS and FTS** (R1,4,7: rotifer samples in tanks, W 2,4,9: water samples in tanks, Lnm: L is larval, n is tank number, m is larval number).

The band richness, diversity and evenness indexes were calculated for each sample based on the peak area values in the spread sheet resulting from Gel2K analysis of the DGGE image (Fig. 3.12). The results are shown in Table 3.9. The bands richness in larvae in RAS had highest fluctuation (8<S<18), and the lowest fluctuation in evenness index (0.6<J'<0.8). While MMS exhibited a stable bands richness (10<S<13), FTS had the highest variation in evenness index (0.4<J'<0.8). However, band richness, diversity and evenness index were not significantly different between the three water treatment systems (p>0.05).

**Table 3.9**: **Band richness (S), diversity index (H') and evenness index (J') for DGGE profiles of larvae samples at 8 dph** (R1,4,7: rotifer samples in tanks; W2,4,9: water samples in tanks, Lnm: L is larval, n is tank number, m is larval number).

| **RAS** | | | | **MMS** | | | | **FTS** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** |
| L9.36 | 8 | 1.29 | 0.62 | L6.31 | 11 | 1.57 | 0.66 | L3.31 | 12 | 1.09 | 0.44 |
| L9.35 | 11 | 1.87 | 0.78 | L6.28 | 13 | 1.86 | 0.72 | L3.29 | 10 | 1.76 | 0.76 |
| T9.33 | 11 | 2.04 | 0.85 | L6.26 | 12 | 1.79 | 0.72 | L3.27 | 12 | 2.00 | 0.81 |
| L8.20 | 11 | 1.59 | 0.67 | L5.21 | 10 | 1.97 | 0.85 | L2.21 | 12 | 1.98 | 0.79 |
| L8.18 | 16 | 2.19 | 0.79 | L5.20 | 12 | 2.02 | 0.81 | L2.18 | 14 | 2.30 | 0.88 |
| L8.16 | 10 | 1.55 | 0.67 | L5.19 | 10 | 1.72 | 0.75 | L2.16 | 12 | 1.86 | 0.75 |
| L7.9 | 18 | 2.37 | 0.82 | L4.10 | 12 | 2.28 | 0.92 | L1.7 | 11 | 1.90 | 0.79 |
| L7.6 | 11 | 1.57 | 0.66 | L4.6 | 12 | 1.79 | 0.72 | L1.3 | 11 | 2.06 | 0.86 |
| L7.2 | 12 | 1.64 | 0.66 | L4.2 | 11 | 2.10 | 0.88 | L1.2 | 11 | 1.75 | 0.73 |
| **Aveage** | **12** | **1.7** | **0.7** | **Average** | **11.4** | **1.9** | **0.7** | **Average** | **11.6** | **1.8** | **0.7** |
| **SE** | **1.0** | **0.1** | **0.0** | **SE** | **0.3** | **0.0** | **0.0** | **SE** | **0.3** | **0.1** | **0.0** |
| W9 | 12 | 1.61 | 0.65 | W4 | 14 | 2.17 | 0.82 | W2 | 11 | 1.99 | 0.83 |
| R7 | 18 | 2.24 | 0.78 | R4 | 23 | 2.70 | 0.86 | R1 | 18 | 2.49 | 0.86 |

The MC of the rearing water in MMS and FTS, as well as MC of feed in all three system appeared to be relative similar at 8 dph, indicated by overlapping points on the ordination plot (W2, W4, F1, F4 and F7; Figure 3.13). However, the MC of the rearing water in RAS seemed to be distinct from the rearing water of other two waters. The points presenting microbial community composition of larvae in RAS were scattered in a large area, and were far away

from to the MC in FTS and MMS. Additionally, the MC of larvae samples in FTS and MMS were disposed in narrow area and were clearly overlapping.

The average Bray-Curtis similarities of larval MCs within and between water treatment systems are presented in Figure 3.14. The average similarities of larval MC within the MMS and FTS were higher than within RAS. Between groups comparisons showed that the larval MC of the MMS and FTS were relative similar. ANOSIM analysis confirmed that the MC of larvae at day 8 post hatching from the three treatment systems were significantly different from each other with p values equal to or lower than 0.0056 (Table 3.10).

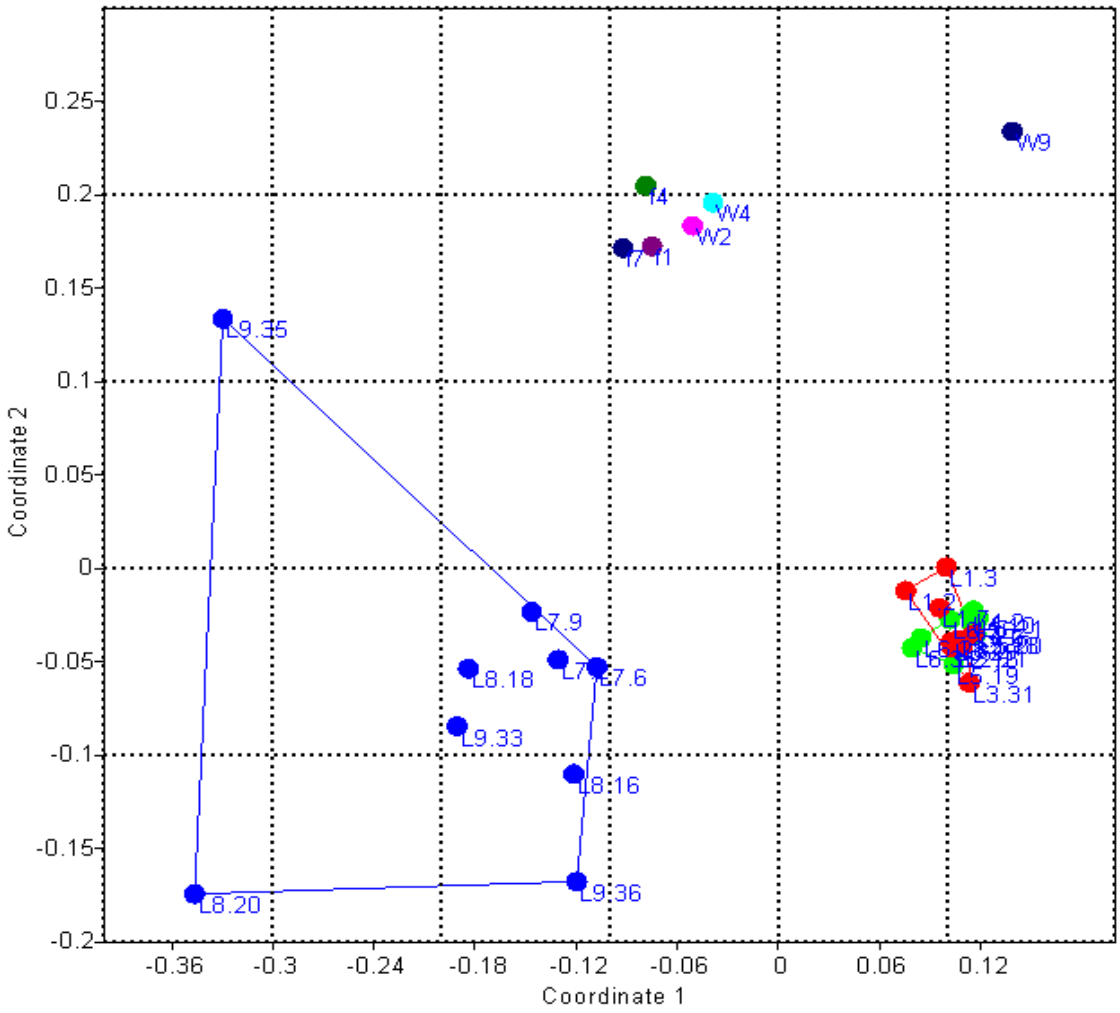



**Figure 3.13: NMS ordination of larval MC at day 8 in three water treatments based on the Bray-Curtis similarities** (the points blue: larvae of RAS, the points green: larvae of MMS, the red points: larvae of FTS, R1,4,7: rotifer samples in tanks, W 2,4,9: water samples in tanks)

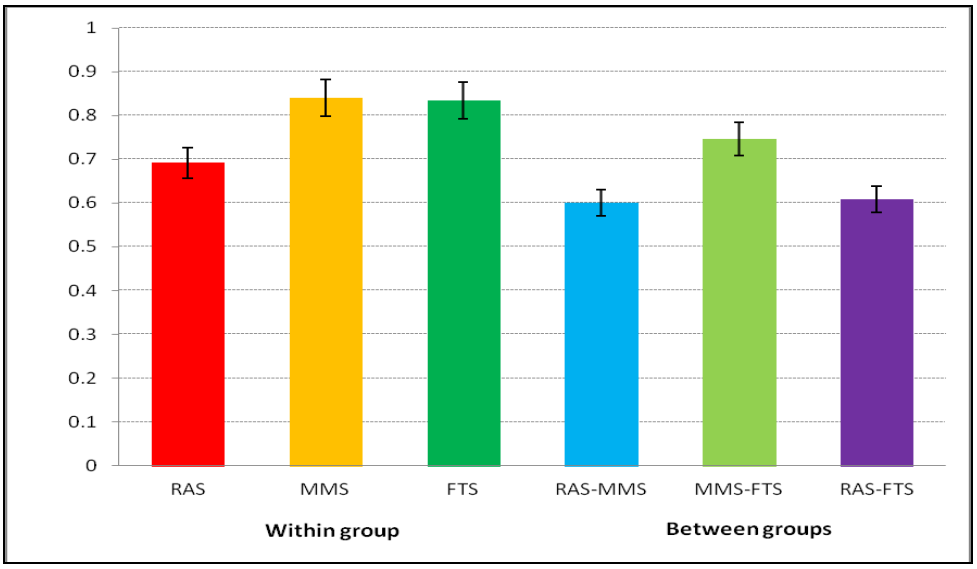



**Figure 3.14**: **Average Bray-Curtis similarities of MC composition of larvae at day 8 post hatching between and within the three different water treatment regimes**

**Table 3.10: ANOSIM analysis for testing the hypothesis of no difference between larval MC at day 8 from the three water treatment systems**

| Overall analysis | | R value | p value |
|---|---|---|---|
| | | **0.5370** | **0.0001** |
| **Between groups** | RAS - MMS | 0.7953 | 0.0002 |
| | RAS - FTS | 0.7767 | 0.0001 |
| | MMS - FTS | 0.2394 | 0.0056 |

### *3.3.2 Larval microbiota at day 17 post hatching*

The bacterial communities of the fish larvae in the three water treatment systems at 17 dph were investigated by PCR-DGGE. The resulting DGGE gel is shown in Fig. 3.15. The DGGE profiles for the larval samples seemed relatively similar to those at 8 dph (Fig. 3.12), indicating that the MC of larvae from the three water treatment regimes generally change little from day 8 to day 17.

Sequencing of six bands identified Proteobacteria and Cyanobacteria in three different water samples from the three treatment systems. Moreover, Proteobacteria was found in larval samples as well, which illustrated this bacterium present was in both the water and the larval.

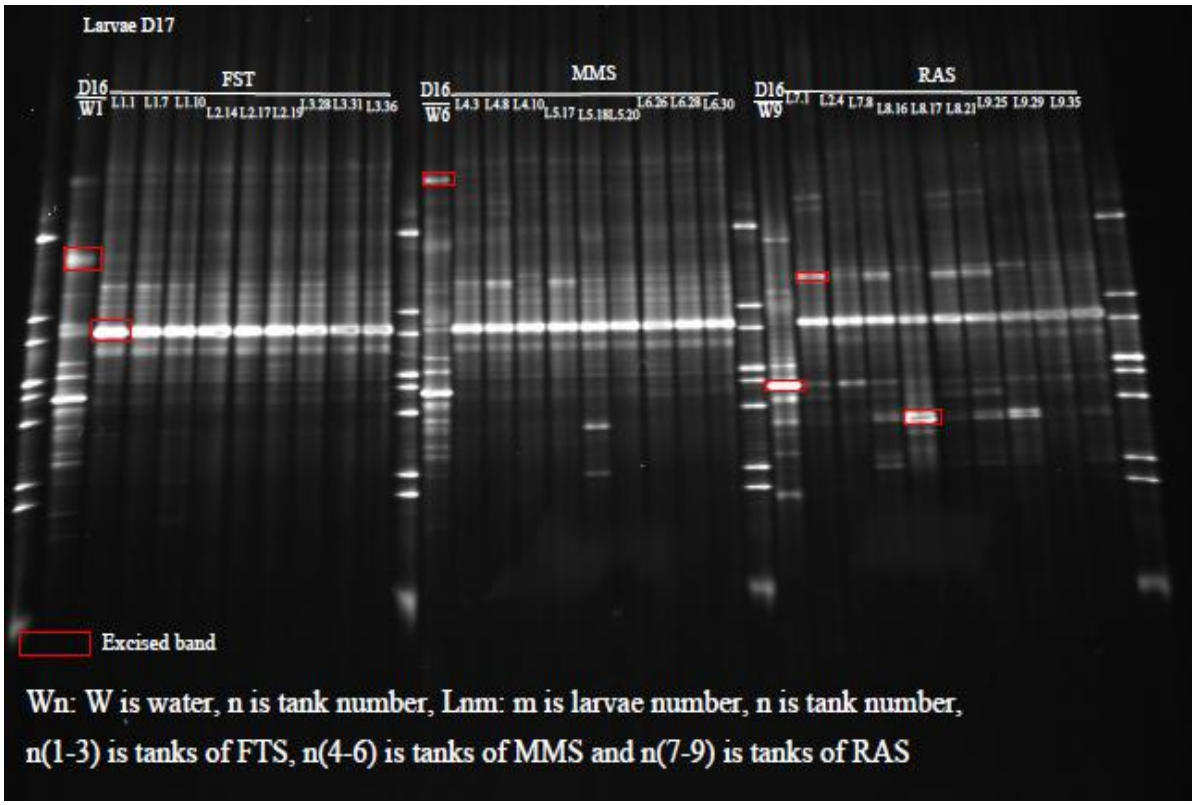



**Figure 3.15**: **DGGE gel image of a 16S rDNA fragment representing the microbial composition of feed, water and larval samples at 17 day post-hatching in RAS, MMS and FTS (**W 1,6,9: water samples in tanks, Lnm: L is larval, n is tank number, m is larval number**)**

The band richness found for the larvae samples from the FTS were relatively equal (12<S<15), while it varied more in RAS (6<S<12) (Table 3.11). ANOVA and Tukey analysis showed that the band richness in RAS was significantly different from that in FTS and MMS ($p<0.05$). The diversity and evenness index of the larval MC were similar among the three water treatment systems ($p>0.05$).

**Table 3.11**: **Band richness (S), diversity index (H') and evenness index (J') for DGGE profiles of larval MC at 17 dph** (Ln.m: L is larval, n is tank number, m is larvae number, W1, 6, 9: water sample in tank 1, tank 6 and tank 9)

| RAS | | | | MMS | | | | FTS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** |
| L9.35 | 8 | 1.51 | 0.73 | L6.30 | 13 | 1.49 | 0.58 | L3.36 | 12 | 1.33 | 0.53 |
| L9.29 | 8 | 1.36 | 0.66 | L6.28 | 12 | 1.63 | 0.66 | L3.31 | 15 | 1.58 | 0.58 |
| L9.25 | 6 | 1.48 | 0.83 | L6.26 | 15 | 1.76 | 0.65 | L3.28 | 13 | 1.53 | 0.59 |
| L8.21 | 10 | 1.64 | 0.71 | L5.20 | 14 | 1.42 | 0.54 | L2.19 | 13 | 1.67 | 0.65 |
| L8.17 | 8 | 1.28 | 0.61 | L5.18 | 17 | 2.02 | 0.71 | L2.17 | 15 | 1.79 | 0.66 |
| L8.16 | 9 | 1.64 | 0.75 | L5.17 | 13 | 1.61 | 0.63 | L2.14 | 15 | 1.73 | 0.64 |
| L7.8 | 12 | 1.89 | 0.76 | L4.10 | 12 | 1.69 | 0.68 | L1.10 | 14 | 1.63 | 0.62 |
| L7.4 | 6 | 1.20 | 0.67 | L4.8 | 16 | 1.86 | 0.67 | L1.7 | 14 | 2.00 | 0.76 |
| L7.1 | 8 | 1.37 | 0.66 | L4.3 | 14 | 1.76 | 0.67 | L1.1 | 15 | 1.73 | 0.64 |
| **Average** | **8.3** | **1.4** | **0.7** | **Average** | **14** | **1.6** | **0.6** | **Average** | **14** | **1.6** | **0.6** |
| **SE** | **0.6** | **0.0** | **0.0** | **SE** | **0.5** | **0.0** | **0.0** | **SE** | **0.3** | **0.0** | **0.0** |
| W9 | 13 | 2.15 | 0.84 | W6 | 16 | 2.43 | 0.88 | W1 | 15 | 2.48 | 0.92 |

Figure 3.16 shows an NMS plot based on the Bray-Curtis similarities for the water and larval samples at 17 dph. The plot indicated that the MCs of the water as well as of the larvae from three treatment systems were different. The MC of larvae in RAS was disposed in a larger area (indicating that the RAS larval MC was more variable among individuals), followed by the larvae in MMS and a smallest area was occupied by the MC of the larvae in FTS (indicating that the larval MC of MMS and FTS was less variable among individuals).

The average Bray-Curtis similarities of larval MCs within and between the three water treatments (Fig. 3.17) revealed that the FTS larval MC showed the highest average similarities, followed by MMS. The RAS larval MC showed the lowest average similarities. These results corroborated those found for the larval MC at day 8. The larvae from the RAS showed more diverse microbial composition compared with FTS and MMS until day 17. Results from ANOSIM analysis confirmed that the larval MCs from the RAS, MMS and FTS at day 17 post hatching were significant difference from each other, with p values equal to or lower than 0.0003 (Table 3.12).

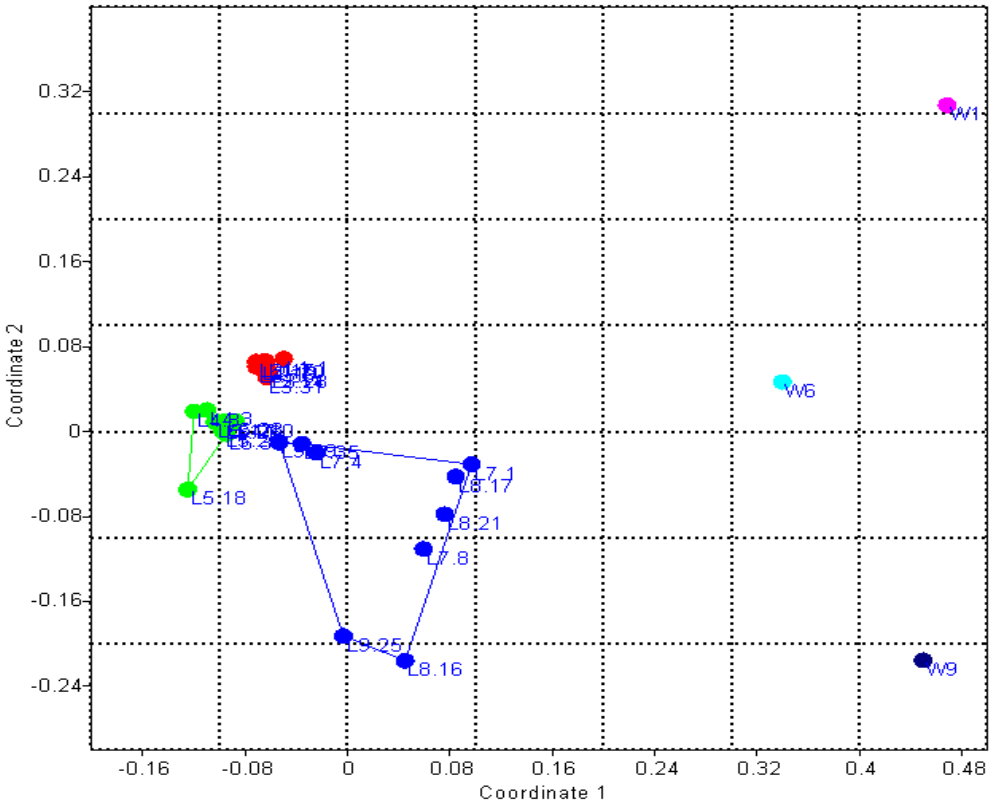



**Figure 3.16**: **NMS ordination of the larval MC based on the Bray-Curtis similarities at day 17 in three water treatment systems** (the points and lines blue: larvae of RAS, green: larvae of MMS, red: larvae of FTS, W9: water of the tank 9 in RAS, W6: water of the tank 6 in MMS and W1: water of the tank 1 in FTS).

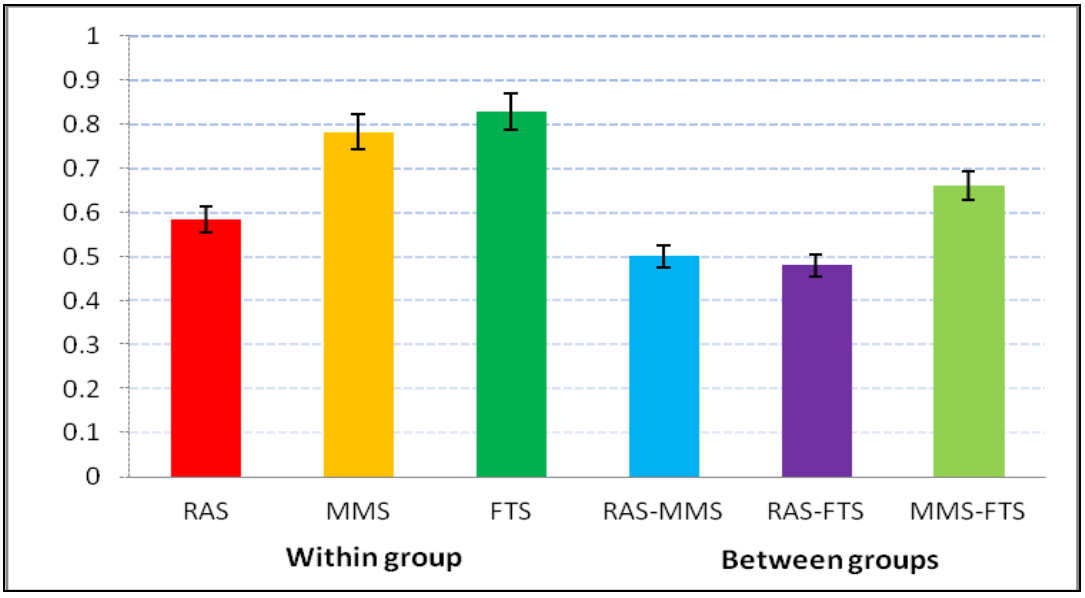



**Figure 3.17: Average Bray-Curtis similarities of MC composition of larvae at day 17 post hatching between and within the three different water treatment regimes.**

**Table 3.12: ANOSIM analysis for testing the hypothesis of no difference between larval MC from the three water treatment systems at day 17 post hatching.**

| Overall analysis | | R value | p value |
|---|---|---|---|
| | | **0.6808** | **0.0001** |
| **Between groups** | RAS - MMS | 0.6298 | 0.0003 |
| | RAS - FTS | 0.727 | 0.0002 |
| | MMS - FTS | 0.8899 | 0.0001 |

### *3.3.3 Larval microbiota at day 30 post hatching*

The larvae and water samples collected from the three water treatment systems at day 30 post hatching were analyzed by PCR-DGGE. The resulting gel is shown in Fig. 3.18. A total of 46 bands were determined in DGGE gel by software program Gel2k. Two bands in total of 46 bands presented mostly in the larvae samples from three water treatments, and one of these two bands was the same as the one dominating at 8 and 17 dph. Some of bands were selected for DNA sequencing and taxonomic assignment as marked with red frames in the gel (Fig. 3.18).

Seven bands in gel were excised, re-amplified and sequenced. The bands, which corresponded with the larval samples, were determined to be Cyanobacteria, Proteobacteria, of which Proteobacteria represented the two dominating bands of all larval MC. The remaining bands, corresponded with the water samples, revealed the two following phyla: Proteobacteria and Bacteroidetes.

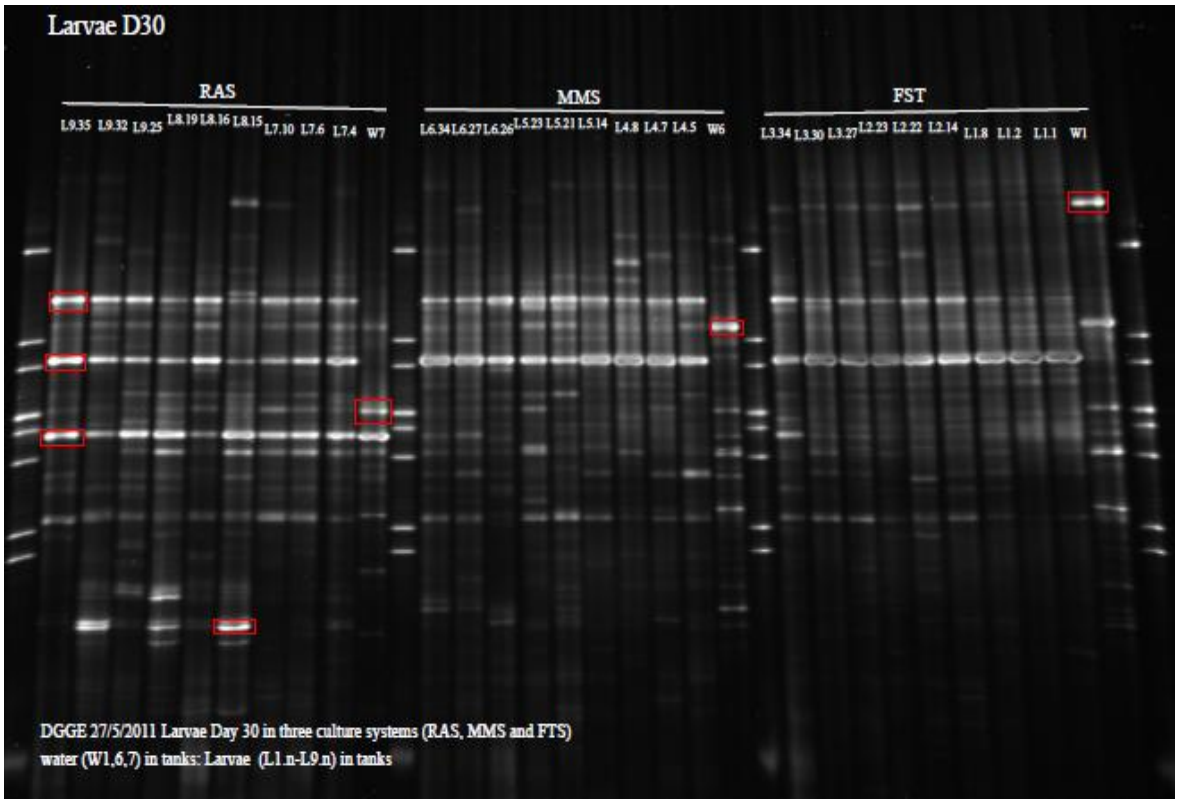



**Figure 3.18: DGGE gel with PCR-amplified 16S rRNA fragments from the MCs of larval and water samples of the FTS, MMS and RAS at 30 dph** (W 1,6,7: water samples in tanks, Lnm: L is larval, n is tank number, m is larval number, red frames: sequencing bands)

The diversity and evenness indexes of MC of the larvae at 30 dph in RAS, MMS, and FTS were similar with average values of approximately 2.1 and 0.8 respectively (Table 3.13). However, the band richness index varied among the systems. RAS larvae MC had the highest average value (16.5±0.8) and FTS larvae MC had the lowest (13.6±1.2) (Table 3.13). Still, none of the indexes (richness, diversity, evenness) for larval MC of the three water treatment regimes were found to be significant difference by ANVOVA and Tukey analysis ($p>0.05$).

**Table 3.13**: **Band richness, diversity and evenness indexes for microbial communities of the larval and water samples at 30 dph** (Ln.m: L is larval, n is tank number and m is larvae number, W1: water sample in the tank 1, W6: water sample in tank 6 and W7: water sample in tank 7)

| RAS | | | | MMS | | | | FTS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** |
| L9.35 | 15 | 2.46 | 0.91 | L6.30 | 10 | 1.71 | 0.74 | L3.34 | 15 | 1.98 | 0.73 |
| L9.32 | 14 | 2.15 | 0.81 | L6.27 | 14 | 1.94 | 0.74 | L3.30 | 12 | 1.99 | 0.80 |
| L9.25 | 18 | 2.33 | 0.81 | L6.26 | 14 | 2.22 | 0.84 | L3.27 | 13 | 2.02 | 0.79 |
| L8.19 | 18 | 2.06 | 0.71 | L5.23 | 15 | 1.92 | 0.71 | L2.23 | 21 | 2.49 | 0.82 |
| L8.16 | 18 | 2.29 | 0.79 | L5.21 | 16 | 2.35 | 0.85 | L2.22 | 11 | 1.99 | 0.83 |
| L8.15 | 21 | 2.42 | 0.79 | L5.14 | 16 | 2.29 | 0.82 | L1.14 | 17 | 2.46 | 0.87 |
| L7.10 | 17 | 2.09 | 0.74 | L4.8 | 14 | 1.82 | 0.69 | L1.8 | 12 | 1.89 | 0.76 |
| L7.6 | 16 | 2.21 | 0.79 | L4.7 | 19 | 2.16 | 0.73 | L1.2 | 14 | 2.18 | 0.82 |
| L7.4 | 12 | 2.04 | 0.82 | L4.5 | 16 | 2.31 | 0.83 | L1.1 | 8 | 1.51 | 0.73 |
| **Average** | **16.5** | **2.2** | **0.7** | **Average** | **14.8** | **2.0** | **0.7** | **Average** | **13.6** | **2.0** | **0.7** |
| **SE** | **0.8** | **0.0** | **0.0** | **SE** | **0.8** | **0.0** | **0.0** | **SE** | **1.2** | **0.0** | **0.0** |
| W7 | 15 | 2.10 | 0.78 | W6 | 14 | 2.15 | 0.81 | W1 | 9 | 1.66 | 0.75 |

NMS analysis based on Bray-Curtis similarities showed the MCs of the water and the larvae from three water systems were fairly different, with no overlap between the different systems in the plot shown in Figure 3.19.

Average Bray-Curtis similarities of larval MC within and between systems are showed in Figure 3.20. As at 17 dph, FTS still had higher average larval MC similarity in compared to RAS and MMS larval MCs. In addition, the microbial compositions of the larval MC in MMS and FTS were more similar than in RAS and MMS larval MC, and the RAS and FTS larval MC.

ANOSIM analysis affirmed that the MC of larvae in three systems were significant different from each other ($R=0.748$, $p<0.0001$, Table 3.14)

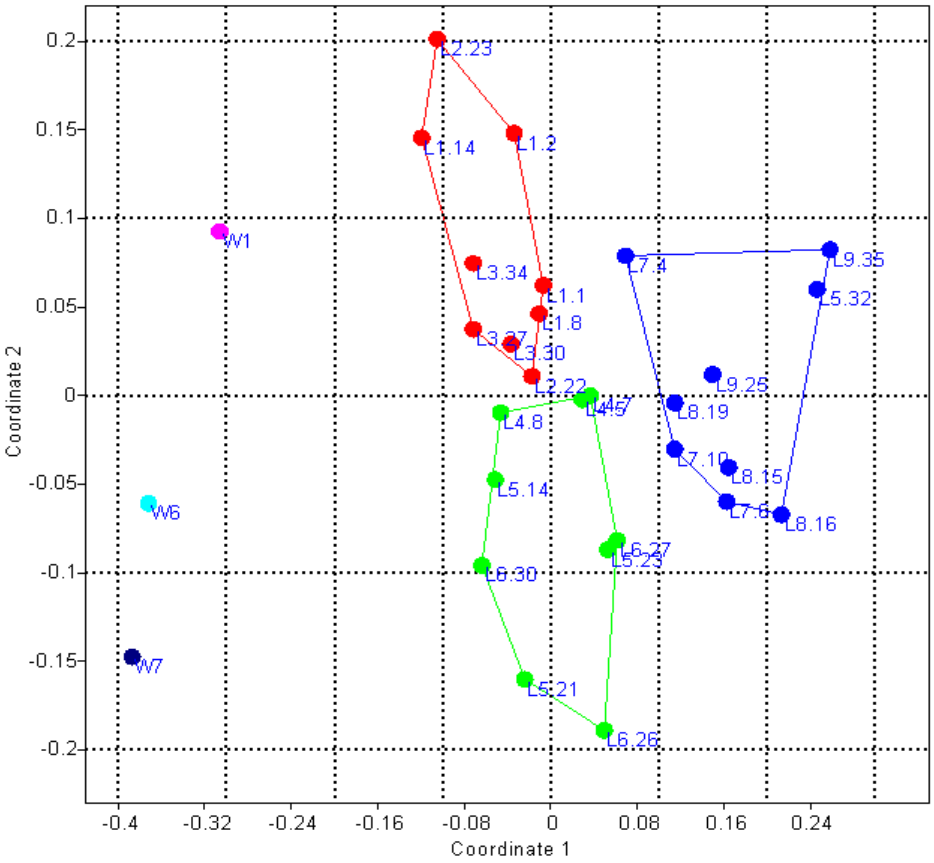



**Figure 3.19: NMS ordination of the larval MC from the three water treatment systems at day 30 post hatching based on Bray-Curtis similarities** (the points and lines green: larvae of MMS, the points and lines blue: larvae of RAS, the points and lines red: larvae of FTS, W7: water of the tank 7 in RAS, W6: water of the tank 6 in MMS and W1: water of the tank 1 in FTS)

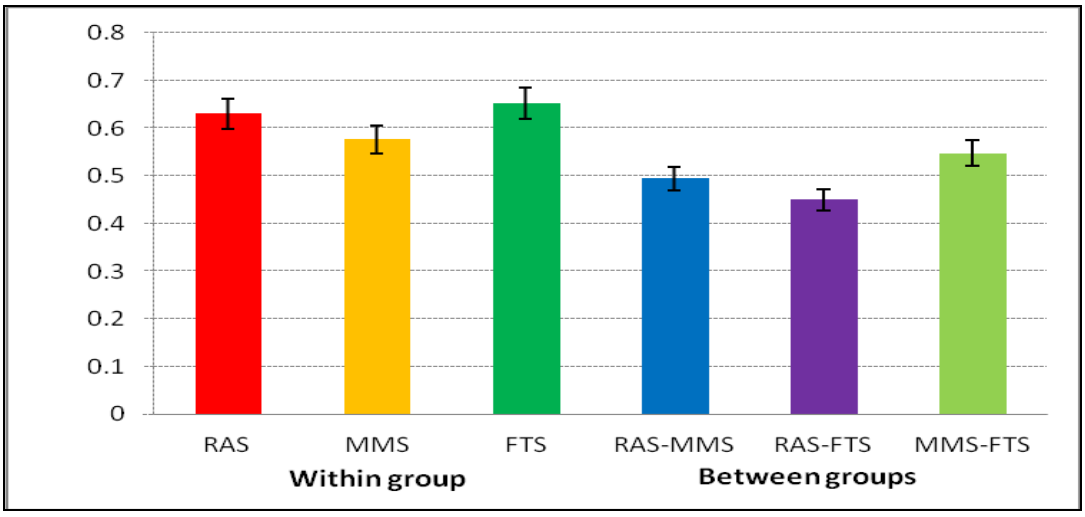



**Figure 3.20: Average Bray-Curtis similarities of larval MC at day 30 post hatching between and within three different water treatment systems.**

**Table 3.14: ANOSIM analysis for testing the hypothesis of no difference between larval MC from the three water treatment systems at day 30 post hatching.**

| Overall analysis | | R value | p value |
|---|---|---|---|
| | | **0.748** | **0.0001** |
| **Between groups** | RAS - MMS | 0.5837 | 0.0003 |
| | RAS - FTS | 0.929 | 0.0003 |
| | MMS - FTS | 0.6845 | 0.0003 |

### *3.3.4 Larval microbiota at day 46 post hatching.*

All tanks received the same water (MMS) from day 31 post hatching to the end of the experiment. At day 46, the larvae were big enough to dissect the gut from the larvae. The MCs of the larval guts were analyzed by PCR-DGGE. Nine larval samples and a water sample from each water treatment system were investigated on a gel, and the result is given in Fig. 3.21. Five bands in red frames, which represented the larval samples, were excised for taxonomic assignments (Figure 3.21). The comparison of the DNA sequencing in the Genbank concluded Firmicutes, Bacteroidetes and Proteobacteria being the bacteria in larva samples.

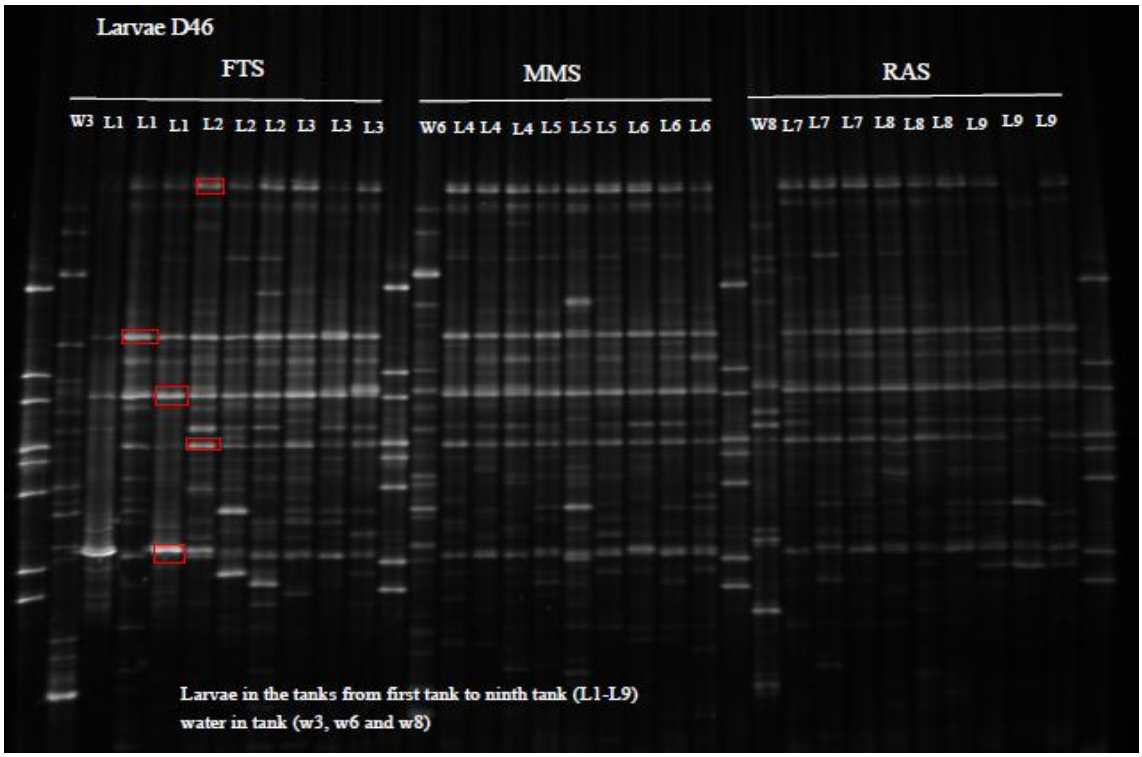



**Figure 3.21: DGGE gel image of the microbial community composition of water and gut in larvae at 46 day post hatching in RAS, MMS and FTS** (W1,3,8: water samples in tanks, L: larval samples in tanks, red frame: sequencing bands)

The band richness, diversity and evenness indexes were calculated based on the peak area values in the spread sheet resulting from Gel2K analysis of the DGGE image (Figure 3.21). The results are given in Table 3.15. The diversity index in three systems was similar with average values around 2.4. The average band richness for the FTS and MMS larval MCs was approximately 19.5, and in RAS the corresponding average value was 16.4. The average evenness for larval MCs in RAS, MMS and FTS was 0.87; 0.84 and 0.80 respectively (Table 3.15). ANOVA and Tukey analysis showed the indexes for the larval MCs from the three regimes were similar, except that evenness of larval MC from RAS and FTS was significantly different from each other ($p<0.05$).

**Table 3.15: Band richness, diversity and evenness indexes of the gut microbial communities of the larvae and water in FTS, MMS and RAS** (W 3,6,8: water samples in tanks, Lnm: L is larval, n is tank number, m is larval number**)**

| **RAS** | | | | **MMS** | | | | **FTS** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** |
| L9.34 | 12 | 2.24 | 0.9 | L6.32 | 17 | 2.44 | 0.86 | L3.32 | 20 | 2.39 | 0.8 |
| L9.33 | 14 | 2.22 | 0.84 | L6.31 | 20 | 2.56 | 0.85 | L3.29 | 20 | 2.39 | 0.79 |
| L9.26 | 14 | 2.36 | 0.89 | L6.28 | 19 | 2.34 | 0.79 | L3.25 | 23 | 2.69 | 0.86 |
| L8.19 | 16 | 2.48 | 0.89 | L5.22 | 22 | 2.59 | 0.84 | L2.24 | 25 | 2.79 | 0.87 |
| L8.18 | 18 | 2.5 | 0.87 | L5.17 | 23 | 2.64 | 0.84 | L2.21 | 23 | 2.57 | 0.82 |
| L8.17 | 18 | 2.53 | 0.87 | L5.15 | 20 | 2.53 | 0.85 | L2.15 | 22 | 2.58 | 0.83 |
| L7.8 | 19 | 2.49 | 0.85 | L4.3 | 19 | 2.52 | 0.86 | L1.9 | 15 | 1.94 | 0.72 |
| L7.7 | 17 | 2.54 | 0.89 | L4.2 | 21 | 2.49 | 0.82 | L1.8 | 19 | 2.28 | 0.77 |
| L7.2 | 20 | 2.59 | 0.87 | L4.1 | 14 | 2.19 | 0.83 | L1.1 | 10 | 1.77 | 0.77 |
| **Average** | **16.4** | **2.4** | **0.8** | **Average** | **19.4** | **2.4** | **0.8** | **Average** | **19.6** | **2.3** | **0.8** |
| **SE** | **0.8** | **0.0** | **0.0** | **SE** | **0.8** | **0.0** | **0.0** | **SE** | **1.5** | **0.1** | **0.0** |
| W8 | 16 | 2.47 | 0.89 | W6 | 24 | 2.89 | 0.91 | W3 | 19 | 2.68 | 0.91 |

Fig. 3.22 showed a NMS plot representing the larval gut MCs in three water treatment systems, based on Bray-Curtis similarities. In the plot, the gut MCs of the larvae from three regimes were now mostly overlapping, except several divergent samples, especially the L1.1 sample in FTS (Fig. 3.22). Hence, it revealed that the larval MC became more similar after receiving rearing water from the same source, and showed the larval MC seemed to be influential from the rearing water MC.

The average similarities calculated for larval MCs within the RAS and the MMS tanks were similar (around 0.7), whereas the average similarity determined for larval MCs within the FTS tanks were lower (around 0.5). Further, average similarities of larval MCs for between system comparisons indicated that the larval MCs of the RAS and MMS were more similar to each other than to larval MC of the FTS.

ANOSIM analysis indicated that there were no significant difference in the larval gut MC from the three treatments systems (R=0.0308, p>0.05, Table 3.16).

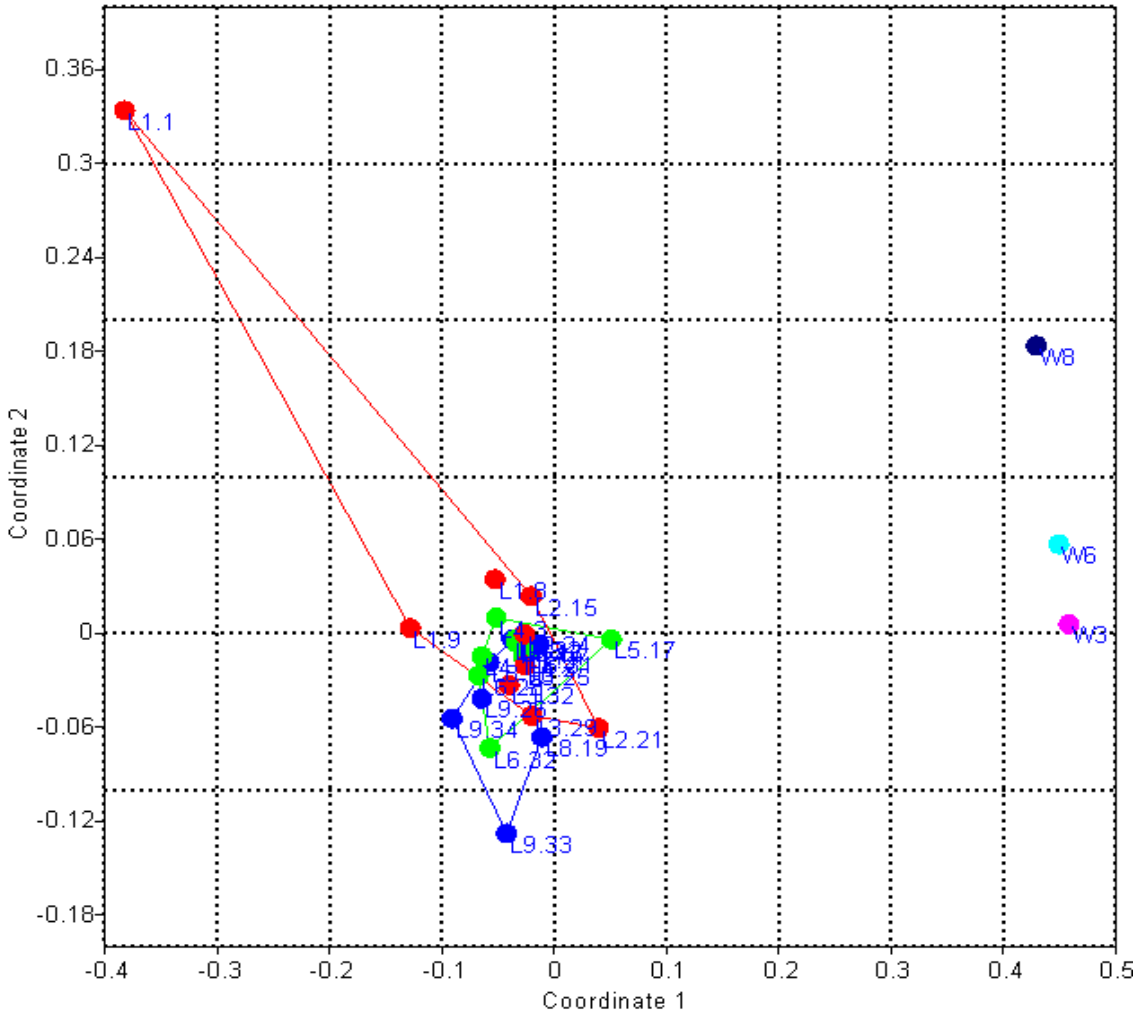



**Figure 3.22**: **Ordination of the larval gut MC from the three water treatments at day 46 by NMS using the Bray-Curtis similarities** (the points green: larvae in RAS, the points blue: larvae in MMS, the points red: larvae in FTS, W3: water in the tank 3 of FTS, W6: water in tank 6 of MMS, W8: water in the tank 8 of RAS).

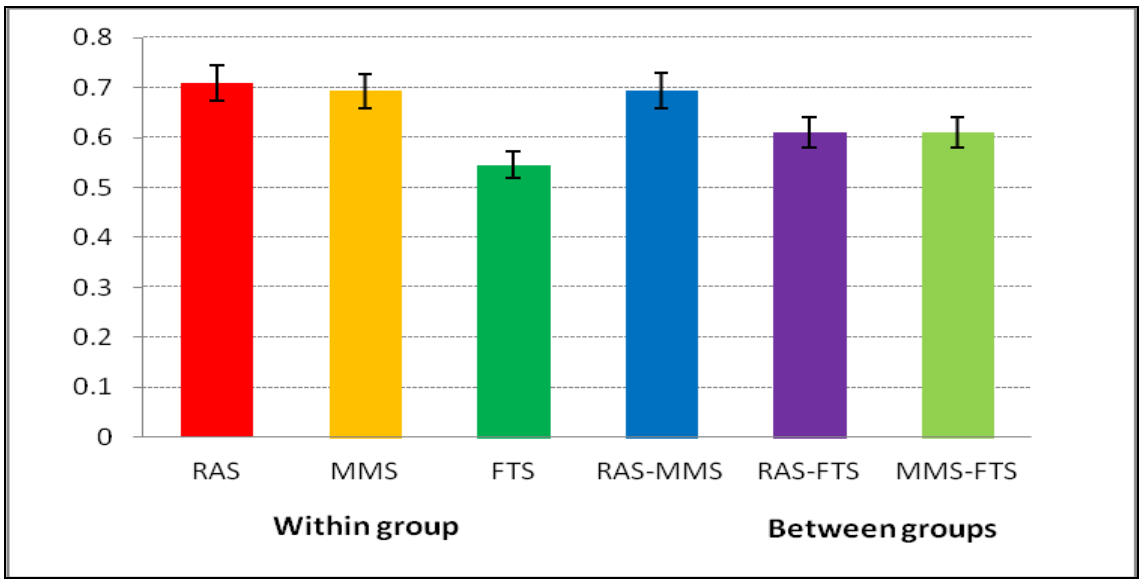



**Figure 3.23: Average Bray-Curtis similarities of larval MC at day 46 post hatching within and between the three water treatment systems.**

**Table 3.16: ANOSIM analysis for testing the hypothesis of no difference between larval MC from the three water treatment systems at day 46 post hatching.**

| | | R value | p value |
|---|---|---|---|
| **Overall analysis** | | **0.03079** | **0.1932** |
| **Between groups** | RAS - MMS | 0.04355 | 0.1993* |
| | RAS - FTS | 0.03498 | 0.2189* |
| | MMS - FTS | 0.00480 | 0.5283* |

*p> 0.05 no significant difference

### *3.3.5 Larval microbiota at day 60 post hatching.*

DNA was extracted from the larvae guts, rearing water samples from the three water systems on the last day of the experiment to investigate the microbial community composition. The result (Figure 3.24) showed that there were 36 bands in the gel, of which two bands found in all of the larval samples. Several bands were sequenced and marked by red frames. These bands were only observed in the larvae samples, and the sequencing result showed that they belonged to three following phyla: Firmicutes, Proteobacteria and Cyanobacteria.

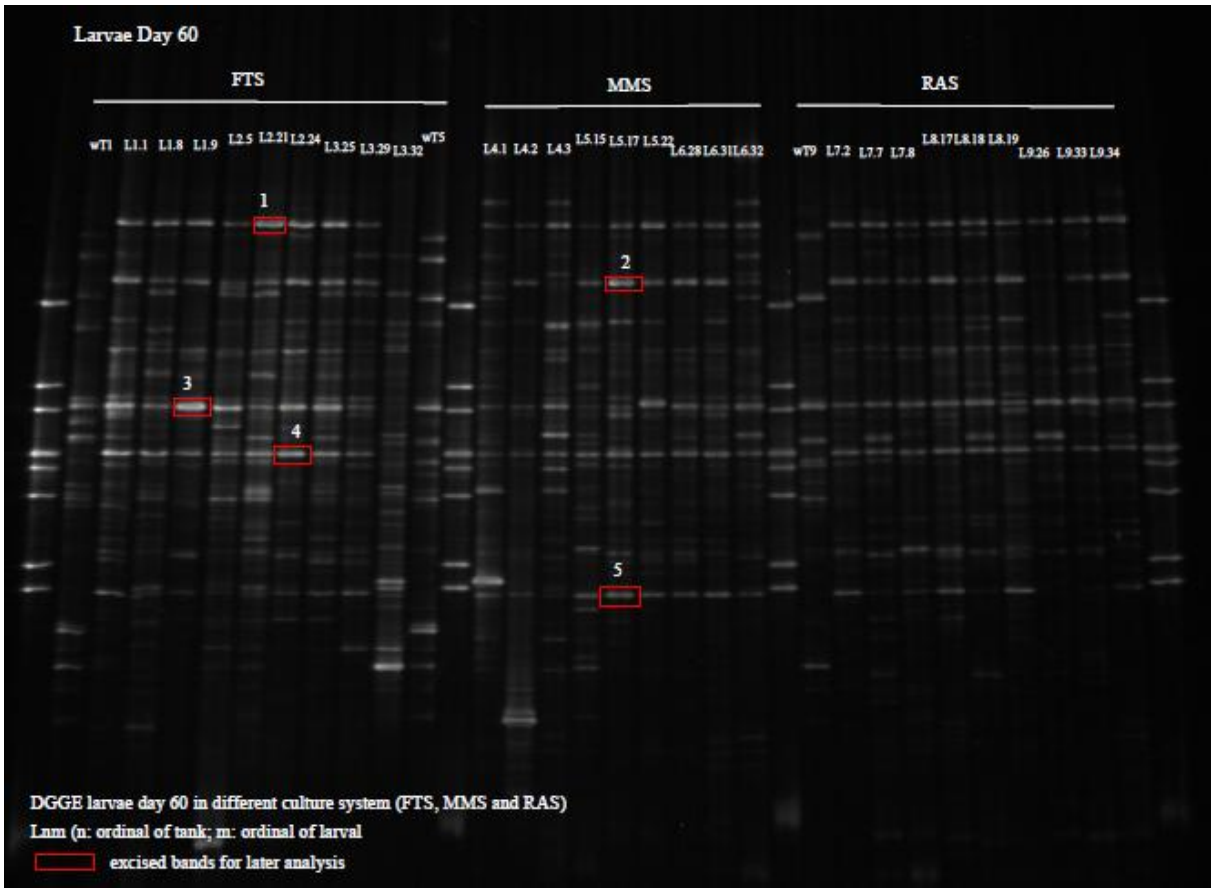



**Figure 3.24**: **DGGE gel with PCR-amplified 16S rRNA fragments from the MCs of larval and water samples of the RAS, MMS and FTS at 60 dph** (Ln.m: L is larval, n is tank number, m is larvae number, W1,5,9: water samples in tanks, red frames: sequencing bands)

The band richness, diversity and evenness indexes were calculate based on the peak area values in the spread sheet resulting from Gel2K analysis of the DGGE image (Fig. 3.24). Further, the average and standard error values for each larval group from the RAS, MMS and FTS were also calculated. These results are given in Table 3.17. Generally, individual larval from the replicated tanks in RAS, MMS and FTS had large fluctuations in band richness, diversity and evenness index. ANOVA and Tukey analyses attested evenness index was not different between three groups (RAS, MMS and FTS). The band richness and the diversity values were significantly different between the FTS and RAS, between FTS and MMS ($p<0.05$).

**Table 3.17**: **Band richness, diversity and evenness index of the gut microbial composition of the larvae and water at 60 dph in RAS, MMS and FTS** (Ln.m: L is larval, n is tank number, m is larvae number, W1, 5, 9: water sample in tanks)

| RAS | | | | MMS | | | | FTS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** | **Samples** | **S** | **H'** | **J'** |
| L9.34 | 9 | 2.01 | 0.92 | L6.32 | 16 | 2.59 | 0.94 | L3.32 | 20 | 2.54 | 0.85 |
| L9.33 | 11 | 2.06 | 0.86 | L6.31 | 14 | 2.32 | 0.88 | L3.29 | 18 | 2.70 | 0.94 |
| L9.26 | 8 | 1.75 | 0.84 | L6.28 | 13 | 2.25 | 0.88 | L3.25 | 21 | 2.66 | 0.87 |
| L8.19 | 17 | 2.59 | 0.92 | L5.22 | 13 | 2.19 | 0.85 | L2.25 | 19 | 2.45 | 0.83 |
| L8.18 | 12 | 2.31 | 0.93 | L5.17 | 19 | 2.44 | 0.83 | L2.24 | 26 | 2.93 | 0.89 |
| L8.17 | 15 | 2.36 | 0.87 | L5.15 | 18 | 2.59 | 0.89 | L2.15 | 21 | 2.55 | 0.84 |
| L7.8 | 15 | 2.46 | 0.91 | L4.3 | 23 | 2.71 | 0.86 | L1.9 | 15 | 2.17 | 0.80 |
| L7.7 | 17 | 2.44 | 0.86 | L4.2 | 10 | 2.03 | 0.88 | L1.8 | 21 | 2.60 | 0.86 |
| L7.2 | 11 | 2.07 | 0.86 | L4.1 | 20 | 2.55 | 0.85 | L1.1 | 19 | 2.57 | 0.87 |
| **Average** | **12.7** | **2.2** | **0.8** | **Average** | **16.2** | **2.4** | **0.8** | **Average** | **20** | **2.5** | **0.8** |
| **SE** | **1.1** | **0.0** | **0.0** | **SE** | **1.3** | **0.0** | **0.0** | **SE** | **0.9** | **0.0** | **0.0** |
| W9 | 10 | 2.02 | 0.88 | W5 | 14 | 2.39 | 0.91 | W1 | 14 | 2.29 | 0.87 |

Figure 3.25 shows an NMS plot based on the Bray-Curtis similarities calculated for the individual larval samples and rearing water samples in three water treatment systems at 60 dph. The plot indicated that the MCs representing individual larval in MMS and FTS seemed to be less similar in comparison to those found in RAS. Because of far distances between sample points, especially the L3.32 sample stood out far from other points in FTS. Conversely, the larval gut MC in RAS had more similarity by the short distance between points (Figure 3.25).

Average Bray-Curtis similarities of larval gut MCs between and within the three different treatment systems were described in Figure 3.26. The larval gut MCs within as well as between groups had high similarities.

ANOSIM confirmed that the larval guts MCs between RAS and MMS was not significant difference (R=0.144, p=0.229). While those found to be significantly different between RAS and FTS (R=0.312, p=0.0004), between MMS and FTS (R=0.144, p=0.018), however was not as clear as that described in day 46 (Table 3.18).

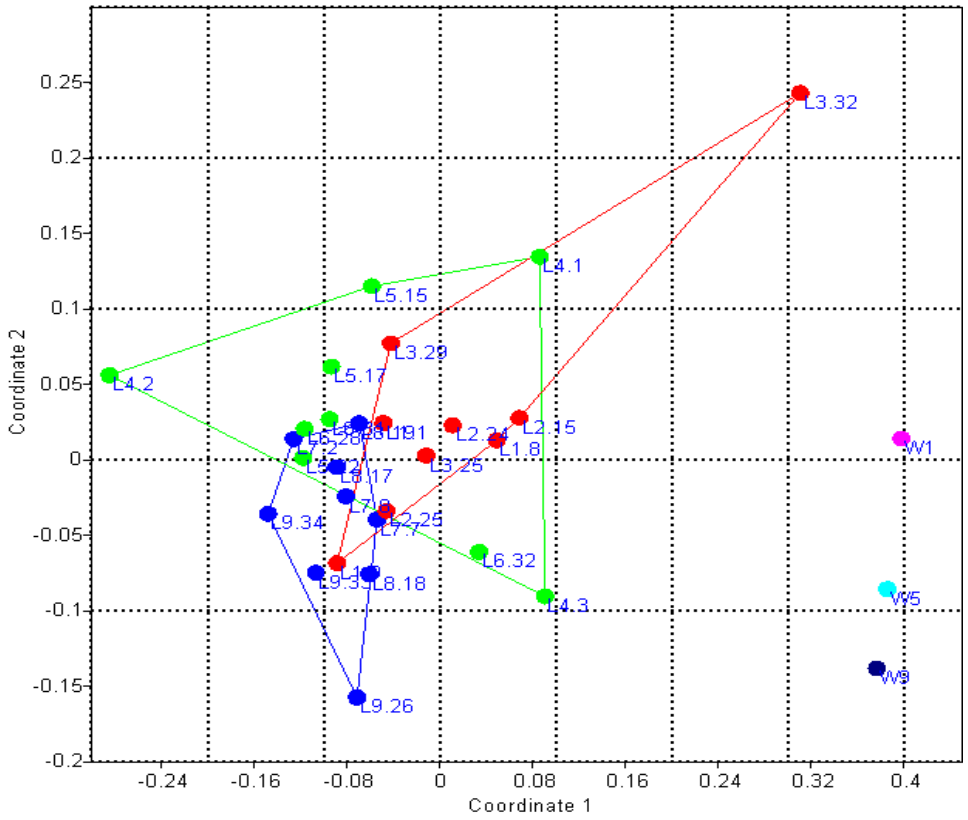



**Figure 3.25**: **Ordination of the larval gut MC from the three water treatment systems at day 60 by NMS using the Bray-Curtis similarities** (the points green: larvae of MMS, the points blue: larvae of RAS, the points red: larvae of FTS, W9, 5, 1 is water in tank 9, tank 5 and tank 1 respectively).

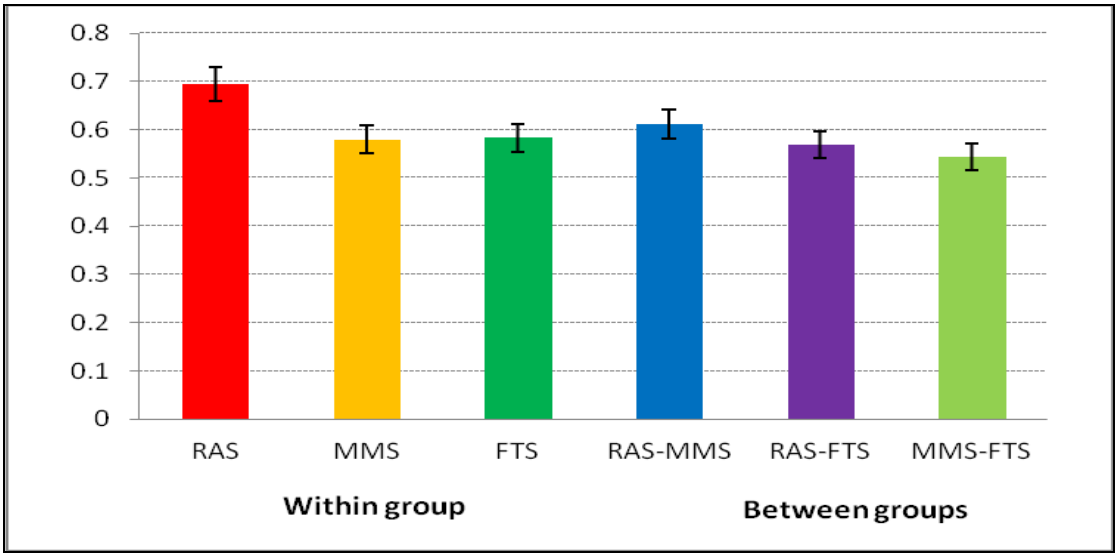



**Figure 3.26: Average Bray-Curtis similarities of the gut MC composition of larvae at day 60 post hatching between and within the three different water treatment systems**

**Table 3.18: ANOSIM analysis for testing the hypothesis of no difference between larval MC from the three water treatment systems at day 60 post hatching.**

| Overall analysis | | R value | p value |
|---|---|---|---|
| | | **0.1974** | **0.0001** |
| **Between groups** | RAS - MMS | 0.1437 | 0.229* |
| | RAS - FTS | 0.3124 | 0.0004 |
| | MMS - FTS | 0.1437 | 0.0176 |

*p> 0.05 no significant difference

### *3.3.6 Similarity of MCs between feed in tank, rearing water and larvae*

To compare the larval MC to the water and feed MCs, the average Bray-Curtis similarities between larval and rearing water MCs and also between larval and feed in tank MCs from the three water treatment systems at day 8 post hatching were determined. The results are shown in Fig. 3.27. Apparently, the similarities of bacteria composition between larvae and feed MCs, and between larvae and water MCs from the FTS, MMS and RAS were similar (around 0.30-0.35). T-test analysis confirmed that at day 8 post hatching, there were no significant differences in the average similarities determined for the larval-water MC comparisons, and those determined for the larval-feed comparisons (p>0.05). This indicates that MC of both the rearing water and of the feed influenced the MC of the larval MC.

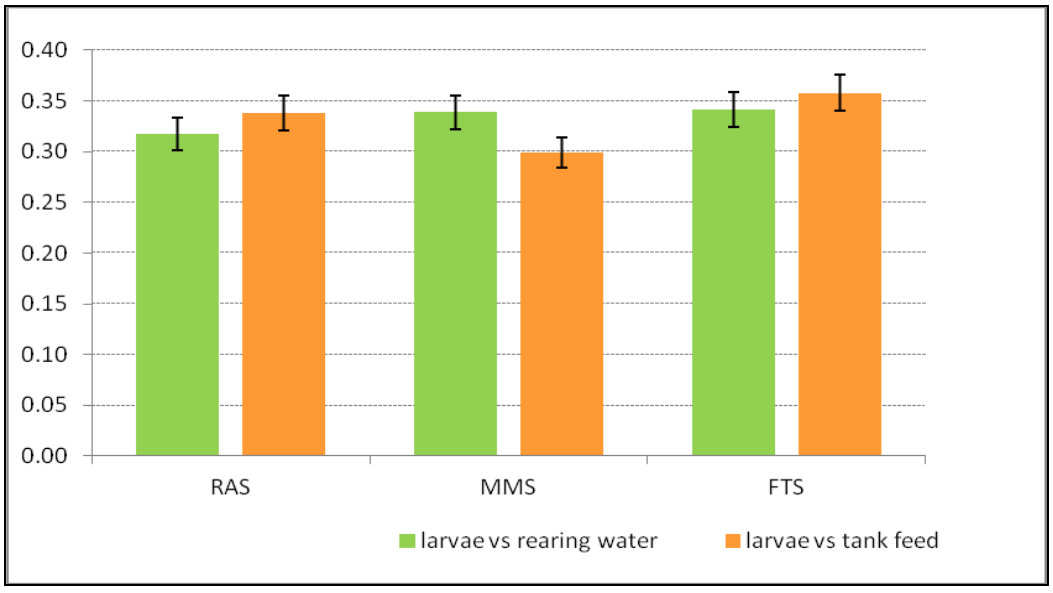



**Figure 3.27: Average Bray-Curtis similarities between larvae and feed MCs, and between larval and rearing water MCs from the three treatment systems at 8dph.**

The average Bray-Curtis between larval and rearing water MCs from the three water treatment systems at day 8, 17, 30, 46 and 60 post hatching were calculated (Fig. 3.28). Generally, the similarities between larval and water MCs in RAS seemed to be stable with values around 0.25-0.32. These similarities values varied more in MMS and FTS, especially in the period from day 8 to day 30. Moreover, the average Bray-Curtis similarities for water-larvae MC in MMS and FTS were higher than in RAS. This indicates that the larval MCs from the MMS and FTS are more influenced by the water MC compared to the RAS.

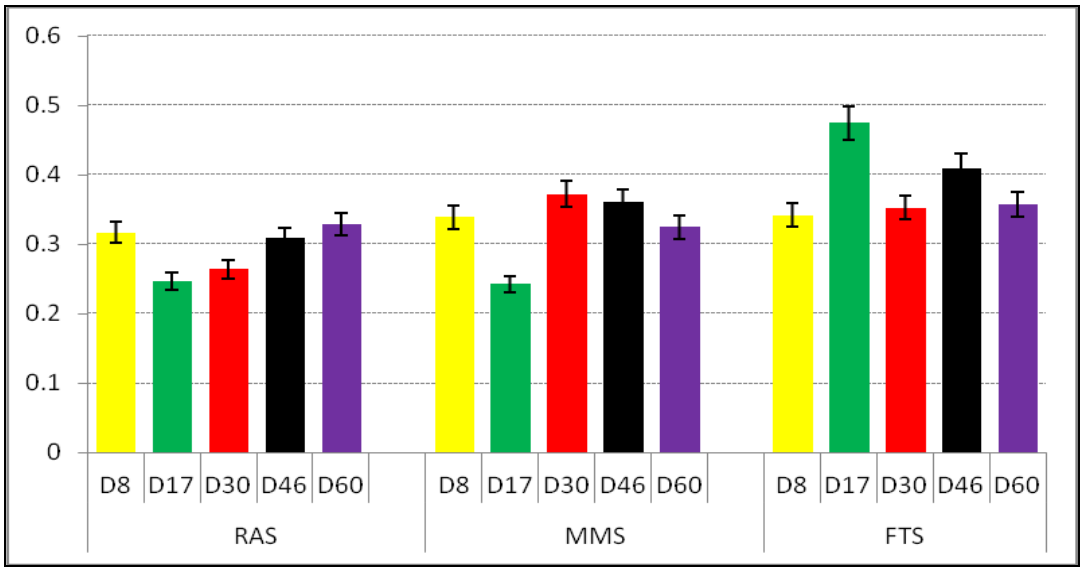



**Figure 3.28: Average Bray-Curtis similarities between rearing water MC and larval MC for three water treatment systems**

### *3.3.7 Taxonomic assignment of DGGE bands*

A total of 56 bands from the DGGE gels were excised to determine the DNA sequences as a basis for taxonomic assignments. These bands included bands from water, feed and larvae samples, with 13, 21 and 22 bands, respectively. For some bands (10, 20, 30% of the larvae, water and feed bands, respectively) taxons couldn't be assigned due to poor quality of sequences.

Proteobacteria, Bacteroidetes, Firmicutes and Cyanobacteria were found in larvae, feed and water samples, in which the Proteobacteria was the most commonly found phylum. Three subclasses including alpha, epsilon and gamma were identified (Figure 3.29), of which, the gamma-proteobacteria were found in all samples; water, feed and larvae with 81%, 58%,

60%, respectively. Epsilon-proteobacteria was 42% in feed, 30% in larvae and 9% in water. Alpha-proteobacteria subclass was not found in feed but in water and larvae approximately at 9% (Figure 3.29)

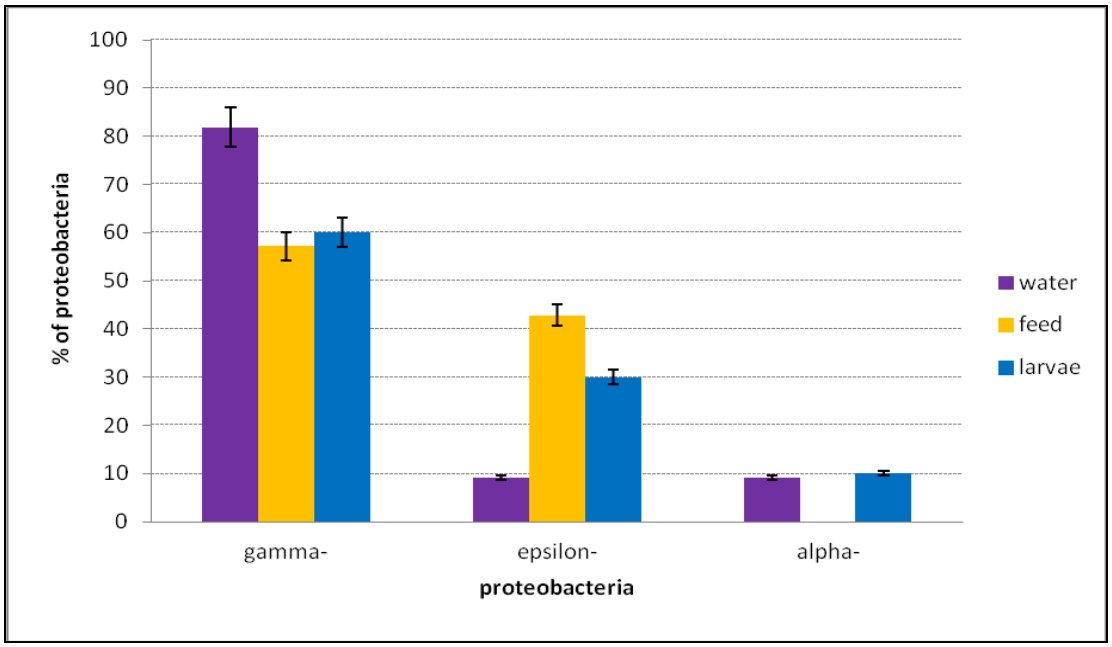



**Figure 3.29**: Ratio of Proteobacteria classes in water, feed and larvae

# 4. Discussion

## 4.1 The different microbial communities from the water treatment systems.

The MCs composition of the incoming water from the three water treatment systems was analyzed using NMS. The resulting plot showed that samples were distributed in three different areas with a clear clustering of samples according to water treatment system (Fig. 3.2). In addition average Bray-Curtis similarities (Fig. 3.3) showed that the similarities of MCs composition between MMS and FTS were higher than the one of between the RAS and the other systems. This indicates that water MCs in RAS were different compared to MMS and FTS. Moreover, ANOSIM analysis confirmed that the MCs composition of the incoming water were significantly different between the three water treatment systems ($p<0.0001$). The significantly difference of water MCs between RAS and FTS were published by Verner-Jeffrey et al. (2004). Another report by Attramadal et al. (2012a) also showed that the RAS developed a different and more stable composition of the MC than the FTS. Until now, no one has investigated and compared the MC of MMS with the MC of RAS and FTS. However, some reports indicate the MMS was applied in aquaculture and fish production in MMS has increased significantly compared to FTS.

The band richness of bacteria of RAS reached the maximum at day 8 and thereafter remained high, whereas the MMS and FTS needed extra 22 days to reach the maximum band richness level (Table 3.1). The MC similarity of samples taken from the RAS incoming water at different time points was higher than those within FTS (Fig 3.3). Moreover, the points corresponding to the bacteria communities of RAS and MMS were distributed in two different area and the points were relatively close together, while FTS points were more scattered (Fig. 3.2). This indicates that the MC of the incoming water was more stable for RAS than for the other systems, especially compared to FTS. There are some reasons to explain stability of the MCs in RAS compared to FTS. For example, water in RAS was reused a long time, which restricted opportunity for invading random bacteria. Moreover, RAS operation can provide opportunities to improve waste water management, and supply more organic matter for the rearing tanks (Martins *et al.*, 2010 and Piedrahita, 2003). These factors may have resulted in the high stability of the microbial community in RAS.

A stable water MCs and high band richness in RAS, can increase the chances of dominance by harmless bacteria (K-strategists), and reduce the growth of harmful bacteria (r-strategists)

in the rearing water. It is indicated that the process of stabilizing the microbial community of the wearing water results in enhance larval growth and survival (Skjermo *et al*., 1997; Vadstein *et al*., 1993).

As the RAS system was most stable and with high richness, this system could improve quality of marine larvae in the first post hatching stage.

### 4.2 The main factor influenced the microbial community of the rearing water

The rearing water MC of RAS was mainly determined by the MC of in-flowing water (Table 3.8, Fig 3.11). For MMS and FTS, the rearing water MCs seemed to be similarly influenced by in-flowing water and feed MCs. These results can be explained based on the disinfection and feeding method.

The disinfection method may have affected the recolonization and development of the microbial community in the rearing water (Hess-Erga *et al*., 2010). For RAS, the microbiota community were not killed by the sand filter, therefore, the abundance of bacteria were introduced into the tanks was high. In the case of high bacteria density and limited nutrient resources, the r-strategist gradually could not compete with the K-strategists (Salvesen, 1999). This reason permitted K-selected species to dominate in RAS (Konneke et al., 2005). For FTS, the UV irradiation was used in the intake water of disinfection process; it means there were very few of the water MC to be introduced into the rearing water. Hence, the high density of fish and live feed caused increasing of organic substances loaded in rearing water, and this reduced the competition among bacteria and promoted the growth of r-selected species (Hess-Erga et al., 2010). For MMS, inlet water passed through a filter UV irradiation, and then passed bio-filter to obtain controlled recolonization of the water under K-selected before it was presented to tanks. Therefore, the K-selected species appeared in MMS tanks but these bacteria density was lower than in RAS tanks.

Besides, the feeding method also affected the rearing water MC. Live feed was added to the tanks 6 times per day. In RAS with friendly water MC, the larval rapidly catch the live feed (Salvesen *et al*., 1999). Therefore, the live feed existed in a short time in the rearing tanks. Thus, the feed MCs had less opportunity for invasion into water. The live feed existence in MMS water was longer than in RAS water because of less K- strategists. For FTS, the performance of larval in the rearing water was reduced because of high abundance of r-

selected species (Salvesen *et al.*, 1999, Skjermo and Vadstein, 1999, Vadstein *et al.*, 1993). This allowed the live feed to stay in the FTS tanks water a longer time than in the RAS and MMS water, so the FTS feed MC had more opportunities to infiltrate into the tank water.

The average Bray-Curtis similarities showed that the MC of rearing water in different replicate tanks was more similar for the RAS than the two other systems (was higher than FTS by 11.6% and MMS by 1.5%, Figure 3.10). The similarity of MC between replicate tanks in FTS was low probably because of strong disinfection and stochastic recolonization (Hess-Erga *et al.*, 2010). That strong disinfection could decrease and destabilize the microbial population in the tank water (Attramadal *et al.*, 2012b). Moreover, the band richness of bacteria of RAS was high stability (Table 3.6), followed by the band richness of MMS bacteria (Table 3.4). The band richness of FTS bacteria was less stability (Table 3.2). Clearly, the condition of water environment in RAS and MMS was more stable compared to FTS.

### 4.3 The factors mainly influenced the microbial community of the larvae.

The larval MC was significantly different between systems during the first 30 days of the experiment (Table 3.10, 3.14 and 3.14). The water system was the only factor that differed among the tanks (feed was identical to all tanks), and therefore the differences in larval MC must be due to the different water qualities. The gastrointestinal microbiota would be established by actively taking up bacteria from the water soon after hatching (Hansen and Olafsen, 1999). The marine larvae need to drink in order to osmoregulate before they start eating. Therefore, this was a good opportunity for the water bacteria infiltrating into intestine of larvae. Hansen et al. (1999) also reported that the primary intestinal microflora was established at the yolk sac stage, and it seemed to be independent of first feeding. Additionally, Reitan et al (1998) showed that fish larvae had an active uptake of bacteria at a rate 100 times higher than the drinking rate. The significant differences of the microbial communities of larvae in RAS compared to FTS were also reported by Fjellheim et al. (2007).

Another experimental factor emphasized the importance of water MC for MC of larvae. From day 31 to the end of experiment, the same water source (MMS) was introduced to all nine tanks. As a consequence, there was no longer any difference between the larval MC of the three systems. At day 46, the larval gut MC of the three treatment regimes were not different ($p>0.05$) any more (Table 3.16). The microbial communities of larvae gut among the three treatment systems as well as within each treatment system at day 46, were more similar than

at day 8 and 17 (Fig. 3.23). This was also supported by NMS analysis, which showed that on that day most points represented larval MCs samples of three systems completely overlapped (Fig. 3.22). Hence, these results indicated that the water MC influenced the larva MC also at day 46. However, we have no data to indicate how fast the MC of larvae changed and the first day the larval MC altered. At day 60, significant differences were found between the FTS larval microbiota and the larval MC of the other systems (Table 3.18). However, in Fig 3.28 it is shown that there was a high similarity between larval and water MC from RAS, MMS and FTS at day 60. The similarities of MC between larval and water in FTS was only different from RAS by 3% and MMS by 6%. Therefore, the significant difference at day 60 can be due only one larval (L3.32; Fig. 3.25). Several reports have shown that the bacteria in the water modulated the microbiota of marine larvae at the early life fish stage (Skjermo *et al*. 1997; Ringø & Vadstein 1998; Fjellheim *et al*., 2011).

In addition, the similarity of the MC between larvae and rearing water as well as between larvae and feed in three water treatment systems were similar (Figure 3.27). This indicated that the larval MC in RAS, MMS and FTS were influenced by the MC of both the water and feed in the tank. However, the water MC was a major determinant for the larval microbiota based on the above discussion. This means that the differences in larval MC that were introduced by the different water systems were not stable. We can make an influence on the larval MC through the rearing water, but we could not control the stability and permanentness of the larval MC.

Regarding to the taxonomic assignment of DGGE bands, the Proteobacteria, Bacteroidetes, Cyanobacteria and Firmicutes were identified from the larval DGGE profiles. The Proteobacteria was predominating in the larval MC at different ages (day 8, 17, 30, 46 and 60). This result is similar to findings in Skjermo et al.'s report (2011), where Cyanobacteria, Firmicutes, Bacteroidetes and Proteobacteria were found in cod larvae at the first days after hatching (Skjermo et al. 2011). These phyla were found in the larvae, but also in the water and feed as well. The predominance of Proteobacteria in water, feed and larvae were also reported by Lauzon et al. (2010) and Thomas et al. (2011). Furthermore, Proteobacteria were found in the waste water of RAS (Jaap and Rijn), and in the marine flow-through cultures (Sandaa et al., 2003, Rocker *et al*., 2012). Edwards et al. (2010) showed that Bacteroidetes and Proteobacteria were commonly found in bio-films. Thus, it is very likely that Bacteroidetes and Proteobacteria appeared in MMS and RAS.

# 5. Conclusion

1. The differences in microbial communities of the water from the three water treatment systems (RAS, MMS and FTS) were significant. Concurrently, the in-flowing water MCs in RAS also was more diverse and stable over time compared to the FTS. Therefore, it is possible to control MC with water treatment systems, and systems with K-selection give more stable and reproducible MCs.

2. The microbial community of the rearing water in RAS was mainly determined by the MC of the incoming water. MMS and FTS appeared to be similarly influenced by the MC of the incoming water and the feed. Moreover, the rearing water MCs were more similar between replicate tanks and more stable over time for RAS. These findings indicate that RAS would be the best water treatment system for obtaining stable and controllable MCs for rearing of fish larvae.

**3.** The larval MC was significantly different from three different water treatment systems. However, when the rearing tanks of RAS, MMS and FTS received the same water, the larval MC became similar. These results show that the water is a major determinant for the larval MC. Therefore water treatment systems can be used for controlling larval MC.

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# Appendices

## *Appendix 1: The protocol for DNA extraction from water, feed samples*

1. Plug the bottom of the Dynaguard with a PCR tube and put this in a new 1.5ml eppendoft tube.
2. Add 90µl enzymatic lysis buffer to the plugged Dynaguard filter
3. Incubate at $37^0$C for 30 min
4. Add 20µl proteinase K and 90µl ATL buffer, mix with the pipette, incubate at $55^0$C for 45 min
5. Transfer the plugged Dynaguard filter upside down to a new eppendoft tube
6. Centrifuge at 8000rpm for 1 min to transfer the liquid to the eppendoft tube
7. Keep the tube on ice
8. Transfer the plugged Dynaguard filter to a new eppendoft, and repeat step 1-6
9. Pool the extracts from the two rounds of lysis, and add 200µl AL buffer
10. Vortex and incubate at $75^0$C for 10 min
11. Add 300µl 96% ethanol and vortex
12. Transfer the solution to the Dneasy column and centrifuge at 8000rpm in 1 min. Discharge the filtrate
13. Add 500µl AW 1 buffer and centrifuge at 8000 rpm for 1 min. Discharge the filtrate
14. Add 500µl AW 2 buffer and centrifuge at full speed (13-15000rpm) for 3 min
15. Transfer the column to an eppendoft tube, add 50µl AE buffer directly on the membrane, incubate for 1 min at room temperature and centrifuge at 8000rpm for 1 min
16. Elute once more with 50µl AE buffer to obtain a total of 100µl extract
17. Quantify amount of DNA with Nanodrop.

## *Appendix 2: Recipe for dye solution for DGGE*

| **Name of reagents** | **Volume** |
|---|---|
| SYBR Gold | 3 µl |
| TAE (50x) | 600 µl |
| Pure $H_2O_2$ | 30 ml |

## *Appendix 3: Protocol for re-amplification and purification PCR product*

### *Re-amplification of DGGE bands*

| | |
|---|---|
| 10 x reaction buffer: | 2.5µl |
| dNTP (10mM each): | 0.5µl |
| $MgCl_2$ (25mM): | 0.5µl |
| BAS: | 0.75µl |
| Primer fwd (10µM): | 0.75µl |
| Primer rev (10µM): | 0.75µl |
| Taq pol.: | 0.125µl |
| $H_20$: | 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:

| | | |
|---|---|---|
| $95^0$C | 3 min | |
| $95^0$C | 30 sec | 40 cycles |
| $53^0$C | 30 sec | |
| $72^0$C | 1min | |
| $72^0$C | 10min | |
| $10^0$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

### *Purification PCR product*

1. Add 5µl buffer PN to 50µl PCR product and mix
2. Place a QIA quick spin column in a provided 2ml collection tube

3. Apply the sample to the QIA quick column and centrifuge for 1min at 13.000 rpm
4. Discard the flow-through and put QIA quick column back into the same tube
5. To wash QIA quick column, add 750µl of buffer PE and centrifuge for 1 min at 13.000 rpm
6. Discard the flow-through and place the QIA quick column back in the same tube and centrifuge foe additional 1 min at 13.000rpm
7. Place QIA quick column in a clean 1,5ml micro-centrifuge tube
8. To elute DNA add 50µl sterile water to the center of the QIA quick membrane and centrifuge the column for 1 min at 13.000 rpm