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Effects of different water treatments on microbial communities when startfeeding European lobster (*Homarus gammarus)*

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Marine Coastal Development
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Abstract

The challenge with poorly performing larvae and high mortality in replicate tanks is a bottleneck in aquaculture. The main objective of this thesis has been to reveal if differences in performance and survival of the reared larvae is caused by instability in the microbial communities in the rearing water, and furthermore to investigate if disinfection devices also contribute to this instability and work against its purpose, which is to increase the level of performance and survival. The European lobster (*Homarus gammarus*) was used in the experiment because this species is subject to a lot of research today.

Two recycling aquaculture systems (RAS), of which one included an ultraviolet filter (UV filter) and one flow-through system (FTS) with no UV filter were compared. The samples were analysed using a PCR (Polymerase chain reaction) and DGGE (Denaturing gradient gel electrophoresis) strategy and tested statistically for variations between the systems. Samples were taken from intake water, rearing water and from whole larvae. There were indications that the water treatment influenced the microbial communities in both water and larvae of the systems. RAS with no UV filter presented the highest rate of survival and showed the most similar microbiota between intake water and rearing water. The RAS with UV filter and FTS both showed significant differences between intake water and rearing water, creating a room for regrowth for bacteria. This study support the hypothesis that r-strategic bacteria will be favoured in unstable systems and create opportunity for pathogens to dominate the waters, resulting in less optimal conditions for the reared organism. It also supports the theory that using disinfection on the water before entering the rearing systems will lower the total bacteria concentration and may create instability and room for re-growth.

The experiment was executed at the Norwegian Unicersity of Science and Technology (NTNU) with participation from, Sintef fisheries and aquaculture and Norsk hummer AS. Live-feed experiment was performed at NTNU Brattøra and the laboratory work was done at NTNU Gløshaugen. The live-feed was produced by Sintef Fisheries and Aquaculture, and the lobster larvae were produced at Norsk Hummer AS, Tjeldbergodden.

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1. Introduction

1.1 Background

Norway has grown into a worldwide provider of farmed seafood. The species with most success in Norwegian aquaculture is the Atlantic salmon (*Salmo salar*) and in 2013 the total volume of exported salmon was 936 969 metric tons (Statistisk sentralbyrå, 2013). Company's rearing other relevant species, such as halibut, cod, turbot and shellfish are still struggling with one or more factors like; disease, deformities and/or high mortality in replicate tanks (Olsen et al., 1999, Verner-Jeffreys et al., 2003, Jensen et al 2004, Magnesen et al., 2006)

European lobster (*Homarus gammarus*) has the potential to become a success in modern aquaculture. In the present market, lobster is one of the highest priced products. There has been cultured lobster in Europe and USA since the mid-1800´s (Nicosia et al, 1999). Land based saltwater systems, however, is a modern approach to increase control over the rearing. A low natural stock and a high demand due to the tasty meat create a hungry marked for reared lobster. The American lobster (*Homarus americanus*) is not as exclusive for the consumers and does not obtain the same high price (Barrento et al, 2009)

All arthropods grow by replacing the exoskeleton. This is very energy demanding process, and is the period very many individuals die. A new exoskeleton develops under the old, and after the moulting, while the shell is soft, the animal increases the volume by pumping fluid under the exoskeleton. The exoskeleton becomes hard, and there is new space to grow.

In the period after the moulting, before the shell becomes hard, the larvae are vulnerable to predation. Other larvae grab hold and start to devour these individuals. The cannibalistic behaviour causes a high juvenile mortality when rearing the larvae in one open tank (Wahle and Steneck, 1991, 1992, van der Meeren, 2000).

One of the key challenges in present aquaculture is to create stabile and satisfying quality of the rearing water and to develop feed which satisfy the nutritional demand for the marine larvae. At the moment, the high mortality and poorly performing larvae is a bottleneck for many species. Studies on the subject have shown that stability in the water microbiota influences survival of the reared species (Vadstein et al., 1993, Attramadal et al., 2011). The theory is that the microbial community plays a vital role in the development of a functioning gastrointestinal tract (GI), and furthermore gives the larvae the needed stimuli for immunological reactions. This is crucial for good growth and survival.

1.2 Biology

The European lobster (*Homarus gammarus*) (Linnaeus, 1758) is an animal in the order *decapod* (ten legs): *nephropidae* family and genus: Homarus (Holthuis, 1991). The European lobster's natural habitat ranges from Lofoten in Norway to Morocco, and also some areas of the Mediterranean Sea (van der Meeren, 1995, Knutsen et al., 2009). Lobsters are omnivorous scavengers, feeding on both live and dead organisms. The natural habitat for lobster is rock or mud bottom, where it can find shelter in caves or between rocks. They can modify the substrate or find natural holes to hide (Howard and Bennet, 1979). Lobsters usually have spawning every second year. Mating occurs shortly after the female moult, while her shell is still soft. The male passes sperm into the female's oviducts. The female carries the eggs externally, on the pleopods, for approximately one year before the eggs hatch. After hatching the larvae does not look much like the parents. The larvae changes for each moulting, until it has metamorphosed into the shape of an adult lobster. The time between moulting is called *stages.* The larvae go through one pre-stage followed by 4 stages (*I, II, III and IV)*.
Newly hatched larvae have a pelagic period until *stage IV*. Their natural diet consists of copepods, larva of mollusc worms, and other pelagic organisms of right size. At *stage IV,* the larvae have metamorphosed into the shape of an adult lobster, and seek towards the bottom for a benthic life (Factor, 1995).

1.3 Recommended rearing conditions

Optimal rearing conditions for European lobster are; temperature 18-22 ^{o}C, where temperatures above or below this will influence growth negatively. The salinity should be 28-35‰ and O_2 >6.4mg/L saturation. The water should have pH 8, where <5 or >9 will be lethal (van Olst et al., 1980, Wickins and Lee, 2002). Nitrifying bacteria in biofilters are pH sensitive and the will not survive pH <6 (Lucchetti and Gray, 1988)
Ammonia must be < 6mg/L, which is the toxic level. It has not yet been possible to provide recommended levels for nitrite and nitrate (Beard and MacGregor, 2004, Jacklin, 2007), but limits of <5mg/L for Nitrite and <100mg/L for Nitrate has been suggested (Estrella, 2002).

1.4 Disease associated with rearing of lobster

Suboptimal rearing conditions are believed to be the cause of a number of diseases known to infect lobster (Battiston et al., 2004, Estrella, 2002). Typical infections are shell disease, fungal diseases, ciliate infections (Beard and MacGregor, 2004, Jacklin, 2007), bacterial pathogens (Battison et al., 2008) such as *Aerococcus viridans* (Battison et al., 2004, Estrella, 2002) and *Anophryoides haemophila* (Greenwood et al., 2005).

1.5 Feed for cultured lobster larvae

When cultivating lobster larvae, the most common feed is frozen copepods of various species. The copepods are caught with nets in the ocean and concentrated in boxes, which is then frozen and shipped to the rearing facilities. The problem with this feed is the degeneration of nutrients and leakage out through the exoskeleton into the rearing water when the copepods are thawed. Using live feed keeps the nutrients intact until the larvae catch the prey. The movement of the prey also generates more feeding activity. The challenge is to develop simple on-site systems with a short transport route between feed production and rearing tanks, for producing live feed in satisfying numbers and quality at an acceptable price.

Many species of copepods have been subject to research as feed in aquaculture. They are found in all marine waters, and they are a part of the natural diet for most pelagic larvae. The northern cold-water species of copepods contain high amounts of the polyunsaturated fatty acids, EPA (Eicosapentaenoic acid), DHA (Docosahexaenoic acid) and Ara (Arachidonic acid). These fatty acids are essential to develop high performing cold water larvae and increase survival. In aquaculture, *Acartia tonsa* is one many species used for research in aquaculture due to its high reproduction and favourable nutritional composition (Støttrup, J., 1997).

1.6 Water treatment for marine cold-water juveniles

In land based aquaculture there are two main methods in water treatment for rearing aquatic organisms, flow-through system (FTS) and recirculating aquaculture system (RAS).
FTS rely on a good water source outside the rearing facilities. In marine aquaculture this source is usually the ocean. The water is treated to meet the requirements of the cultured species. The treatment usually consists of particle removal, temperature regulation, aeration/degassing and disinfection.
The RAS can be designed to meet the requirements of the reared animal and create more stabile conditions. There is no need for a source of water close to the rearing facilities. The water flows in a loop, and there is less need for energy to heat the water, or powerful pumps to transport water as there is with a FTS. The same water is re-used over time, and after the water passes the rearing tank it gets treated in various ways to meet the requirements of the reared organism.
A common RAS consist of a rearing tank, a retaining tank and biofilters breaking down ammonia, nitrite and organic matter. One of the challenges in RAS is to control Nitrogen

waste products. Components like ammonium is a bi-product from metabolism and will be harmful if the levels get too high. The ammonium is reduced to nitrite and then to nitrate, which is a less harmful compound to the animals in the rearing tank. To keep the nitrogen levels low is one of the challenges in a RAS. Because of more complex treatment, the RAS has a higher maintenance cost, and requires a higher level of competence in the operator.

1.7 Biofilm

Biofilms occur on any surfaces in connection with water. Bacteria in planktonic form will change their gene expression and settle as sessile forms, many as ultramicrobacteria (Costerton et al., 1999, de Beer et al., 1994). Some invertebrates (Armstrong et al., 2000, Gil-Turnes and Fenical, 1992) and biofilms (Bewery et al., 1996) are able to regulate the colonization of surfaces. Studies done by Gil-Turnes and Fenical in 1992, found that 2.3-idolenedione compound produced by bacteria prevent the growth of fungal infections of *Homarus gammarus* embryos. Experiments on biofilm development in rearing tanks with tropical rock lobster, *Panulirus ornatus* show that up to day 20 the biofilm was dominated by α- and γ- Proteobacteria. After day 20 to 24 the biofilm was dominated with the gram-negative bacteria Vibrio. A Vibrio harveyi strain isolated from the biofilm late in the experiment, which were introduced to the larvae in a small-scale phyllosoma survival study, demonstrated increased mortality (Bourne et al., 2006). This means that bacteria will colonize a surface and possibly counteract any water treatment.

1.8 Microbial environment

One of the main challenges in modern aquaculture is the production of larvae. Great differences have been observed between replicate tanks on development and survival. Common problems are poor growth, deformities, and low survival. A healthy larva is crucial for the performance in later stages. Factors like egg quality, nutrition, chemical composition in the rearing water, temperature, light, genetic characteristics and the microbial composition are all connected to the performance of marine larvae. Even replicated systems with sibling groups typically display large variations in performance. One hypothesis is that detrimental microbial communities in the rearing water are the cause of this instability (Vadstein, O., 1997, Vadstein et al., 2004). This theory has been supported in other studies using antibiotics (Munro et al., 1994, Skjermo et al., 1997, Verner-Jeffreys et al., 2004).

Larvae in first feeding systems are highly vulnerable due to their lack of a specific immune system. They have to rely on the innate immune system, which makes them vulnerable to changes in the system. Before the larvae have established a permanent microbial community

on the skin and in the gastrointestinal tract, a stable microbial community in the rearing water is important. In first feeding of marine larvae, the establishment of a specific immune system is more important than the risk of pathogens in the water (Vadstein et al., 1993, Vadstein, O., 1997). The exposure to bacteria in the first feeding may be contributing to the development of immune tolerance (Hansen and Olafsen, 1999).

Microbial communities can be divided into two groups, K-strategic and r-strategic. K-strategic bacteria are slow growing, and are found in stabile microbial communities close to the carrying capacity (CC) limit where resources is limited. The CC-limit is the maximum amount of bacteria the system can support over time with the available resources. The r-strategic bacteria are fast growing with a high demand for resources, dominating in unstable systems with a lot of resources for the bacteria. Microbial maturation of water is defined as selective promotion of K-strategic bacteria, which increases the stability of the microbial community and depresses the r-strategic bacteria (Skjermo et al., 1997, Salvesen et al., 1999, Skjermo and Vadstein, 1999). Experiments indicate that in microbial maturation of water, the r-strategic bacteria cannot compete with the K-strategic bacteria, because they utilize the resources better (Salvesen et al., 2000). A system with instability will favour r-strategic bacteria. Recirculating system with an ozone or UV-filters before the inlet water will lower the total amount of bacteria going into the rearing tank. It is expected that the microbial community will re-establish and grow until it reaches the CC-limit. This will create room for r-strategic bacteria with the risk of pathogens, which will influence growth and survival. It has been shown that larvae perform better in a system with K strategic bacteria, compared to a system dominated with r-strategic bacteria (Attramadal et al., 2012).

Rearing water contain both autotrophic and heterotrophic bacteria. Heterotrophic bacteria feed on the organic matter in the system and consume oxygen in this process.

Dissolved organic matter (DOM) comes from the live feed and defecation from the larvae. The bacterial density in the rearing water can reach high numbers ($>10^8$ cells mL^{8-1}**)** **(**Vadstein et al., 1993). This makes the rearing water an important source of bacteria, as the larvae drink, feed and live in the water. Studies have shown that the microbial composition in the rearing water seems to be more important than the total number of bacteria in the system (Salvesen et al., 1999, 2000, Verner –Jeffereys et al., 2004).

1.9 Development of microbiota associated with larvae

Larvae demand microbial interactions for the development of a functioning digestion system and for the establishment of a good immune defence. Research on the subject with cod (*Gadus morhua*), indicate that the microbial organisms associated with the feed are less important than the microbiota in the rearing water, which has a greater impact on the microbiota found in the gastrointestinal (GI) tract of larvae and furthermore gives higher growth and survival (Bakke et al., 2013). Biocontrol agents is a term used for microbes that are antagonistic to pathogens, but which are not to be found present in the GI tract, either transiently or residentially (Maeda et al., 1997). The term "probiotics" in aquaculture are defined as microbes that are associated with health promoting properties. Prebiotics are non-digestible fiber compounds that pass undigested through the upper part of the gastrointestinal tract, stimulating the growth and/or activity of advantageous bacteria that colonize the large bowel by acting as substrate for microbiota (Spanggaard et al., 2001, Irianto and Austin, 2002).

1.10 Disinfection

Biosecurity procedures can be implemented to reduce the risk of introducing pathogenic organisms into the rearing system. In landbased aquaculture the most used barrier to prevent pathogens entering the system with the intake water is ultraviolet light (UV). The UV light has a wavelength of 100-400 nm. UV light is divided into tree groups; UV-A (315-400nm), UV-B (280-315nm) and UV-C (100-280nm). UV light in the range 254nm creates photo-induced dimerization to DNA and RNA. The level of damage depends on time and strength of the light, which the bacteria are exposed to (Liltved and Landfald, 1996). The use of UV filter in RAS is commonly used to disinfect the water before entering the rearing system, or to stop pathogens from entering when introducing new water to the rearing system. Experiments on the use of UV filter in RAS show that the effect of bacteria removal is reduced with increasing load of particles, both biotic and abiotic (Hess-Erga et al., 2008).

1.11 Particles

Microbial communities may be reduced in a recirculating system by removal of solids. An effective control of particle removal will minimize the level of soluble organic compounds and ammonia released by decomposing waste feed and fecal matter. Waste feed and fecal matter newly released into the water can easily be removed from the system with various filters. Smaller particles that are not removed can accumulate, and they represent the majority of the organic solids within RAS (Chen et al., 1993, Patterson et al., 1999, McMillan et al.,

2003). Periodic flushing of the system will reduce the load of organic matter and contribute to lower the reservoir of opportunistic pathogens (Summerfeldt et al., 2001).
A protein skimmer is often placed in a RAS to remove particles. It creates bubbles in a chamber, which the rearing water flows through. The particles in the water stick to the membrane of the bubbles and are carried up, creating foam on the surface, which is then removed from the system.

1.12 Gasses

In RAS, it is important to have a gas composition close to the levels found in the natural habitat of the reared animal. Pumps and turbulence in the pipes may alter the gas composition in the water. To compensate any oversaturation, a degasser is often used. This device creates a vacuum, which increases the gas exchange over the water membrane, preventing oversaturation (Barrut et al., 2012).

1.13 pH in rearing water

In a saltwater aquatic system, the pH will usually be stabile due to the CO_2-$CaCO_3$ system (Rebello and Moriera, 1982). When the water is re-used this may change the physiochemical composition and cause the pH to increase or decrease. The cultivated species have optimal levels of pH, and too low or too high pH will influence performance negatively and increase mortality.

2. Aims

In this study, the aim was to investigate how different water systems influence the rearing of *H. gammarus*.

More specifically the objective for this study was:

- Investigate if water the treatment has an effect on survival and growth when startfeeding European lobster (*Homarus gammarus*).

- Investigate if different water treatment has an effect on the microbial community in rearing water.

- Investigate if the microbial community in the rearing water influences the composition of microbiota associated with larvae.

3. Material and methods

3.1 Ethical statement

All the animals used in this experiment were treated with respect and care as demanded by the Norwegian animal welfare law -2009-06-19-97. Rearing and sampling was executed with focus on reducing the discomfort and stress to a minimum. The number of larvae samples were kept to a minimum needed for scientific statistical analysis.

3.2 Experimental design

The experiment was carried out at NTNU Sealab`s facilities at Brattøra. Contributing institutes was Sintef fisheries, providing live feed (*Acartia tonsa*) reared on the marine algae Rhodomonas, and the lobster hatchery Norsk hummer AS providing Stage I lobster larvae (*Homarus gammarus*) from wild lobster carrying eggs, caught in Norway. A total of 360 *stage I* larvae were introduced to 3 water systems with different water treatments. Temperature, pH, salinity and O_2 levels and feed were kept identical.
Larvae were hatched over night at Norsk hummer, Tjeldbergodden and shipped to NTNU Brattøra the following day. The larvae were distributed to the respective systems. Each system had 120 larvae placed in two parallel tanks, and the larvae were kept in separate chambers with a size of 75x 37 mm to eliminate any cannibalistic behaviour. The three different water treatment systems were:

System 1: Recirculating water with UV filter (RASUV)
System 2: Recirculating water without UV filter (RAS)
System 3: Flow through system without UV filter (FTS)

A schematic presentation of the three systems are illustrated in figure 3.1.

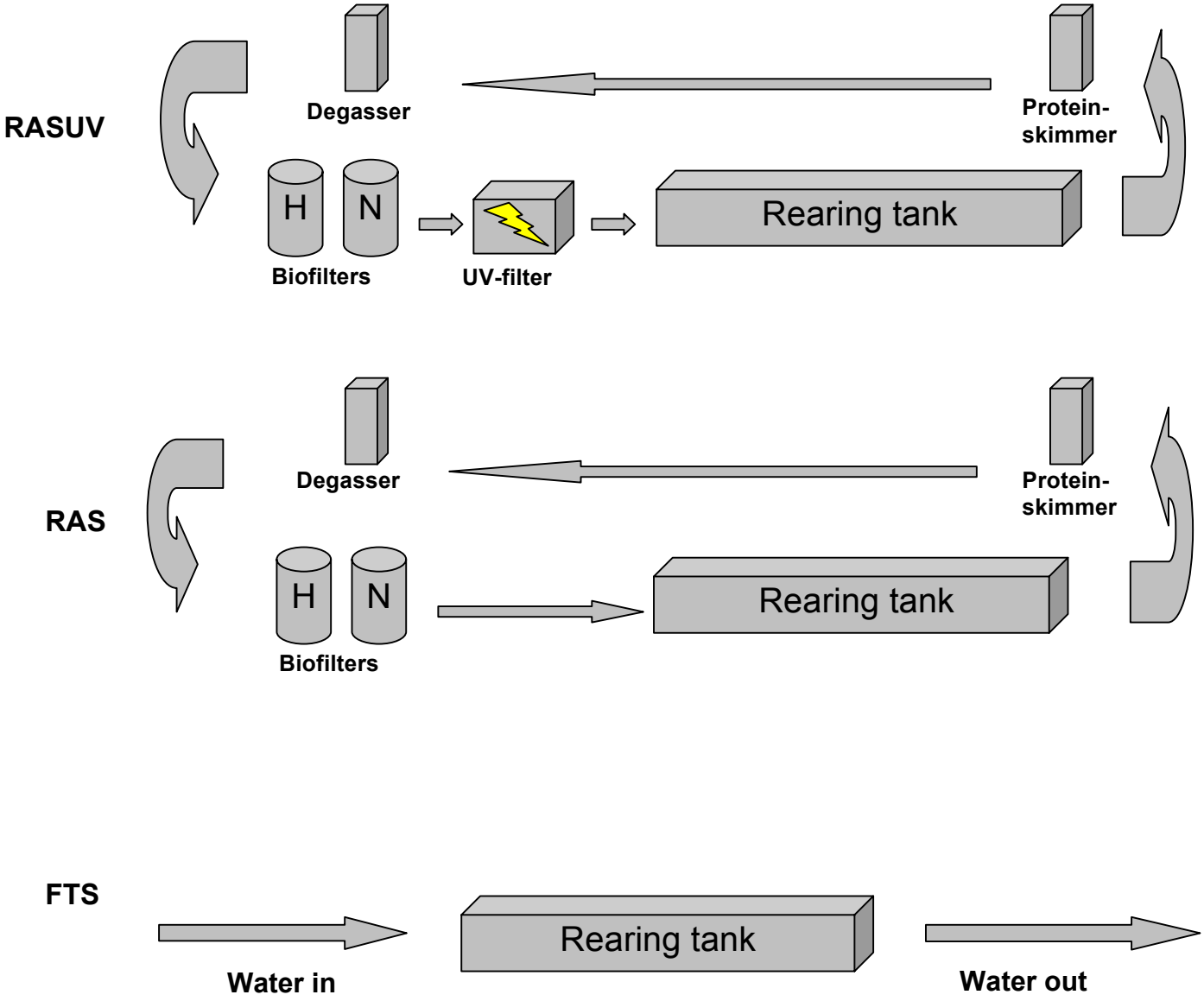


Figure 3.1. Illustration of the different rearing systems. One recirculating water system with UV filter and one recirculating water system without UV filter. The third system was a flow-through system without UV filter.

3.2 Rearing systems

3.2.1 Flow through system

The seawater used in the FTS was pumped in from the Trondheims fjord at a depth of 90 meters, 700-800 meter outside of Brattøra research centre. The water was processed through a sand filter without maturation before entering the rearing tank of 2 x 37 litres. The water exchange rate was set to 150ml/min, exchanging the all water in the rearing tank 3 times a day. The same exchange rate was used for all three rearing systems.

3.2.2 RAS - recirculating aquaculture system

The RAS used in this experiment had two biofilters containing 230 litres water, each with corrugated plastic sheets, increasing the surface area for bacteria growth to reduce organic matter and to break down ammonium. The first biofilter (H) was fed organic matter to stabilize the heterotrophic microbial community to favour K-selective bacteria. One week before introduction of lobster larvae, this biofilter was fed 3 grams per day of fish feed granulate to increase the amount of heterotrophic bacteria in the biofilter, breaking down organic components.

The second biofilter was fed 3 grams per day of ammonium chloride to increase the microbial community of nitrifying bacteria. This feeding was also carried out 1 week prior to the experiment start-up. The RAS was set up with a degasser holding 73 litres of water, and the rearing tanks were 2 x 37 litres. Behind the rearing tank the water went into a tank, containing 86 litres water, and from there into a protein skimmer holding 63 litres. The pipelines in had a total volume of 30 litres. The whole system had a maximum capacity of 784 litres.

9.5% of external water was added from a reservoir tank to the RAS to compensate for lost water. This amount of new water ensured that the RAS never got too diluted. The water flow was controlled and adjusted several times during the experiment.

3.3 Rearing conditions in tanks

The measurement regime is shown in table 3.1. YSI multiparameter was used to measure temperature, O_2 and pH. To control the accuracy of O_2 levels, the instrument was calibrated in a bucket containing seawater with high aeration. Each rearing tank had aeration downstream of the larvae chambers. Salinity was measured using a refractometer, calibrated in fresh water with the identical temperature as the rearing tanks. In the recirculating systems there was a problem with high salinity due to evaporation. Adding fresh water to the systems compensated for this effect. Fresh water was added in the first biofilter, containing heterotroph bacteria to maximize the mixing before going into the rearing tank.

Dph	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Temperature	x	x	x	x	x	x	x	x	x	x	x	x	x	x
O_2	x	x	x	x	x	x	x	x	x	x	x	x	x	x
pH					x					x			x	
Salinity	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Nitrogen				x							x			x
Sampling of water				x				x				x		x
Sampling of larvae				x				x				x		

Table 3.1: Water measurements and sampling intervals for the experiment.

3.4 Live Feed

Feed used in the experiment was produced at the facilities of Sintef Fisheries and Aquaculture, Brattøra, where a test production of the marine copepod *Acartia tonsa* was carried out. Density in the rearing tank for the copepods was 7 copepods per ml. In a similar experiment with communal rearing of larvae, the copepod density was 3 copepods per 1ml. (Evjemo, O., 2013). The larvae were fed enough to ensure the feed not being a limiting factor for growth or survival. Each chamber was given an average number of 336 copepods each day. Since the copepods migrated freely between chambers and out into the rearing tank, some additional feeding was done to chambers with little feed.

3.6 Sampling of larvae

Sampling of larvae was executed at 4, 8, 12 days post hatching (dph).

The animals were put in a cup containing seawater and placed on ice. Secondly the animals were transferred to a cup of sterile milli-Q water to rinse of any salt. Finally, animals were placed individually in eppendorf tubes, snap-frozen in liquid nitrogen, and then stored at -20

°C. For the other sampling days there was taken 8 larvae from each system, giving a total of 72 samples of larvae for microbial analysis.

3.7 Sampling of water

Samples of the water were taken at 1, 4, 8, 12 and 14 dph. Water samples were taken from the inlet to the tanks in each system, and from both sides of the rearing tanks. Using a filter, particles larger than 120µl were removed and the water was filled in 50ml glass bottles with a silicone tube. Samples were fixated with 25% glutaraldehyde to a final concentration of 2% and stored at 4 °C. Parallel samples of the waters were taken using Dynagard filters (Microgen). Using a 50ml syringe, 40ml of water was pressed through the 0.22µm filters. The filter was stored at -20 °C.

3.8 Monitoring of larvae

Mortality was recorded with a schematic presentation of the chambers in the rearing systems. The table in figure 3.3 shows the mortality and sampling during the experiment.

	A					B					C			
	Ras+uv					Ras					FTS			
	A1		A2			B1		B2			C1		C2	
1	16,7	16,7	13,7	15,7	1	13,7	13,7	13,7	15,7	1	17,7	17,7	14,7	levende
2	13,7	16,7	13,7	14,7	2	14,7	16,7	levende	15,7	2	13,7	14,7	8,7	13,7
3	9,7	12,7	8,7	8,7	3	13,7	14,7	14,7	13,7	3	12,7	8,7	8,7	12,7
4	levende	13,7	13,7	13,7	4	7,7	11,7	14,7	15,7	4	14,7	11,7	8,7	14,7
5	13,7	levende	9,7	15,7	5	13,7	14,7	15,7	14,7	5	12,7	16,7	9,7	16,7
6	11,7	8,7	13,7	12,7	6	15,7	11,7	8,7	14,7	6	7,7	16,7	13,7	8,7
7	levende	16,7	13,7	10,7	7	14,7	12,7	15,7	12,7	7	13,7	levende	11,7	7,7
8	12,7	11,7	13,7	15,7	8	13,7	levende	16,7	12,7	8	8,7	12,7	11,7	11,7
9	16,7	14,7	13,7	16,7	9	12,7	levende	levende	13,7	9	13,7	11,7	13,7	16,7
10	8,7	14,7	11,7	Levende	10	7,7	8,7	levende	11,7	10	14,7	16,7	14,7	16,7
11	17,7	14,7	13,7	13,7	11	14,7	14,7	11,7	15,7	11	11,7	12,7	12,7	14,7
12	16,7	12,7	14,7	12,7	12	16,7	16,7	levende	11,7	12	13,7	levende	10,7	14,7
13	levende	levende	9,7	14,7	13	13,7	14,7	12,7	8,7	13	14,7	16,7	12,7	16,7
14	13,7	14,7	9,7	levende	14	13,7	levende	levende	16,7	14	7,7	6,7	13,7	11,7
15	14,7	13,7	13,7	16,7	15	6,7	15,7	levende	13,7	15	16,7	7,7	8,7	16,7
16	levende	13,7	12,7	14,7	16	13,7	14,7	levende	13,7	16	11,7	7,7	15,7	7,7
17	7,7	8,7	14,7	14,7	17	8,7	levende	levende	13,7	17	13,7	levende	8,7	7,7
18	13,7	levende	8,7	13,7	18	13,7	levende	levende	7,7	18	14,7	7,7	13,7	14,7
19	13,7	levende	13,7	levende	19	7,7	levende	levende	13,7	19	13,7	14,7	14,7	12,7
20	12,7	16,7	13,7	8,7	20	18,7	levende	levende	13,7	20	13,7	8,7	14,7	16,7
21	14,7	14,7	13,7	14,7	21	12,7	8,7	levende	levende	21	12,7	7,7	12,7	16,7
22	13,7	13,7	13,7	15,7	22	7,7	levende	levende	12,7	22	16,7	7,7	14,7	16,7
23	8,7	levende	12,7	14,7	23	12,7	6,7	levende	14,7	23	14,7	18,7	13,7	8,7
24	17,7	14,7	13,7	14,7	24	14,7	levende	12,7	13,7	24	8,7	12,7	9,7	levende
25	levende	12,7	13,7	16,7	25	6,7	levende	17,7	8,7	25	11,7	16,7	16,7	levende
26	levende	tapt prøve	14,7	10,7	26	8,7	12,7	16,7	15,7	26	14,7	levende	12,7	9,7
27	15,7	levende	13,7	16,7	27	18,7	18,7	levende	13,7	27	17,7	16,7	15,7	7,7
28	levende	levende	13,7	levende	28	17,7	levende	8,7	14,7	28	13,7	18,7	13,7	16,7
29	16,7	levende	7,7	15,7	29	16,7	16,7	levende	7,7	29	16,7	13,7	15,7	levende
30	levende	13,7	16,7	levende	30	levende	levende	17,7	16,7	30	16,7	15,7	17,7	16,7
	8	8	0	5		1	12	16	1		0	4	0	4
	inlet water													
	Dead													
	Sample													
	Living													

Figure 3.2. Control sheet for the larvae in the rearing chambers. Red = dead, yellow = sampling and green = larvae alive when the experiment ended.

3.9 Analytic procedures

3.9.1 DNA extraction

Whole animals were used in the microbial analysis. Extraction of total DNA from larvae was carried out using Powersoil DNA Isolation kit provided by MOBIO Laboratories Inc. First the sample was crushed using a glass rod to mechanically grind the exoskeleton of the larva. The homogenized larval samples were then transferred to the lysis tubes for bead-beating included in the kit, and the protocol given by the manufacturers were followed (complete protocol given in Appendix X. The DNA extracts were stored at – 20 ^{o}C. The DNA concentrations were measured using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

3.9.2 Amplification of bacterial rRNA gene associated with larvae

The PCR amplification of the V3-region of the bacterial 16S rRNA gene for the larvae was performed in a two step procedure called nested PCR (Bakke et al., 2011) developed for samples dominated by eukaryotic DNA. Primers EUB8F/984yR where used for the external amplification and primers 338F/518R for the internal amplification. PCR primers sequences are found in table 3.2. Using just internal primers creates a risk of amplifying the corresponding region of the eukaryotic 18S rRNA, which contains conserved regions with sequence similar to the bacteria 16S rRNA. (Bakke et al., 2011). A GC-clamp connected to the primer 338F was used to keep the PCR products from complete denaturation during the DGGE (See below).

External primers:	Eub8F, 5′-agagtttgatcmtggctcag-3′
	984yR, 5′-gtaaggttcytcgcgt-3′

Internal primers:	338F, 5′-actcctacgggaggcagcag-3′
	518R, 5′-attaccgcggctgctgg-3′

Table 3.2. External and internal primers used in the amplification of the bacteria rDNA from the samples.

Total volume for each PCR reaction was 25µl. 20ng/1µl of DNA extracts were used as template in the PCR reactions. 1µl template was added to 24µl mastermix consisting of 2mM $MgCl_2$, 0.2 mMnNTP, 0.3µM of each primer (forward and reverse) and Taq polymerase (QIAGEN) with reaction buffer.
The PCR reaction cycle for amplifying of samples are given in table 3.3 and 3.4. Cycling step 2-4 was repeated 22 times each for external and for internal amplification.

Cycling step	**Temperature**	**Time**
1	95 °C	3 min
2	95 °C	0.5 min
3	50 °C	0.5 min
4	72 °C	1 min
5	72 °C	10 min
6	10 °C	∞

Table 3.3. PCR cycling program for the external amplification of the V3 region of the bacteria 16S rDNA.

Cycling step	**Temperature**	**Time**
1	95 °C	3 min
2	95 °C	0.5 min
3	53 °C	0.5 min
4	72 °C	1 min
5	72 °C	30 min
6	10 °C	∞

Table 3.4. PCR cycling program of the internal amplification of the V3 region of the bacteria 16S rDNA.

Some PCR reactions did not successfully amplify and the amount of template had to be adjusted in those samples, from 0.5µl – 2µl of the DNA extracts per reaction.
Given the low content of eukaryotic DNA, the water samples were amplified using only internal primers 338F/518R, with 38 cycles, and the rest as described for the PCR of the larval samples above. 1µl of DNA extracts was used as template in a 25µl reaction.

3.9.3 DGGE (Denaturing Gradient Gel Electrophoresis)

Denaturing gradient gel (DGGE) system from INGENYphorU (INGENY, Netherland) was used for separation of PCR sequences. For the casting of the gel, two solutions of 8% acrylamide containing denaturants urea and formamide was used (recipe can be found in appendix 2). The difference in the concentration of denaturing agents defines where in the gel the PCR sequence will denaturate and stop migrating. The GC clamp holds the two strains together even after the target sequence denaturate due to the extra hydrogen binding between G (Guanine) and C (Cytosine) compared to A (Adenine) and T (Thymine) which only has two. A sequence with high ratio of GC to AT base pairs will travel further down the gel before denaturating compared to a sequence with fewer GCs.

The hardware for this system consists of two glass plates, one spacer and a 48 well comb. Mounting the two glass plates on top of each other with the spacer between created a room for casting the gel. The glass plates were put in a cassette and locked firmly with screws pressing the glass plates against the sealing of the cassette. The gel used in separation of PCR product contained 8% acrylamide with a denaturing gradient from 35% - 55%. The stock solutions with 0% and 80% denaturing agents were made after recipe found in Appendix 2. 35% and 55% denaturation solutions together with stacking gel were prepared from the stock solutions. The 80% denaturing acrylamide solution was filtered to remove precipitate of urea prior to addition. To initiate the polymerization process, tetramethylenediamine (TEMED) and ammonium persulfate (APS) was added to the solution. Solutions were transferred to a gradient mixer and pumped into the casting frame through a hose giving high denaturation in the bottom (55%), and a gradient towards the top to a final concentration of 35% in the top area of the gel. When the solution reached 1cm from the comb the gradient mixer was stopped and the system was cleansed with milli-Q water before stacking gel was added to the gradient mixer. Stacking gel was added until the solution reached the top of the glass plates. The comb was re-fitted and the gel was put aside for 2 hours for complete polymerization. Before running the gel, 0.5x TAE buffer was filled into the gel tank. The gel with cassette was lowered into a buffer tank with 0.5µl TAE preheated to 60 ^{o}C. When placing the gel into the buffer tank, no bubbles of air could occur underneath the gel, obstructing vertical migration of the samples. Air bubbles obstruct an even distribution of the electrical circuit though the gel, changing the vertical migration of the samples towards the sides (smiling effect). Some smiling effect always occurs at the sides of the gel, and because of this, 6 wells at each side of the gel were not used.

The amount of PCR sample applied to each well varied from 5-15µl depending on the concentration of the PCR product, and was added 2-4µl 6x loading dye (Fermentas) to make the sample visual and able to sink into the well. The amount of sample to each well was determined by the intensity of the bands representing the PCR products in an agarose gel from PCR. To achieve a complete migration of the samples, the DGGE gel was run for 20 hours at 60 ^{o}C with an electric current set to 100 volts.
When the process was finished, the gel was taken out from the glass plates and stained with a mixture containing 3µl Sybr ® Gold (Invitrogen), 600 ml 1xTAE and 30 ml milli-q water. After staining the gel for one hour, the gel was rinsed with milli-q water and placed under UV- light to be photographed.

3.10 Statistical analyses

Images taken of the DGGE gels where formatted and analyzed using Gel2k (Norland, 2004). Gel2k is used for transforming the DGGE band profiles into histograms. Each peak of the histogram corresponds to a band on the gel, and the peak area reflects the bands intensities. The peak area data were transferred to a Microsoft Excel sheet, and normalized by dividing each peak area by the total peak area value of all the bands in the lane, creating a fractional peak area by using the formula in 3.1.

$$p_i = \frac{n_i}{N} \tag{3.1}$$

Where p_i = normalized values of the intensity in the bands, n_i = intensity of a single band and N = sum of all the intensity values.

Band richness (K'), Shannon's diversity index (H') and Pielou's evenness-index (J') was calculated from the normalized values of the peak area.
Band richness is the sum of bands in the DGGE-profile and reflected the richness of species in the sample.

Shannon's diversity index is one of many methods to calculate diversity. Diversity index describes the diversity in a sample, taking in consideration both number of DGGE bands richness and the relative intensity in the bands (evenness) (Peet, 1974).
Shannon index (H´) is calculated using the formula in 3.2.

$$H' = -\sum_{i=1}^{k} (p_i \ln p_i) \quad (3.2)$$

Where K = band richness and p_i = normalized band intensity value.

Pielou's Evenness index (J') is another diversity index which calculated the evenness distribution between different species in a given sample or area (Peet, 1974). J' is calculated using the relationship between the observed diversity (H') and the maximum diversity (H'^{max}) (Peet. 1974, van Dyke 2008). J' is constrained between 0 and 1. The less variation in communities between the species, the higher the J' is. High values (1 > J') describe a community with an even distribution between the species. J' was calculated using the formula in 3.3.

$$J' = \frac{H'}{H'_{\max}} \quad (3.3)$$

The program PAST (Hammer et al., 2001) was used for statistical analyses. One-way ANOVA and Tukeys HSD (Honestly Significant Difference) test was used to investigate if there were any significant differences in diversity indices between different groups of samples.

Bray-Curtis similarities were calculated by using square root transformed fractional peak areas resulting from the DGGE analyses, in order to down-weight abundance in the data Bray-Curtis similarities range from 0 to 1. If the samples are identical, the index is 0, and if the samples are totally different the index is 1. Bray-Curtis similarities are calculated from 1 minus Bray-Curtis unevenness index, and the samples will be identical when the evenness index is 1 and totally different when the index equals 0 (Bray and Curtis, 1957).

In order to compare groups of samples, a nonmetric multi dimensional scaling (NMMDS) plot based on Bray-Curtis similarity matrices was created. NMMDS is an ordination method for representing objects in a multi dimensional presentation. The distance between the points representing the samples in the plot, will be have more distance, the less similar the samples

are. The method produces a value of stress (goodness-of-fit), where the samples in the plot will reflect the exact relationship between the bacteria in the microbial communities from the samples. Generally, the result from the NMMDS should not be interpreted unless the stress value is < 0.2 (Bray and Curtis, 1957, Clarke, 1999).

To test the significance of differences observed between groups of samples in the NMMDS plots, one-way Analysis of Similarity (one-way ANOSIM) based on the Bray-Curtis similarity measure was used (Clarke, 1993, Clarke and Ainsworth, 1993).

4. Results

4.1 Physiochemical water quality in rearing water

Lobster larvae were reared in tree systems with different water treatment; RASUV, RAS and FTS. To evaluate the quality of the rearing water in the different systems, physiochemical properties of the water were investigated.

4.1.1 Nitrogen species

The amount of nitrogen species in the rearing systems was measured three times. Through the experiment there were never harmful levels in any of the systems. Ammonia (TAN) was 0.30 ± 0.10, 0.30 ± 0.03 and 0.06 ± 0.03 mg L^{-1} (average ± standard deviation) in the RASUV, RAS and FTS, respectively, and significantly lower in the FTS than in the other two systems (one-way ANOVA, p = 0.004).

Nitrite (NO2-N) was 0.0045 ± 0.0027, 0.0053 ± 0.0016 and 0.0032 ± 0.0008 mg L^{-1} (average ± standard deviation) in the RASUV, RAS and FTS, respectively, and there were no significant differences between the systems (one-way ANOVA, p = 0.422).

Nitrate (NO3-N) was 0.30 ± 0.10, 0.30 ± 0.03 and 0.06 ± 0.03 mg L^{-1} (average ± standard deviation) in the RASUV, RAS and FTS, respectively, and there were no significant differences between the systems (one-way ANOVA, p = 0.551).

4.1.2 pH

pH was measured at 5, 10 and 13 dph during the experiment. Average values with standard variation was; 8.46 ± 0.2, 8.45 ± 0.032 and 8.35 ± 0.05 in RASUV, RAS and FTS, respectively. The differences in mean values among the treatment groups were not great enough to exclude the possibility that the difference was due to random sampling variability; no statistically significant difference was observed (P = 0,133).

4.1.3 Temperature

There was a temperature gradient across the rearing room. This caused differences in the rearing tanks of 1- 1.5 °C throughout the whole experiment. As shown in figure 4.1, the differences in temperature were the same between the systems during the whole experiment. Average temperature was 20.7 ± 0.4, 20.0 ± 0.5 and 19.3 ± 0.5 mg L^{-1} (average ± standard deviation) in the RASUV, RAS and FTS, respectively, and there were significant differences between all tree systems (one-way ANOVA, p =0.001).

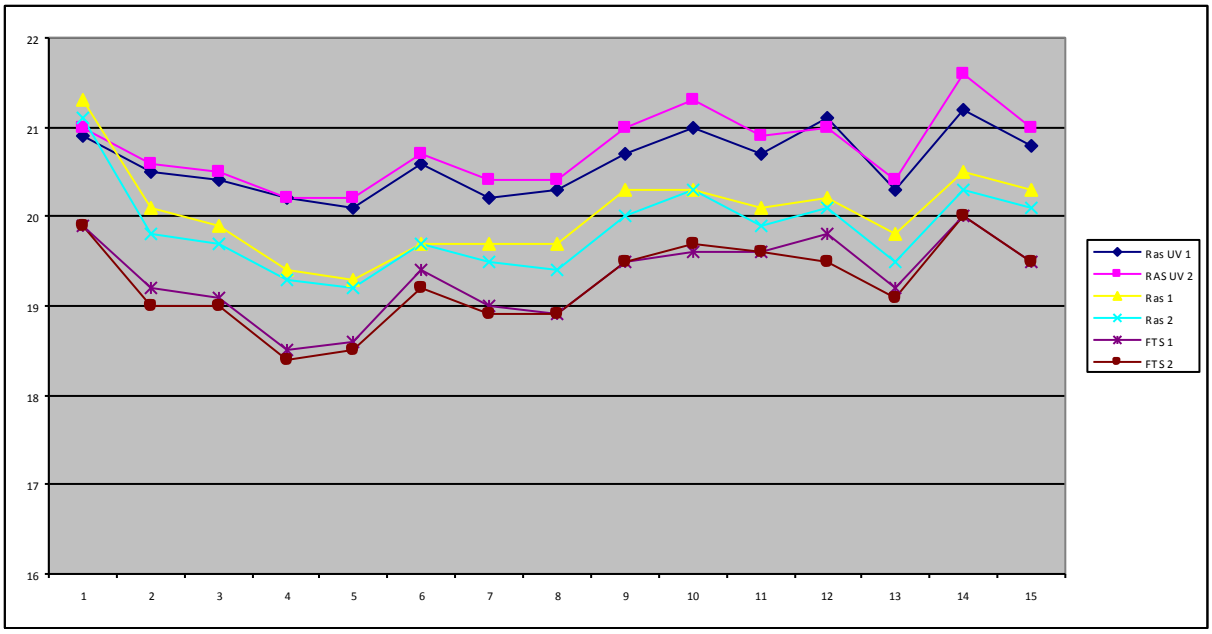


Figure 4.1. Temperature for left and right side (1 and 2) in the rearing tank of RASUV, RAS and FTS for each day during the experiment.

4.1.4 Salinity

Salinity was 37.8 ± 1.3, 38.2 ± 1 and 37.1 ± 0.5 g L^{-1} (average ± standard deviation) in the RASUV, RAS and FTS, respectively, and a significant difference was observed between RAS and FTS (one-way ANOVA/Tukey test, p = 0.004).

4.1.5 Oxygen

The oxygen concentration in the rearing water was within the recommended levels during the whole experiment. The temperature was recorded daily as can be seen in figure 4.2.

Oxygen was 7.62 ± 0.12, 7.80 ± 0.11 and 7.75 ± 0.12 mg L^{-1} (average ± standard deviation) in the RASUV, RAS and FTS, respectively. There were significant differences between RASUV vs RAS and RASUV vs FTS (one-way ANOVA/Tukey test, p=0.001 for both). No significant difference was detected between RAS vs FTS.

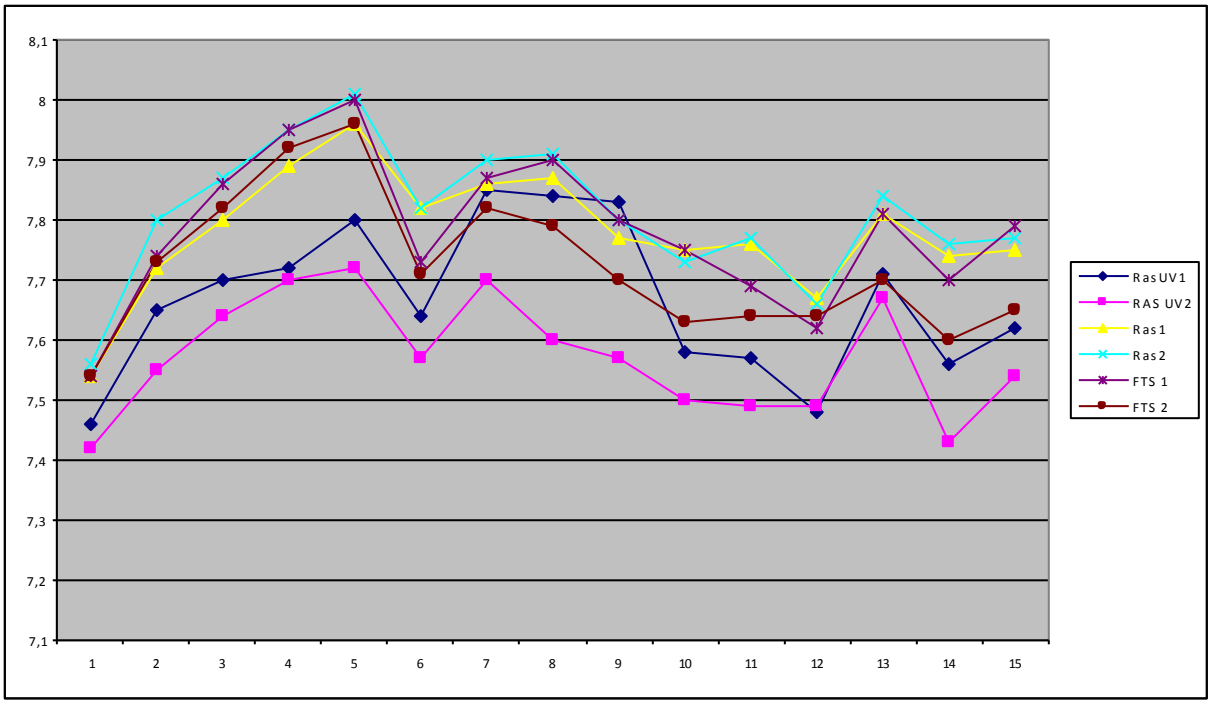


Figure 4.2. The oxygen concentration in mg/l^{-1} for the left and right side (1and 2) of the rearing tank in RASUV, RAS and FTS for each day during the experiment.

4.2 Analyses of microbial communities

Analyses of the microbial communities found in samples of water and larvae from this experiment were examined using a PCR/DGGE strategy.

To investigate if the differences in rearing waters had an effect on the microbiota in the rearing water and connected to the larvae, we examined the bacterial v3 region of the bacterial rRNA connected with the larvae using nested PCR together with the rearing water from the same day at 4, 8 and 12 dph.

4.2.1 Microbiota in water at 4, 8 and 12 dph

The DGGE gel obtained for water samples (figure 4.3) indicated that there were differences between the samples. A non-metric MDS plot based on Bray-Curtis similarities was made based on the values from the DGGE produced in Gel2K shown in figure 4.4. A one-way ANOSIM test was carried out to see if there were significant differences in microbiota between the systems, observed in the gel and the nm-MDS plot.

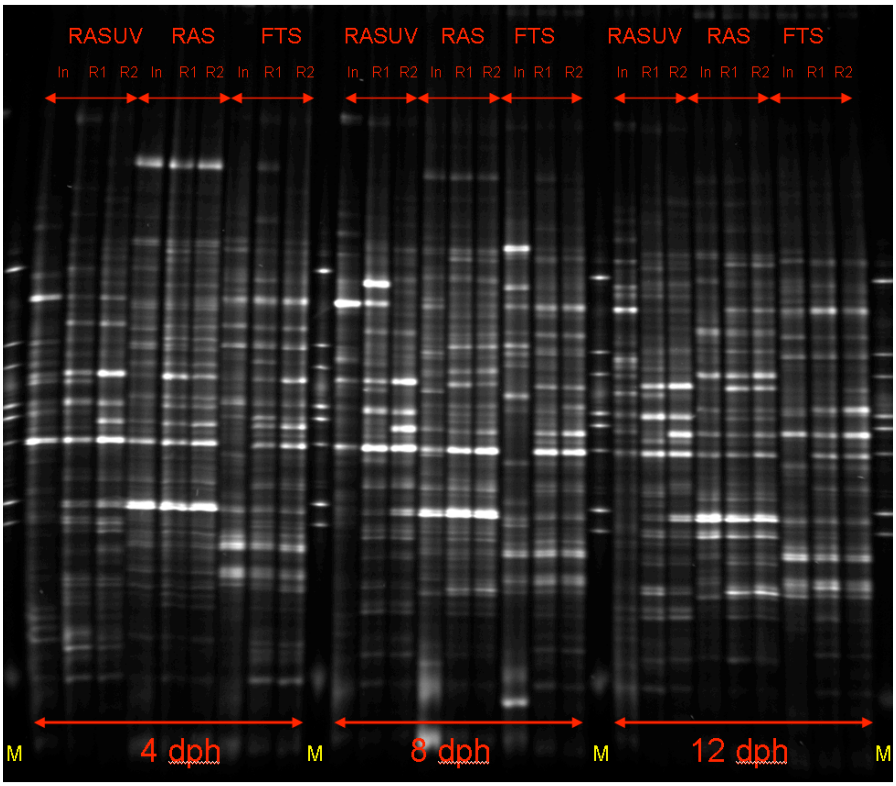


Figure 4.3. DGGE gel with PCR-products of the V3 region of bacterial rRNA gene from water samples of RASUV, RAS and FTS at 4, 8 and 12 dph (In = intake water. R = Rearing water. M = marker).

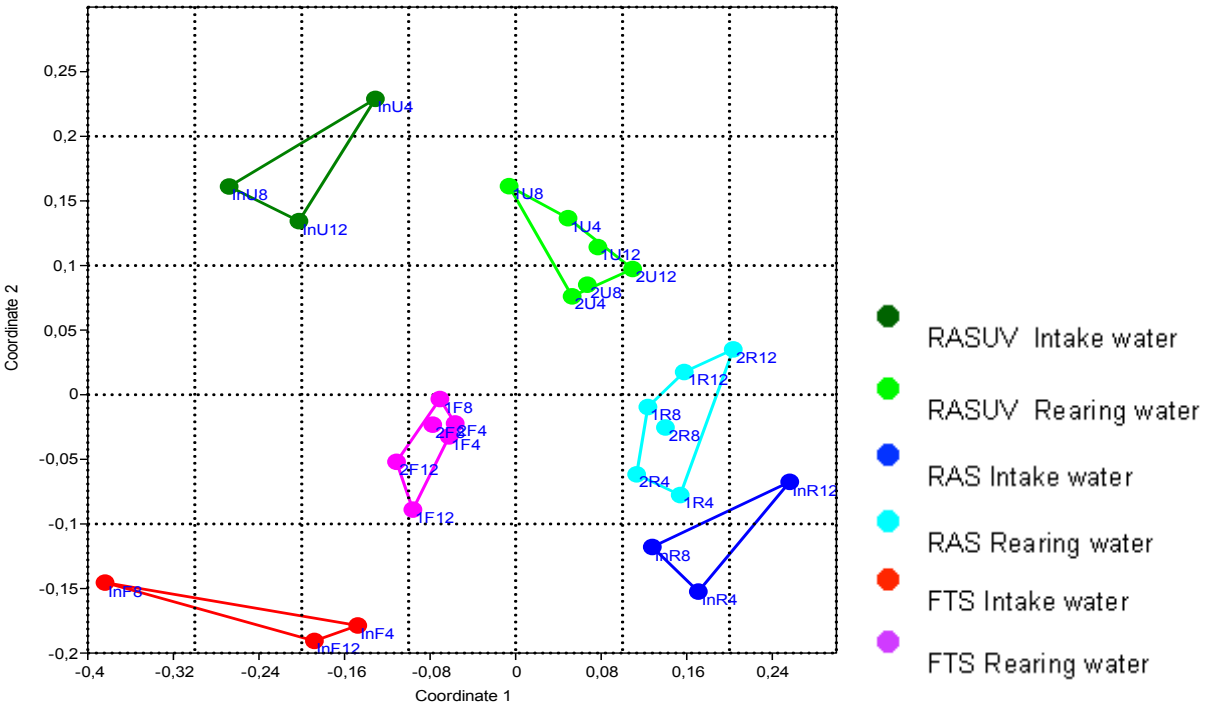


Figure 4.4. Non-metric MDS plot based on Bray-Curtis similarities of microbial communities from inlet- and rearing water in RASUV, RAS and FTS at 4, 8 and 12 dph (RASUV=U, RAS=R and FTS=F).

The one-way ANOSIM test of Bray-Curtis similarities with Bonferroni corrected p values showed no significant differences in the microbiota between the inlet waters RASUV vs FTS, RASUV vs RAS and RAS vs FTS (p = 0.295, 0.298 and 0.298, respectively). There was however significant difference between the rearing waters of RASUV vs FTS, RASUV vs RAS and RAS vs FTS with p=0.0069, 0.0066 and 0.0057, respectively. Significant differences were observed in the microbiota between the intake water and rearing water for the RASUV and FTS (one-way ANOSIM, p=0.013 and 0.015), but there were no significant differences in the RAS systems microbiota between the intake water and the rearing water (one-way ANOSIM, p= 0.62).

To examine the stability of the microbiota in the water systems over time, average Bray-Curtis similarities with standard deviation were calculated for the inlet- and rearing water from the respective systems (Figure 4.5). The similarity among intake water samples over time from RAS is higher than those for the other systems, indicating a more stable microbiota. FTS intake water portrayed the highest standard deviation of the water samples. The microbiota of the rearing water in all the systems was similar in stability over time.

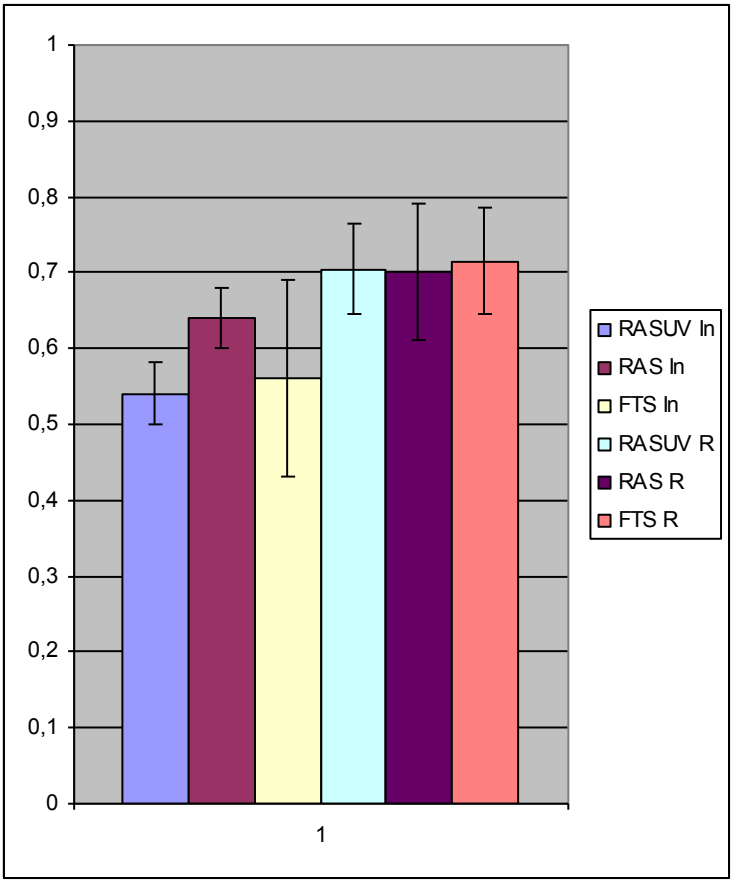


Figure 4.5. Average Bray-Curtis similarities of the microbiota in intake- and rearing water within the RASUV, RAS and FTS (In = Intake water. R = Rearing water). Error bars indicate standard deviation.

4.2.2 Richness, diversity and evenness of rearing water microbiota.

Band richness, Shannon`s diversity and Pielou`s evenness were determined for the DGGE profiles of all water samples (Table 4.1). Band richness for RAS inlet and rearing water microbiota was higher than in RASUV and in FTS inlet and rearing water microbiota.
A one-way ANOVA/ Tukey test of band richness for the microbiota for intake and rearing water showed that band richness was significantly higher for RAS rearing water microbiota compared the microbiota from RASUV rearing water, FTS intake water and RASUV intake water (one-way ANOVA with bonferroni-corrected, p values, p= 0.035, 0.029 and 0.013, respectively).

Shannon`s diversity index values were similar between systems, and the one-way ANOVA test could not rule out the possibility that the observed variation was a result of random variation (p = 0,590).

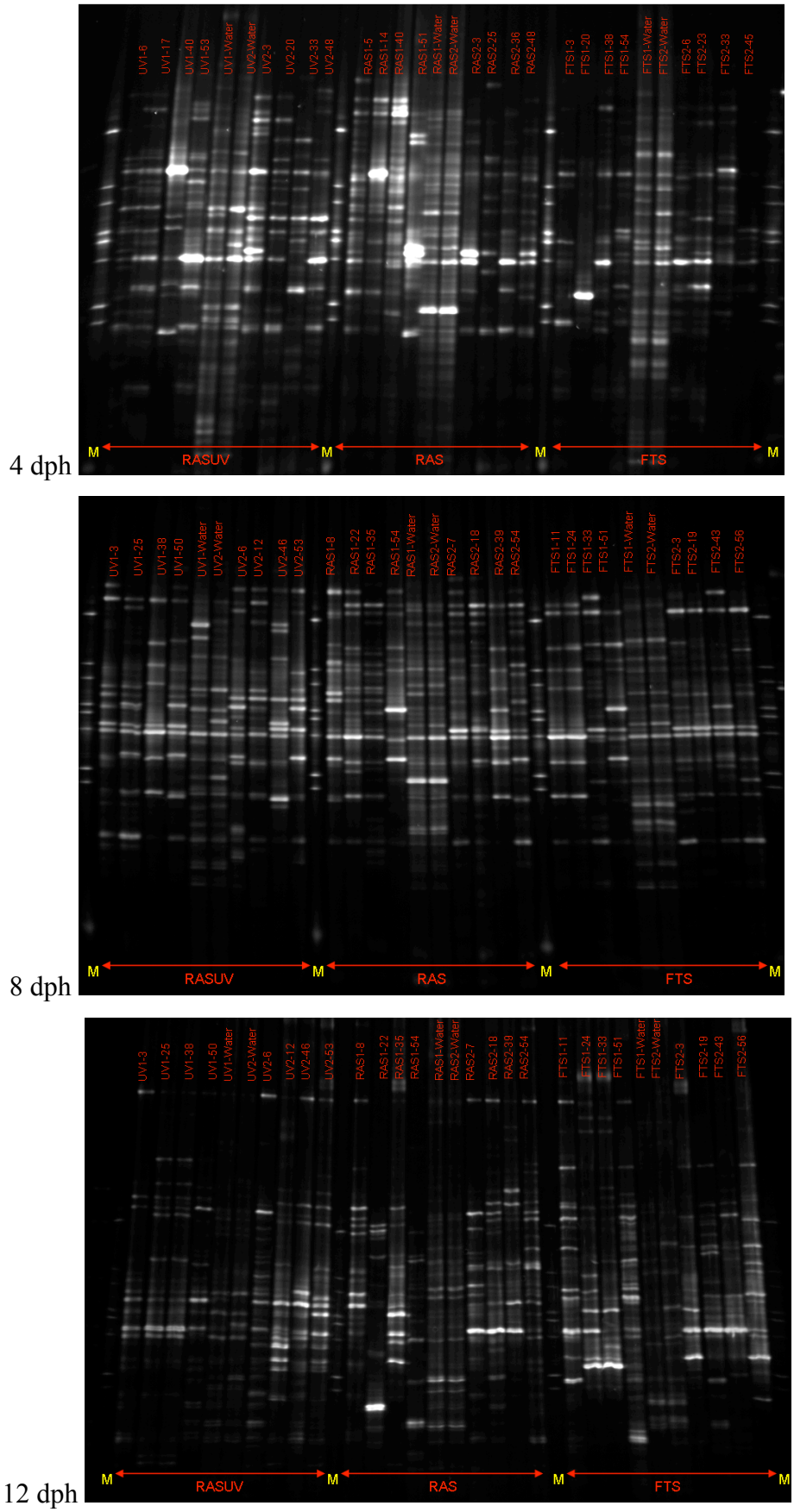
No significant differences in Pielou`s evenness was found between the water systems when testing the results with a one-way ANOVA (p = 0,117).

	Band richness (K`)	Shannon W (H`)	Evenness (J`)
FTS Tank	31 ± 5	2.84 ± 0.19	0.83 ± 0.03
FTS In	28 ± 3	2.69 ± 0.04	0.81 ± 0.03
RAS Tank	39 ± 4	2.76 ± 0.14	0.76 ± 0.04
RAS In	36 ± 5	2.72 ± 0.27	0.76 ± 0.05
RASUV Tank	33 ± 3	2.78 ± 0.20	0.79 ± 0.05
RASUV In	29 ± 3	2.58 ± 0.34	0.76 ± 0.08

Table 4.1. Average values with standard deviation on band richness, Shannon`s diversity and Evenness index for microbiota for the three rearing systems. In = intake water and Tank = rearing water.

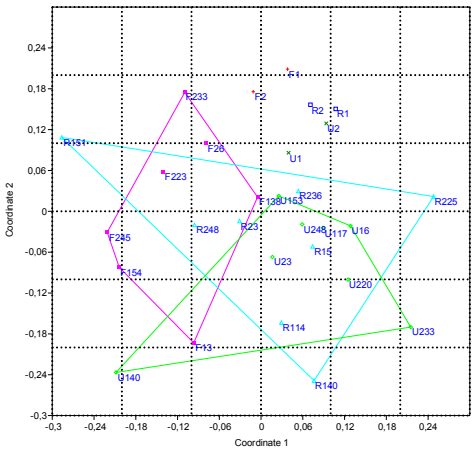
4.3 Microbial communities associated with the lobster larvae

Microbial communities in larvae were examined with the same approach applied to the water samples. 8 samples from larvae and 2 samples of rearing water from the 3 systems were used in each of three gels representing the sampling times 4, 8 ans 12 dph. The gels (Figure 4.6) show some bands on the same position in the denaturing gradient, indicating that they have similar DNA sequences. There is also a lot of variation in the band profiles between the gels, indicating that the microbial communities developed through out the experiment.

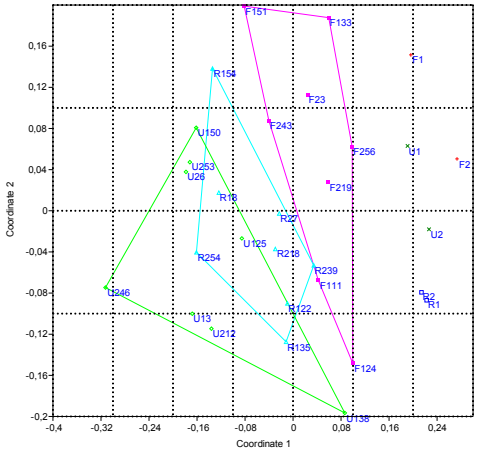


Figur 4.6. DGGE gel with PCR product of the v3 region of the bacterial rDNA from 4, 8 and 12 dph larvae and water in RASUV, RAS and FTS (M = Marker).

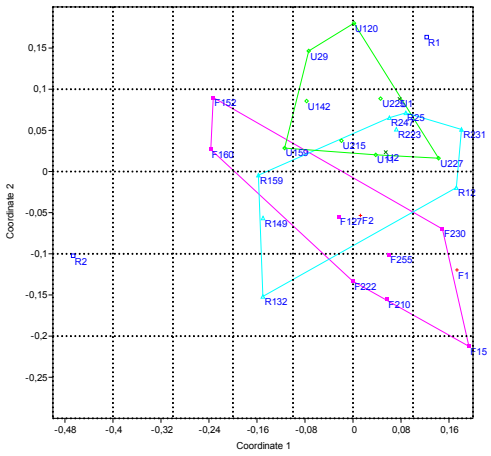
Non-metric MDS ordinations based on Bray-Curtis similarities between bacteria DGGE profiles shown in Figure 4.7, to compare the microbiota associated with the larvae and the rearing water of the three systems at 4, 8 and 12 dph.



4 dph.



8 dph.



12 dph

Figure 4.7. Non-metric MDS plot based on Bray-Curtis similarities for v3 rRNA bacterial sequences in samples from lobster larvae and rearing water from the respective systems at 4, 8 and 12 dph (RASUV=U, RAS=R and FTS=F).

4.3.1 Similarities between the microbiota in rearing water and larvae

Average Bray-Curtis values were calculated to compare the larval microbiota to the rearing water microbiota for each system at 4, 8 and 12 dph (Figure 4.8). The FTS and RASUV displayed similar patterns, where the similarity between larval and rearing water microbiota went from more similar at 4 dph and became less similar at 8 dph before the microbiota becomes more similar at 12 dph. In the RAS, the larval and rearing water microbiota becomes less similar throughout the experiment.

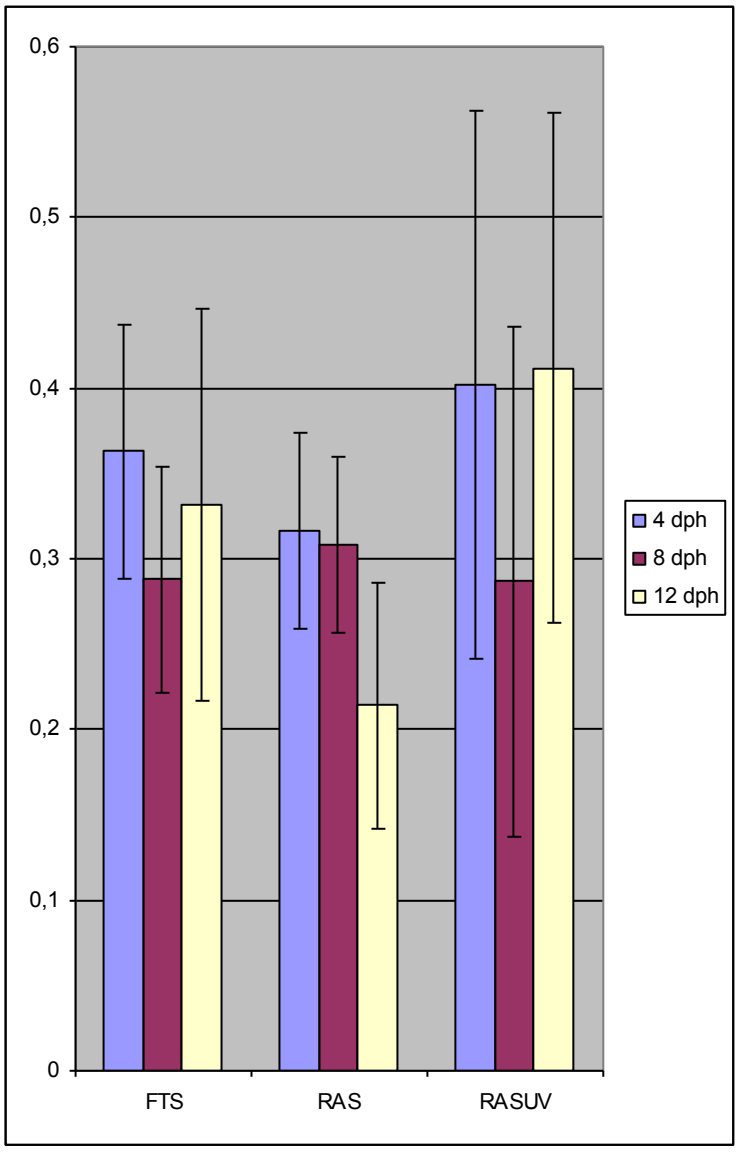


Figure 4.8. Average Bray-Curtis similarities with standard deviations between larval and rearing water microbiota for the three rearing systems.

A one-way ANOSIM test was carried out to examine if there were significant differences in the microbial communities of larvae compared to the rearing water in each rearing system. At 4 dph no significant differences between the larval and rearing water microbiota were found in RASUV, RAS or FTS, (bonferroni corrected p values: p=0.3301, 0.0701 and 0.1364, respectively). At 8 dph there were found significant differences between larval and rearing water microbiota for all systems (bonferroni corrected p values of 0.0210, 0.0199 and 0.0234, for RASUV, RAS and FTS, respectively). At 12 dph the one-way ANOSIM test found significant differences between larval and rearing water microbiota in the RAS and FTS but not in the RASUV (bonferroni corrected p values: p=0.0223, 0.0226 and 0.0657, respectively).

4.3.2 Similarities in the larval microbiota between systems

A one-way ANOSIM test was performed to investigate if the larval microbiota were different between the rearing systems (See table 4.2). Significant differences were found between larval microbiota in FTS and RAS, and FTS and RASUV for all sampling days. When we compared the larval microbial communities from RAS and RASUV there were no significant difference at 4 dph and 8 dph (p= 0.652 and 0.3987). However at 12 dph there was a significant difference found between the two recirculating systems (p= 0.0483).

4 dph		FTS	RAS	RASUV
	FTS	0,0000	0,0135	0,0003
	RAS	0,0135	0,0000	0,6522
	RASUV	0,0003	0,6522	0,0000
8 dph		FTS	RAS	RASUV
	FTS	0,0000	0,0051	0,0018
	RAS	0,0051	0,0000	0,3987
	RASUV	0,0018	0,3987	0,0000
12 dph		FTS	RAS	RASUV
	FTS	0,0000	0,0027	0,0009
	RAS	0,0027	0,0000	0,0483
	RASUV	0,0009	0,0483	0,0000

Table 4.2. ANOSIM test with Bonferroni corrected p-values for comparison of larval microbiota between the rearing systems at 4, 8 and 12 dph.

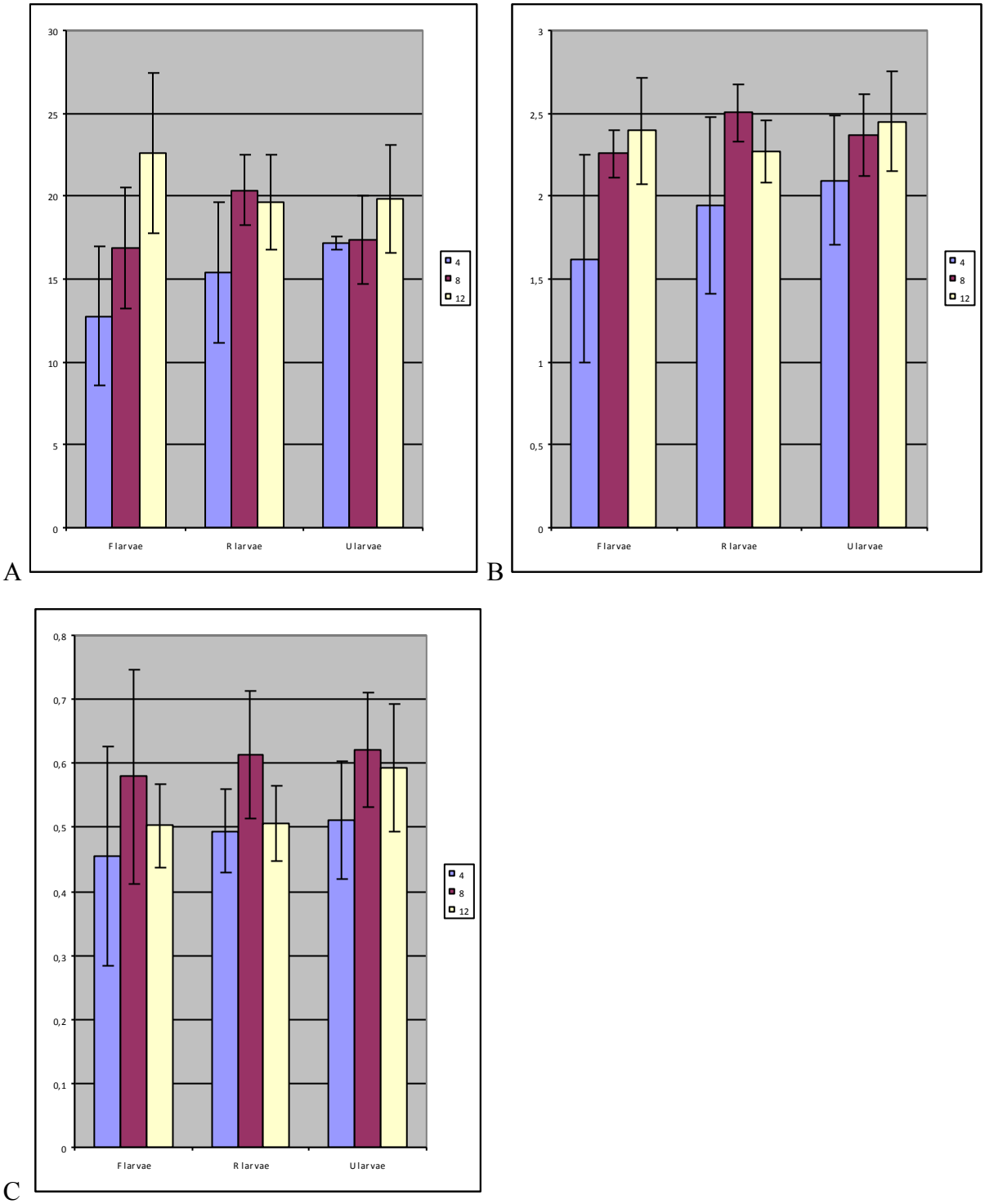
4.3.3 Richness, diversity and evenness in larval microbiota.

Band richness, Shannon`s diversity index and Pielue`s evenness was determined for all larval DGGE profiles (Figure 4.9; A, B and C). There were not any clear trends when comparing the results. To examine if there were significant differences between the groups at 4, 8 and 12 dph, a one-way ANOWA test was performed.

The one-way ANOVA test did not find any significant similarities when comparing the mean values for band richness among the FTS, RASUV and RAS. The differences were not great enough to exclude the possibility that the difference was due to random sampling variability ($p = 0,059, 0,062, 0,259$).

A one-way ANOVA test on Shannon`s diversity did not reveal any significant differences at 4 and 12 dph in the mean values among the treatment groups. The differences was not great enough to exclude the possibility that the difference was due to random sampling variability; there was not a statistically significant difference ($p = 0.207$ and 0.394, respectively).
On 8 dph, the one-way ANOVA test showed that the differences in the average Shannon's diversity among the treatment groups were greater than would be expected by chance; there was a statistical significant difference found between RAS and FTS ($p= 0.037$)

A one-way ANOVA test was used to investigate if there were significant differences between the mean values among the treatment groups. The differences was not great enough to exclude the possibility that this was due to random sampling variability any further more there was no statistically significant difference ($p = 0,802, 0,504, 0,501$).



Figur 4.9. A- Average band richness, B- Shannon diversity index and C- Pielou`s evenness index with standard deviation for larval DGGE profiles at 4, 8 and 12 dph. F= FTS, R=RAS and U=RASUV.

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4.4 Larval growth and survival

The mortality for the entire experiment is shown in Figure 4.12. The mortality increased at 3dph in FTS, and from 9 dph the mortality was high. At the end of the experiment the RAS and RASUV had a less larval mortality, compared to the FTS. The survival in RAS, RASUV and FTS were, 47.5%, 37.5% and 27.5%, respectively. No statistical analyses were done because of the low number of replicate samples. The results from the replicate experiment done in 2014 can be viewed in Figure 4.13. The survival in the RAS and FTS were and RASUV, 65.7 and 57.4 and 44.5%, respectively. In both experiments the RAS has the highest survival at the end of the experiment.

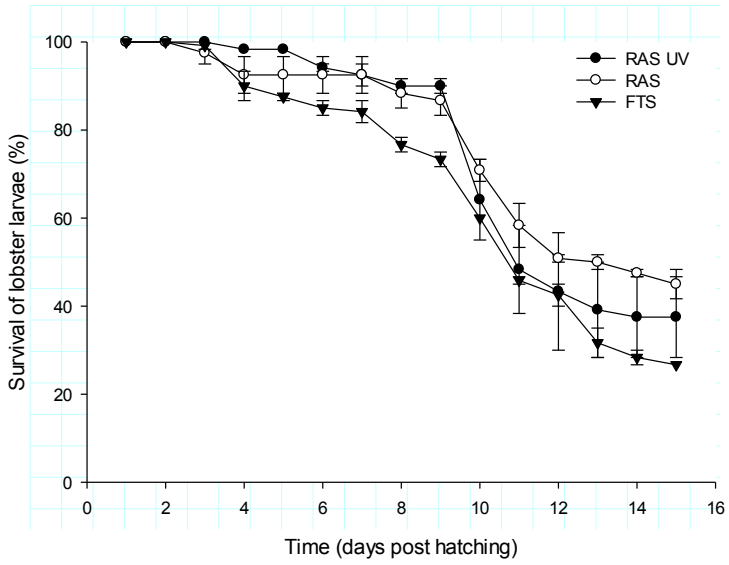

Figure 4.12. Relative larvae survival with standard deviation for RASUV, RAS and FTS.

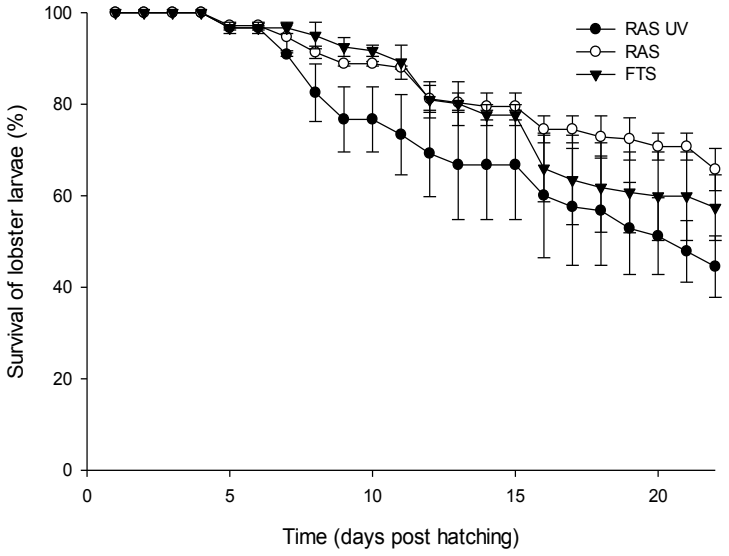

Figure 4.13. Relative larvae survival with standard deviation for RASUV, RAS and FTS from the replicate experiment in 2014.

4.5 Dry weight

Dry weight was measured two times during the experiment (Table 4.3); first when the larvae arrived from the hatchery at *stage I*, and a second time at the last day of the experiment using animals that had reached *stage IV*. No significant differences in growth between RAS and RASUV were found. No measurements were done for FTS due to high mortality of the larvae. Statistical analyses were not performed because of the similarity in dry weight between the recirculating systems.

	Stage I	Stage IV (mg/ind)	
	mg/ind	With UV	No UV
1	2,2	9,4	8,8
2	2	9,1	9,3
3	1,7	8,8	9,4
4	2	9,1	8,7
5	2,2	9,7	9,9
6	2,1	8,9	9,5
7	1,9	8,5	9,3
8	2,1	9,9	9,7
9	2,4	10	8,7
10	1,9	9,3	9
Avg	1,86	8,34	8,33

Table 4.3. Lobster larvae weight in mg/individual at experiment start and end.

5. Discussion

The present wild population of *H. gammarus* is over exploited, and there is a need to develop better methods to cultivate lobster for re-populating old habitats as well as to utilize new species in modern aquaculture. The recirculating system combined with new techniques for control and better water treatment methods creates interesting possibilities. The sum of factors that influences the microbiota in rearing water is complex. Rearing conditions like optimal feed, genetic manipulation, chemical and microbial composition in the rearing are all areas that need to be further investigated to ensure a reliable industry. Especially how new bacteria introduced in the rearing water influences the established microbial community is of great importance. There is also a need for a better understanding of how the microbial communities interact with the reared species. The focus area of this study was how the water treatment influenced the microbial communities in the rearing water and furthermore how it affected growth and survival of the lobster larvae. There are many unknown factors to this study, but the conditions were kept as identical as possible. The 3 different water systems had only two parallels each, which is limiting for quantitative analyses. There is still possible to say something about the trends in each respective system and between the rearing systems.

5.1 Rearing system

Evaporation from the water surfaces became a problem in the RAS used in this experiment, and it had to be adjusted for during the experiment by adding of freshwater to compensate for lost water. The physiochemical values in aquaculture systems should be recorded daily to ensure good rearing conditions.

There was a great loss of copepods escaping through the walls of the rearing chambers. This would represent an extra cost for the farmer. A solution would be to use bigger prey or smaller openings in the chamber walls. Smaller openings in the walls will most likely have a negative effect on particle removal from the cage. Using bigger prey to feed the larvae will be favourable. Since the larvae hunt each other, size up to the larvae`s own carapace length may be suitable. Further work need to be done on this area to optimize feed and minimize loss of feed.

Organic matter settling on the bottom of the rearing tank was also a problem that had to be dealt with. This may cause anoxic conditions, which may create habitat for anaerobe pathogens and production of H_2S gas. For the rearing systems used in this experiment, it was possible to use a tube and remove the particles manually. With bigger systems this could be solved with a mechanical device cleaning the bottom of the tank, or creating a current to

transport the particles away from the rearing tank. There was a 24 hours light regime in this experiment. The species H. gammarus are in general nocturnal as an adult, and the pelagic pre-metamorph larvae will possibly follow the migration pattern of zooplankton. 24-hour light may influence the feeding behaviour or create stress. This experiment was done in laboratory size and the result may be different if executed in an industrial large-scale farming (Johnsen. 2007, Sigstadstø, 2007).

5.2 Physiochemical water quality

While the FTS will have the stabile water quality from the ocean and a short retention time in the system, the RAS will re-use the water and change or use up, one or more essential components. As stated in the work of Rebello and Moreiro (1982), the buffer capacity of CO_2 in seawater depends mostly on the available concentration of magnesium. Furthermore this will influence the CO_2-$CaCO_3$ system and the pH buffer will be less efficient. For an animal with a calcium-based exoskeleton this may create stress and influence survival and growth due to extended energy loss if pH is outside the recommended values. Seawater will usually have a good buffer capacity, and pH is rarely a problem in FTS, regarding high or low pH. In a RAS the recirculating water may go through chemical changes and if the pH drops below pH 6 the nitrifying bacteria will not survive (Luccetti and Gray, 1988) It is important to bear in mind that the conclusion may differ if some chemical values are not recorded and implemented in the total evaluation of the results. In a longer experiment this will be of greater importance than in a short laboratory experiment like the one discussed here. Measurements of the rearing water showed that the level of ammonia (TAN) was significantly higher in the RAS compared to FTS. This was as expected since the water is only used once in FTS. The mortality in the RAS was lower than in the FTS, indicating that the elevated ammonia and nitrite levels did not have negative effect on the larvae. In this experiment there was a low biological pressure on the systems. A FTS will manage higher biomass by just increasing the water flow, while the RAS will have to be designed more specific for the biomass the farmer want to keep in the rearing tank (van Olst et al., 1980, Estrella, 2002, Wickins and Lee, 2002).

The experiment was repeated, but only the survival is compared in this thesis. In the other experiment, the mean temperature was 17^{o} C. A difference on around 3^{o}C caused to prolong the development of the larvae to reach *stage IV* with weeks. For the experiment in this thesis there was significant difference in temperature between the rearing systems (average1-2 ^{o} C). At the last day of the experiment none of the larvae in FTS had developed to stage IV. There

was a significant difference in salinity between the RAS and FTS. The measured values were higher than the recommended rearing conditions and this may have influenced the survival. RAS had a higher salinity compared to the FTS, but the RAS had better survival. This could mean that the RAS larvae might have performed even better because of the energy demanding process connected with osmoregulation.

5.3 Growth and survival

The larvae development was similar in both of the recycling systems. In the FTS the number of surviving larvae was not enough for measuring dry weight. The final stage for startfeeding of lobster larvae is at *stage IV*, when the larvae metamorphose into the shape of an adult. There were no larvae reaching *stage IV* in FTS. The temperature in this system was lower during the whole experiment, possible resulting in slower growth.

There was little mortality up to 3 dph (5%). This was around first moulting. The second clear increase in mortality was at 9 dph (10%) and also connected with moulting. This was the point where the systems started to clearly separate from each other when comparing the survival. There was a clear trend that the mortality increased when moulting occurred. This is a demanding process and the mortality can be a result of nutritional deficiency or not enough reserves as a result of energy loss from a hostile environment. In the RAS, which had the highest survival rate, more than half of the larvae died. In the replicate experiment, which is not yet published, there was less mortality among the larvae. The higher survival may be a result of better quality of the larvae received from the hatchery, or that the rearing temperature of 17 ^{o}C had a positive effect on the larvae. In both experiments the larvae in the RAS without UV filtration had the highest survival. In the first experiment the FTS showed the lowest survival and in the second experiment the RASUV had lowest survival.

All animals used for dry weight at the second time were all stage 4. Since arthropods grow by shedding of the old shell and expanding the new one there is not expected to be great differences in these results. In retro perspective it would have been more correct to take dry weight of all larvae or counted the amount of stage 4 to get a better picture of growth in the respective system.

5.4 Microbial communities in water

RAS without disinfection before the water enters the rearing tank seemed to have the most similarity in the microbiota when comparing intake- and rearing water. The MDS plot based on average Bray-Curtis similarities of the microbiota showed that RAS with out UV filter had more similarity between intake water and rearing water of RAS, compared to the RASUV and

FTS. This may be caused by less interference on the microbial community so it can establish stability in all parts of the system. This will most likely be beneficial for the larvae. The results also indicated that putting a UV filter right before the water enters the rearing tank may create instability in the microbial community and possible will be negative for survival of the larvae. This study corroborates the findings of Vadstein et al., 1993, Attramadal et al., 2012. This theory has not before been tested on lobster larvae.

The differences between intake water and rearing water were tested statistical with ANOSIM. Significant differences were observed in both RASUV and FTS. No significant differences were observed in between intake water and rearing water in RAS. This indicates that the RAS have similarity between intake water and rearing water when it re-enters the rearing tank, creating more stability. A limited number of samples from the intake water may have influenced the ANOSIM test, which makes it hard to conclude that other factors may have influences the results.

The ANOVA test on band richness on microbiota between different waters showed significantly higher richness in the rearing water from RAS compared to intake water and rearing water in RASUV and FTS. This indicates more maturation in the microbial community compared to the RASUV and FTS. A more matured microbiota is often more established and has a higher amount of K-strategic bacteria.

The disinfection may have affected the recolonization and influenced the development of the microbial community in the rearing water (Hess-Erga et al., 2008).

These results indicate that using simple methods like biofilter and particle removal to treat the water will increase the survival of larvae. To decrease the total bacterial amount using disinfection on the intake water will only create room for r-strategic bacteria.

The work of Bourne et al., 2006 showed that biofouling in a RAS became populated with strains of Vibrio sp. after day 21. An interesting subject to research would be to investigate if cold water RAS running over a long period of time can create pathogens in the biofilm covering surfaces. An interesting question will be to find out if the biofouling will be dominated with r-strategic bacteria, having altered their phenotype to ultramicrobacteria as a survival strategy.

5.5 Microbial communities associated with larvae

The ANOSIM test described in table 4.2 indicate that the microbiota connected with the larvae and rearing water becomes less similar over time when comparing the results for 4, 8 and 12 dph. As described in the paper of Gil-Turnes and Fenical from 1992, it is possible for

invertebrates to regulate the colonization of surfaces. Since the lobster larvae shed its shell with only days apart, the bacteria do not have a long time to colonize the surface, means that it will be important to have a stabile microbial community in the rearing water.
The one-way ANOSIM test on microbial similarities in larvae between the rearing waters show that there is changes in the microbiota over time in water, and furthermore this also seems to influence the microbiota associated with the larvae. The average Bray-Curtis similarities showed that from 4 to 8 dph, the similarity became less in RAS, RASUV or FTS. However, at 12 dph, the similarity between larval and rearing water microbiota became less in only the RAS.
The result of the ANOSIM test when we compared the microbial communities associated with the larvae in RAS and RASUV, revealed no significant difference at 4 and 8 dph. The test did find significant differences in the larval microbiota between RASUV and RAS at 12 dph. This may be a result of the UV-filter, creating differences in the microbial composition. The mortality of larvae was higher in the RASUV and FTS compared to RAS in this experiment and in the replicate experiment.
One explanation to these results is that the differences observed in the microbiota from the larvae are connected to the differences between the microbiota of the rearing water. This is important because it indicates that it is possible to influence the microbiota in the larvae with the water treatment. Greater variation between the samples in FTS inlet water over time was observed when compared with the RAS systems. A reason for this may be that the water in the fjord has some variations over time.

5.6 Future work

There is a lot of uncertainty connected to the work with creating optimal rearing conditions in aquatic rearing systems. To strengthen the knowledge of which factors influence the performance of marine larvae, a better understanding of the microbial composition in the rearing water should be obtained.
A good approch would be to find out which species of bacteria are dominating the microbial community in the water and which bacteria are beneficial to the larvae development. To analyse wild individuals, microbiota would be useful. The next step would be to implement these microbial communities in artificial rearing systems. The survival should not be the only area of focus. The bacteria may create immunological reactions, which may serve the larvae in a positive way in the later stages. It will also be of interest to investigate which other factors that may influence the growth and survival of the larvae.

5.7 Conclusion

- Growth and survival was affected by the water treatment. The microbial communities seemed to play a vital role in the survival, but temperature seemed to be of greater influence regarding growth of the larvae in this experiment.

- Different water treatments were found to create significant differences in the microbial communities in the rearing water. There were indications that these differences were closely related to the maturation time of the water and the treatment methods.

- This master thesis indicates that microbial communities in the rearing water have an influence on the microbiota associated with the larvae.

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Appendix 1. DNA isolation protocol

Experienced User Protocol

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.
    Note: A total of three loads for each sample processed are required.
16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Thank you for choosing the PowerSoil® DNA Isolation Kit.

Appendix 2: Recipies

50 x TEA

- 242g Tris-base
- 57.1 mL glacial acetic acid
- 100 mL EDTA (0.5 M, pH 8)
- Add dH_2O until total volume is 1000mL
- Autoclave

1% agarose gel with GELRED

- 4g agarose
- 400mL 1xTAE
- 20µL GELRED
- Heat solution to dissolve agarose

Deionized formamide

- Add 7.5 g DOWEX RESIN AG 501X8 in 200 mL formamide to deionize the solution
- Stir for 1 hour

0% denaturing acrylamide solution

8% acrylamide, 5.6 M urea, 32% formamide in 0.5x TAE (per 250 mL)

- 50 mL 40% acrylamide solution (Bio-Rad Laboratories
- 2.5 mL 50 x TAE
- 84 g urea
- 80 mL deionized formamide
- Add dH_2O until total volume is 250 mL
- Stir to dissolve urea
- Store the solution at 4^oC, not exposed to light
- Solution must be filtered before use

SYBR Gold staining solution

- 3µL SYBR Gold

- 600 µL 50 x TAE
- 30 mL dH_2O