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Temporal Development of the Gut Microbiota in European Lobster (*Homarus gammarus*) Juveniles Exposed to Two Different Water Treatment Systems

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Abstract

Aquaculture today exploits only a limited number of species for production and there is a need to utilize novel species. One such species that could contribute to meeting the demands of the market is the European lobster. However, efficient and profitable rearing has thus far not been achieved due to high mortality during larval and juvenile stages. One approach in seeking a solution to this problem is to examine the microbial communities in the lobster gastrointestinal tract, as these have proved important for nutrition, the immune system and resistance to disease in many different host species, especially during early life stages. Our understanding of the microbial communities in the gut of the lobster is, however, very limited. The purpose of this study is to provide an insight into the gut microbial communities of ½-, 1- and 2-year-old lobster juveniles by studying (1) temporal development, (2) the differential effects of water treatment systems by the application of a flow-through system (FTS) and a microbially matured system (MMS) and (3) the similarity between microbial communities in feed/water and the lobster gut. To study the gut microbiota of lobster juveniles, DNA was isolated from faecal samples and PCR was applied to amplify the variable 3 region of the bacterial 16S rRNA gene. For the microbial community analysis, denaturing gradient gel electrophoresis (DGGE) was used, and community dynamics were investigated by means of statistical analysis.

The gut microbial community composition of ½-year-old lobster juveniles was significantly different from that of 1- and 2-year-old juveniles. However, no differences in the composition of the gut microbiota were found when comparing 1- and 2-year-olds lobsters. Over a time period of 3 months no temporal development was found in the microbial community dynamics in the gut of 1- and 2-year-old lobster juveniles, indicating a stable gut microbiota at these life stages. When the effect of water treatment systems on the gut microbiota was examined, significant differences in composition were found between FTS and MMS individuals 6 weeks post introduction to the water treatment systems. The bacterial community composition of both feed and water was found to be highly dissimilar to the composition of the gut microbiota in both 1- and 2-year-old lobster juveniles, indicating strong selection inside the host. In conclusion, these results appear to indicate that a stable gut microbiota, which is highly dissimilar to water/feed microbiota, has been developed by the time lobsters have reached one year of age. This suggests that host selection structures the gut microbiota. However, the results further indicate that water treatment systems have an effect on microbial community composition in the gut of juvenile lobsters.

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1 Introduction

Since the 1600s, the European lobster (*Homarus gammarus*) has been caught in Norway, and as with many other species having pelagic larvae the catch quantity has varied over the years. From the end of the 1800s to 1930 the volume of the catches ranged between 300 and 600 tons per year, reaching a maximum of 1313 tons in 1932. However, after the 1960s, catches declined steadily despite the regulation of lobster fishing by law. In 1993 only 30 tons of caught lobster were registered, indicating that the population of European lobster in Norwegian waters has declined significantly (Van der Meeren et al., 1995). This decline has led to an interest in rearing the European lobster in order to meet the needs of the market. Compared to the American lobster (*Homarus americanus*), the European lobster is considered more flavourful and is consequently more expensive. Despite the progress of *H. americanus* into the European market, there is still a high demand for increased volumes of *H. gammarus* (Lee and Wickins, 2002).

Gut microbiota has proven important for health and development in many species, and it is likely that it affects the lobster similarly. In order, therefore, to develop optimal systems for rearing lobsters, an understanding of microbial community dynamics in the lobster gut is needed. It is also necessary to investigate how microbes in the environment (feed/water etc.) affect the gut microbiota, and furthermore, whether different water treatment systems affect the composition of the GI microbiota in the lobster. Stabilization of water microbiota through water treatment has been shown to have positive effects on growth and survival in marine fish larvae (Skjermo and Vadstein, 1999), but it is not known how water treatment affects growth, survival and the gut microbiota of the lobster. An increased understanding in these areas could contribute to the efficient rearing of the European lobster for mass production.

1.1 Gastrointestinal microbial communities and their influence on health and development

Bacteria have been widely studied due to the pathogenic effects many of the bacterial strains possess, but they are also crucial for the survival of higher organisms. Bacteria colonize both the outer and inner body surfaces of humans and animals, where they act in symbiosis with the host. An example of this is bacteria living in the gut of animals, where they help break down nutrients, enabling the host colon to absorb and transfer them into the bloodstream.

These nutrients would otherwise be excreted, due to the host lacking the required enzymes for their degradation (Ray et al., 2012). The composition of microbial communities in the gut not only affects the metabolism, but also the development of the adaptive and innate immune systems and the digestive tract (Kanther and Rawls, 2010, Rungrassamee et al., 2013).

The role that bacteria play in the development of the immune system in higher organisms is complex. Studies on axenic mice in comparison to conventional mice have revealed the importance of bacteria to the host. It was found that axenic individuals were far more susceptible to infections by pathogenic species. Reduced activity of macrophages in the intestine, low counts of lymphocytes, impaired T-cell function, reduced cytokine production and levels of immunoglobulin were contributing factors to this susceptibility. In addition, muscle wall thickness and vascularity were reduced in axenic individuals, which further weakens the immune system (Fraune and Bosch, 2010). The commensal microbiota of the intestines also contribute to the defense against pathogenic species through competition for adhesion sites and nutrients, which prevents the colonization of pathogens. Several by-products produced in bacterial metabolism also display anti-microbial effects, which further reduces the accumulation of pathogenic species. Disturbances in the normal microbiota can enable the growth of opportunistic and potentially pathogenic bacterial members in the microbial community, and subsequently cause disease in the host. The host is also more susceptible to ingested pathogens becoming established in the intestinal tract whenever the balance of the normal microbial communities is disturbed (Sekirov and Finlay, 2009).

In the case of gut development, microbes could possibly be seen as an integral part of the host body. In the case of zebra fish, for example, the presence of bacterial signals is required for the completion of gut development. Studies of these fish have shown that specific genes, influencing gut development, are affected by the present microbiota, and that the absence of these causes the arrest of several aspects of differentiation. Hence, the gut was not fully developed. However, when bacteria were introduced at later stages, the effects could be reversed (Bates et al., 2006). These findings support the importance of bacterial communication with host processes, and point strongly to the critical role of bacteria in the development of the gastrointestinal tract. Overall, the literature indicates that the presence of microbial communities is crucial both for normal ontogeny and the continued maintenance of the immune and digestive systems.

1.2 Establishment and development of microbial communities in marine species

Unlike terrestrial organisms, aquatic organisms are in constant contact with the surrounding seawater and the diversity of bacteria it contains. After hatching, marine larvae ingest bacteria present in the water both by drinking and feeding (Hansen and Olafsen, 1999). Subsequently, the gastrointestinal tract will become colonized, and commensal or mutualistic relations are formed between bacteria and the host (Kanther and Rawls, 2010). The epidermis mucus layer in the gut of marine species provides adhesion sites for bacteria, and is important in the establishment of a healthy gut microbiota. This process of adhesion and colonization is complex, and is influenced by a range of factors including environmental conditions, properties of the epithelial cell surface of the host, the nature of bacterial species, adhesion chemo attractants and adhesion inhibitors (Hansen and Olafsen, 1999). While the initial colonization of the gastrointestinal tract in marine species is thought to be mainly affected by bacteria present in the water (Hansen and Olafsen, 1999, Reitan et al., 1998, Sun et al., 2013, Bakke et al., 2013), but studies have shown that diet also has a significant influence on the composition of the gut microbiota both in marine larvae and adults (Ingerslev et al., 2014, Hansen and Olafsen, 1999, Meziti et al., 2010). Little is known, however, of how bacteria present in feed and water affect the gut microbiota in the colonization process.

Research has also provided indications that the species diversity of the microbial communities in the gut increases with age, and that younger individuals display a less diverse gut microbiota compared to juveniles and adults (Bakke et al., 2015, Bakke et al., 2013, Yan et al., 2012, Eddy and Jones, 2002). Furthermore, the microbiota of the gut in larvae is assumed to be transient, while the microbiota of individuals at later life stages is relatively more stable (Eddy and Jones, 2002, Olafsen, 2001). In humans, the gastrointestinal microbiota of neonates and infants display a low degree of stability and differ in this respect from adult microbiota. However, during the first two to five years the microbial communities develop and become similar to the microbiota of adults (Robinson et al., 2010, Rodriguez et al., 2015). Very little temporal development occurs from infancy to adulthood in humans, but it is not known whether this is also the case for marine species.

In aquaculture, negative interactions between marine larvae and bacteria have been shown to cause fluctuations in survival rate and growth (Skjermo and Vadstein, 1999). Hence, an increased understanding of microbial community establishment, development and

composition could help improve the production of marine species. To the best knowledge of the author, there is currently no reported research into the initial colonization and development of the microbiota in the gut of lobsters, and more work needs to be done to enhance our understanding of this process.

1.3 Biology and cultivation of the European lobster (Homarus gammarus)

The European lobster (*Homarus gammarus*) is a clawed lobster, which belongs to the genus *Homarus*, family *Nephropidae* and order *Decapoda* (Holthuis, 1991). It can be found in most areas of the Mediterranean Sea, the Atlantic Ocean, and in some parts of the Black Sea (Agnalt et al., 2009). The clawed lobster has a simple and short larval period, which consists of four stages. Growth is accompanied by moulting, which involves growth of body mass inside the shell and shedding when the shell becomes too small. Furthermore, larvae at stage I-III are pelagic, and become benthic after the fourth moulting (stage IV). At this point, the appearance of the lobster is similar to adult lobsters and it is referred to as a juvenile (Cobb and Phillips, 2012). Larvae and juveniles are vulnerable to predation, and juveniles are thought to seek shelter in rock crevices, cobble or burrows on the seabed as soon as they become benthic (Wahle and Steneck, 1992).

At the larval stages, the lobster usually feeds on planktonic species available in the water column and are, along with juveniles and adults, omnivorous predators (Cobb and Wahle, 1994, Cooper and Uzmann, 1980). The opportunistic and scavenging behaviour of the clawed lobster causes it to consume a wide variety of prey, including its own species (Cooper and Uzmann, 1980). It is enabled to consume this wide range of prey due to its feeding mechanism and the structure of its mouth. A set of small claws named pereiopods is used to grab and lead the prey to the mouthparts. Multiple specialized appendages in close proximity to the mouth are then used to secure the food, and pieces are torn from the main mass and ingested (Barker and Gibson, 1977). The digestive system is complex but can be broadly divided into the foregut, midgut and hindgut. The foregut includes the oesophagus and a large fraction of the stomach, which harbours a gastric mill. The hard masticating appendages of the gastric mill grind the consumed food, allowing the lobster to digest a range of prey. Enzyme secretion mainly occurs in the midgut, which is shaped like a straight tube leading to the hindgut and anus. In newly hatched larvae, the digestive system is undeveloped and

merely a straight tube. However, it quickly develops and is fully functional and segmented post-metamorphosis (Barker and Gibson, 1977, Ceccaldi, 1989).

H. *gammarus* is considered as an attractive candidate for cultivation, but the requirement of individual confinement due to cannibalism reduces the economic viability. Several approaches to the intensive rearing of the lobster have been applied, including intensive and a combination of intensive and extensive rearing (Kristiansen et al., 2004). In intensive rearing, the approach differs for larval stages compared to juveniles and adults. Larvae are commonly reared communally in large cylindrical tanks with cone shaped bottoms, which are heavily aerated or applied with water streams to distribute the larvae and to avoid cannibalism. When the larvae have reached the 4^{th} stage, they are transferred to individual compartments, which are often placed side by side in rearing raceways (Burton, 2003, Kristiansen et al., 2004). The lobsters may then be raised to market size in this fashion, or grown to an appropriate size for sea ranching (extensive system). In sea ranching, the lobsters are labelled and released in an area with suitable seabed structures for shelter, and is subsequently collected when they have grown to the desired size (Lee and Wickins, 2002). The method of sea ranching is possible because the lobster is very territorial and does not travel over long distances (Kristiansen et al., 2004).

A major bottleneck in the intensive mass production of the European lobster is the previously mentioned high mortality rate during larval and juvenile stages, which is caused by cannibalism and other unknown factors. However, recirculation systems for larval rearing have shown promise with increased growth rate and survival (Øien, 2014). Furthermore, the requirement of individual confinement reduces the economic viability of intensive lobster rearing due to increased needs in terms of space and labour (Kristiansen et al., 2004). Extensive rearing avoids this issue, but young lobsters are subject to predation by a wide variety of predators, and large numbers of individuals must be released to ensure satisfactory production. An increased understanding on the effect of water treatment systems on lobster performance, especially at early life stages, could enable more efficient and cost effective rearing of both larvae, juveniles and adults for intensive and extensive production.

When rearing lobsters, water quality is of crucial importance for optimal growth and survival (importance of water quality is elaborated in 1.4). Under natural conditions, lobsters are subject to temperatures varying from 1-25°C, but the optimal temperature is in the range of

18-22°C. If the temperature drops below 5°C, the induction of moulting is blocked. The lobster will not moult until an increase in temperature occurs. It is lethal to the lobster if temperatures drop below 1 or go above 31°C (Kristiansen et al., 2004, Van Olst et al., 1980). Lobsters can tolerate levels of dissolved oxygen at 0.2 mg/L in 5°C seawater, but the natural range is 4.0-7.4, with the optimal level being 6.4 mg/L. Water which is supersaturated with oxygen can cause severe damage due to the formation of gas bubbles in the hemolymph and subsequent restriction of blood flow. With respect to pH levels, in natural environments the lobster is subjected to a pH varying between 7.8-8.2, where 8.0 is the optimal level. If the pH drops below 5.0 or above 9.0 it is lethal to the lobster (Van Olst et al., 1980). The optimal level of ammonia (NH_3) is below 0.14 mg/L, and in nature it usually ranges from 0-0.3 mg/L. Levels above 1.4 mg/L will be lethal (Kristiansen et al., 2004, Van Olst et al., 1980)

1.4 Importance of water quality in the rearing of marine species

In their natural habitat, marine species are able to relocate to other areas whenever the water quality is unfavourable to the organism. This is clearly not possible for reared marine species, and it is therefore important to adjust the water quality to a point as close as possible to the optimum for the species. Many land-based rearing facilities are situated in close proximity to the ocean and pump seawater in to the water systems. However, this inlet seawater may not provide the optimal conditions for the reared organism, and must therefore often be treated before it is added to the rearing tanks. Water quality parameters interact with and influence each other, and it is therefore important to maintain balance in the system (Timmons and Ebeling, 2013). The treatment steps, which are often applied, include degassing and aeration, disinfection, particle removal, regulation of temperature and microbial maturation.

The temperature of the culture water has a substantial impact on many aspects of overall water quality and on the reared organisms. It affects the oxygen concentration, pH, toxicity of nitrogen compounds and CO_2, and many other factors. The metabolism of the organism increases with increasing temperature, and similarly decreases with decreasing temperature. As a consequence the growth rate is also affected, increasing with warmer water. The optimal temperature for growth depends on which organism is reared, and is adjusted by heating or cooling the water before it enters the rearing tanks (Van Olst et al., 1980, Timmons and Ebeling, 2013).

The amount of consumed oxygen varies with the level of respiration by fish, bacteria and other small organisms such as live feed. If the temperature of the water increases, the oxygen concentration will decrease due to increased organism metabolism and lower oxygen diffusion through the water surface from the air. Also, if the reared organisms are stressed they will consume more oxygen. It is common to aerate or oxygenate the water if oxygen levels are low (Timmons and Ebeling, 2013).

In seawater the pH is usually 7.5-8.4 and is relatively stable. In aquaculture rearing systems however, it is necessary to keep the pH stable at a level that is beneficial to the reared organism. Metabolism of both the reared organism and bacteria cause the accumulation of CO_2 in the culture water due to respiration, and the levels increase as the organism grows. In systems where a biofilter is applied, the pH must be kept at a level that is beneficial to the nitrifying bacteria in the filter (7.5-8.0). Below pH 6, these bacteria die. Other factors also affect the pH, such as formation of nitric acid by ammonia nitrification (Timmons and Ebeling, 2013). If the pH is too high, substances such as sodium bicarbonate can be added to decrease it (Loyless and Malone, 1997).

Depending on the reared organism, various levels of nitrogen compounds accumulate in the rearing tanks. The reared organism itself excretes nitrogenous waste through gill diffusion and cation exchange, and through faeces and urine. In addition, excess feed and dead organisms are degenerated by bacteria, also adding nitrogen compounds to the culture water. Two forms of ammonia exist, unionized (NH_3) and ionized (NH_4^+), NH_3 being the form most toxic to the reared organism (Meade, 1985, Timmons and Ebeling, 2013). To remove ammonia from the water, a biofilter is often applied to the system. Nitrifying bacteria within the biofilter convert ammonia to nitrite (NO_2^-) and then nitrate (NO_3^-) by oxidation. Nitrite is the intermediate product in the nitrification process and is toxic to reared animals because it negatively affects the oxygen carrying capacity of the haemoglobin. It is therefore important that the nitrite to nitrate conversion in the biofilter is efficient, preventing the build-up of nitrite. Nitrate is the final product of the nitrification of ammonia and is the least toxic nitrogen compound. To limit the toxicity of nitrogenous waste products, it is important to regulate levels of pH as toxicity increases with increasing levels of pH (Timmons and Ebeling, 2013).

The microbial conditions for aquatic species are very different in the wild compared to those pertaining in aquaculture, as the water in rearing facilities often will contain high levels of

bacteria, a higher concentration of opportunists and pathogens, and have a different bacterial composition in general. The microbial stability of rearing water is thought to have a significant effect on the reared species and studies have shown increased performance of marine fry reared in systems with microbially stable rearing water (Skjermo et al., 1997, Attramadal et al., 2012, Salvesen et al., 1999). The *r*/*K*-selection theory (MacArthur and Wilson, 1967, Andrews and Harris, 1986) is thought to be the basis of these observations (Skjermo and Vadstein, 1999). Rearing tanks often contain high densities of organic matter from feed and the reared organisms, hence microbial carrying capacity in the tanks is high. This type of environment favours the growth of fast-growing, opportunistic, *r*-strategic bacteria, including pathogens. It is thought that a high level of opportunistic *r*-strategists cause more negative larvae-microbe interactions, and is hence unfavourable (Skjermo and Vadstein, 1999). *K*-strategic bacteria grow more slowly, and occupy small niches in a crowded environment, resulting in a more stable water microbiota (Attramadal et al., 2012). The development of a community of *K*-selected specialists results in the depression of opportunistic bacteria as resources become limited, and it is thought that this kind of environment has positive effects for the reared organism, especially during start-feeding (Attramadal et al., 2012, Attramadal et al., 2014). The selective promotion of *K*-strategic bacteria in water treatment is defined as microbial maturation of water (Skjermo and Vadstein, 1999). Microbial control and stability of rearing water in aquaculture systems have been receiving an increasing amount of attention but more studies are required to determine the importance of microbial stability and how the bacterial communities in the rearing water affect the reared species. Several approaches to water treatment systems are found in land-based aquaculture today, but systems that recycle the water are becoming more frequently used.

1.5 Water treatment in aquaculture systems

Today there are two common systems that are used to adjust water quality in rearing tanks: Flow-through systems and recirculating systems. In flow-through systems (FTS) the inlet water is treated, while in recirculating systems (RAS) the same water is recycled and restored (Blancheton, 2000).

In a FTS a continuous flow of inlet water is required and this must be treated before it is added to the rearing tanks. It is common to disinfect the water in order to remove potentially

pathogenic bacteria, to regulate the temperature, to remove particles and to aerate/degas. The stages in this kind of treatment are adjusted to the requirements of the cultured organism. The benefits of using a FTS are that the initial costs are relatively low and it is not as complex as a RAS. Another benefit is that a high level of ammonia is usually not a problem as the water is exchanged before it can accumulate to toxic levels (Blancheton, 2000, Lekang, 2008). There are, however, some disadvantages in using a FTS: it requires more energy in order to maintain the right temperature, and the volume of water needed is large. Another major concern is the dependence on a continuous water source, which results in vulnerability to unstable water quality. Even though the initial costs of building a FTS are low, they may be high over the longer term due to the energy required to treat the water.

In a RAS system, culture water from the rearing tanks is continuously recycled and treated before entering the tanks (Summerfelt, 1996). The degree of water reuse can vary but often only a small percentage of new water is added per day. Water coming out of the rearing tanks contains waste products and can be oxygen deficient due to the metabolism of the organisms. It is therefore often necessary to remove the waste to avoid build-up and to add oxygen. Often this includes the removal of dissolved particles, bacteria, carbon dioxide, nitrite and ammonia. Biofilters are commonly used to remove excess ammonia from the water before it is added to the rearing tanks (Timmons and Ebeling, 2013, Lekang, 2008).

Compared to the FTS, a RAS has some important advantages. The volume of inlet water required is low so a good water source does not dictate the location of the system. This is beneficial in areas where water resources are limited. The continuous reuse of culture water lowers the costs of temperature regulation, especially in areas with cold winters. However, one of the most important benefits of RAS is the increased level of control of water quality and treatment and a higher degree of microbial control compared to FTS (Attramadal et al., 2012). The high retention time of water in RAS, in addition to the use of a biofilter, favours a stable and *K*-selected water microbiota. Finally, compared to a FTS the RAS is more complex, and hence the initial costs are higher but in many cases the RAS will be less expensive in the long run.

A modified version of the FTS system is the microbially matured system (MMS), which is a strategy for achieving a more stable water environment in aquaculture systems. The difference lies in a biofilter and increased retention time of water before entry to the rearing

tanks, which is also the case in RAS. The purpose of the biofilter is mainly conversion of ammonia to nitrate, but it also involves some other important benefits regarding microbial control. In FTS systems without a biofilter the retention time of water is relatively short, which favours the growth of *r*-strategists in the rearing tanks (Attramadal et al., 2012). In the MMS the water retention time is increased, which will allow the development of a community of *K*-selected specialists. The MMS hence provide a more diverse and stable water microbiota, close to carrying capacity, compared to the FTS (Attramadal et al., 2012). As the RAS require high investment costs, the MMS can be applied as an approach for achieving increased stability of rearing water as it provides some of the same benefits connected to RAS.

Regarding the European lobster, few studies have been conducted on the effect of water treatments systems on lobster performance. One masters thesis study of the effect of RAS on European lobster larvae showed increased survival rate in RAS compared to FTS (Øien, 2014), but no studies have investigated the effect of water treatment systems and water microbiota on the composition of the gut microbiota of the lobster. This study will thus be the first to investigate this relationship.

1.6 Methods for microbial community characterization

Classical approaches to investigating the composition of microbial communities have previously been based on microscopy and culture-dependent techniques, and have led to extensive amounts of information on microbial communities in the environment (Gilbride et al., 2006). However, these conventional methods do not provide an accurate representation of the composition and diversity of microbial communities in an ecosystem. Under the microscope, only a small fraction of bacteria can be distinguished from one another, making microscopy unsuited for the characterization of a bacterial population (Gilbride et al., 2006). Cultivation techniques provide more information, but are accompanied by several biases. Depending on the culture media, the growth of some groups of microorganisms is favoured over others, and some species are more easily cultivated. This is especially true for fast-growing R-strategists. Hence, the diversity, composition and abundance of species in a sample is very often not reflected when applying culture-dependent methods (Sanz and Köchling, 2007). The development of culture-independent methods for microbial community

characterization has, however, led to an enormous increase in our knowledge of composition, dynamics and function in microbial ecology (Sanz and Köchling, 2007).
Revolutionary progress towards an increased understanding of microbial communities was made with the application of ribosomal RNA and DNA isolation and amplification by PCR (Wintzingerode et al., 1997). The 16S rRNA gene is frequently applied for taxonomic analysis, as it has been conserved in evolution and is universal for all bacteria but contains highly variable regions that differ between bacterial species (Nikolaki and Tsiamis, 2013). The design of primers for the conserved regions of the 16S rDNA has enabled the amplification of the gene in all bacterial species present in a sample, allowing subsequent analysis and comparisons of microbial communities (Malik et al., 2008).

Denaturing gradient gel electrophoresis (DGGE) is a method that has been widely applied for microbial community profiling by separating rDNA amplicons according to base pair composition in a polyacrylamide gel with a DNA denaturing gradient (Malik et al., 2008, Muyzer and Smalla, 1998). The number a bands in the resulting gel reflects the number of dominant species in the applied samples (Sanz and Köchling, 2007), which provides an indication of diversity. Furthermore, the intensity of individual bands allows the comparison of abundance of the different species present in the sample. DGGE is chosen when highly accurate taxonomical information is not required but the method still provides medium resolution phylogenetic determination of dominate species in a community (Sanz and Köchling, 2007, Malik et al., 2008). There are, however, limitations to DGGE. As some bacteria possess multiple copies of the rRNA gene, one strain may display several bands on the gel. This may cause an over-estimation of the number of dominant species in a community (Muyzer and Smalla, 1998). High band intensity may also represent several copies of the gene, but this may be mistakenly perceived as a high abundance of the particular species (Malik et al., 2008). Another problem is that sequences from different species may denature at the same location in the gel, which can be interpreted as only one band. However, when the aim is to monitor changes in microbial community structure that are temporal or spatial, DGGE is an appropriate, relatively inexpensive, fast and simple investigative tool (Malik et al., 2008, Gilbride et al., 2006).

The DGGE approach to investigating microbial communities is becoming less commonly applied as next generation sequencing (NGS) techniques can analyse more DNA sequences per time unit (high-throughput) and provide high-resolution phylogenetic information

(Nikolaki and Tsiamis, 2013). These NGS techniques are also becoming less expensive, more reliable and are relatively fast but they produce large quantities of data that must be appropriately analysed (Di Bella et al., 2013). In microbial ecology, the common strategy has been to sequence 16S rDNA amplicons, but metatranscriptome analysis is being increasingly applied for studies of microbial communities (Gilbert and Hughes, 2011). NGS techniques such as Pyrosequencing and Illumina sequencing are two different approaches to sequencing bacterial DNA for microbial community analysis, which both apply the sequencing-by-synthesis principle. The pyrosequencing platform Roche 454 was the first commercial NGS platform and applies emulsion PCR (emPCR), whereby single template molecules are attached to beads and amplified. The beads are then transferred to a picolitre plate, where each well holds one bead. Pyrosequencing reagents flow across the plate, sequencing the template molecules in a parallel fashion. The method is based on the release of pyrophosphate groups on nucleotide incorporation for termination, which produces detectable light that allows the machine to identify the incorporated base (Nikolaki and Tsiamis, 2013, Di Bella et al., 2013). Illumina was the second commercial NGS platform that was developed. The method captures single template molecules on a glass slide and amplifies them to small clusters by bridge PCR. The clusters are subsequently sequenced by an approach similar to Sanger sequencing but termination is based on fluorescent dye-labelled nucleotides. Compared to 454 sequencing, Illumina provides higher throughput, higher accuracy and lower cost, and is now on the verge of replacing the 454 sequencing technique in microbial ecology (Nikolaki and Tsiamis, 2013).

1.7 Hypothesis and objective of the study

The hypothesis of this master's thesis study is that the faecal microbiota of European lobster juveniles is affected by age and water treatment systems. More specifically:

- The microbial community composition of the lobster juvenile gut changes over time, and is different in ½, 1 and 2 year old lobster juveniles.

- Water treatment systems affect the composition of lobster gut microbial communities.

- Microbial community composition in rearing water and feed are different from the community composition in the gut of lobster juveniles.

The main objectives of this study are to investigate the microbial community dynamics in the gut of European lobster juveniles by a PCR/DGGE strategy, more specifically to:

- Investigate whether there are differences in gut microbiota between ½-, 1- and 2-year-old lobster juveniles.

- Study the gut microbiota dynamics of 1- and 2-year-old lobster juveniles over a two-month time period in order to reveal potential temporal changes.

- Investigate whether water treatment affects the microbial community structure in the lobster gut microbiota by the application of a flow-through system and a microbially matured system.

- Compare the microbiota of the lobster juvenile gut to the microbiota of feed and rearing water.

2 Materials and methods

2.1 Ethical statement

The lobsters used for this experiment were treated in such a fashion as to reduce stress and discomfort as demanded by the Norwegian animal welfare law. Handling time was kept to the minimum required to conduct the samplings and sick or dying individuals were euthanized by humane methods to avoid suffering and pain.

2.2 Experimental design

2.2.1 Experiment 1 (1/2-year-old lobsters)

Fourteen European lobster juveniles originating from the lobster hatchery Norsk Hummer at Tjeldbergodden in Møre and Romsdal were cultivated in a FTS raceway at SINTEF Sealab in Trondheim. At day 1 of the experiment (07.11.13) the juveniles were 4 months of age and the experiment was run for 3,5 months (end date 20.02.14). Each individual was placed in separate 7,5 x 4 cm compartments in the raceway to avoid cannibalistic behaviour. The structure of the compartments had multiple holes, allowing water to flow freely through them and preventing oxygen deficiency and build-up of organic matter.

2.2.2 Experiment 2 (1- and 2-year-old lobsters)

Forty-four European lobster juveniles were transported from Norsk Hummer at Tjeldbergodden to SINTEF Sealab in Trondheim, where the experiment was carried out. The lobsters were placed in two separate raceways with FTS (R1 and R2), where R1 contained twenty-two 1-year olds (ID1-22 group 1y) and R2 contained twenty-two 2-year olds (ID1-22 group 2y). Plastic installations were applied to create compartments of 17 x 17 cm for each lobster to avoid cannibalistic behaviour. To allow water to flow through the compartments, a structure with multiple openings for each compartment was chosen. It was also ensured that the walls of the structure were high enough (20 cm) to avoid the lobsters of climbing over to neighbouring compartments.

The total number of days for the experiment was 90 (12.02.14 – 12.05.14). After the lobsters had been in the FTS for 50 days, half of the individuals from each age group were switched

between the two raceways and raceway 2 was changed into a MMS. Of the 1 year-olds, ID1-10 was moved to the MMS, while ID11-22 remained in FTS. Of the 2 year-olds, ID1-11 was moved to raceway 1 (FTS), and ID12-22 remained in raceway 2 (MMS) (Fig 2.1).

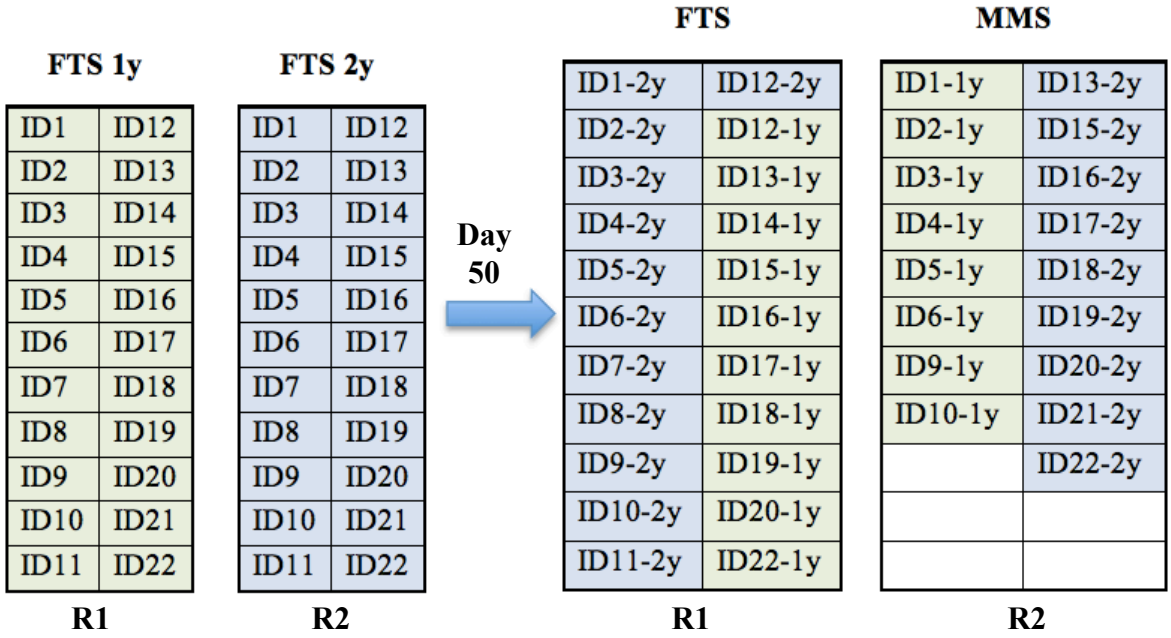


Figure 2.1: Schematic overview of the distribution of 1- and 2-year-old lobster juveniles in two rearing raceways (R1 and R2) before and after a change in water treatment system. At experiment start up, all 1 year olds (1y) were reared in R1, and all 2-year-olds (2y) were reared in R2, where both systems were FTS. After 50 days, R2 was transformed to a MMS and half of the individuals from each age group were switched between the raceways. Diseased individuals are omitted of the illustration.

2.3 Water treatment systems

For general rearing of lobsters, and for investigations on the effects of water treatment system on gut microbiota in lobster juveniles, FTS and MMS were applied. As RAS involves high costs and is more laborious, MMS in combination with flow-through was applied instead as an approach to obtain stability and microbial control.

Seawater from the fjord of Trondheim was pumped into the facility at 90 meters depth and approximately 700-800 metres out from the shoreline. A sand filter was applied to filter out particles present in the inlet water. In the FTS, the filtered water entered the raceways directly beyond this point. In the MMS, however, the sand filtered water entered a biofilter with long retention time to allow the maturation and stabilization of microbial communities in the water. The biofilter contained corrugated plastic sheets to increase the surface for colonization

by bacteria. The matured water subsequently entered the rearing raceway. Outlet water from both systems was returned to the ocean (Fig. 2.2).

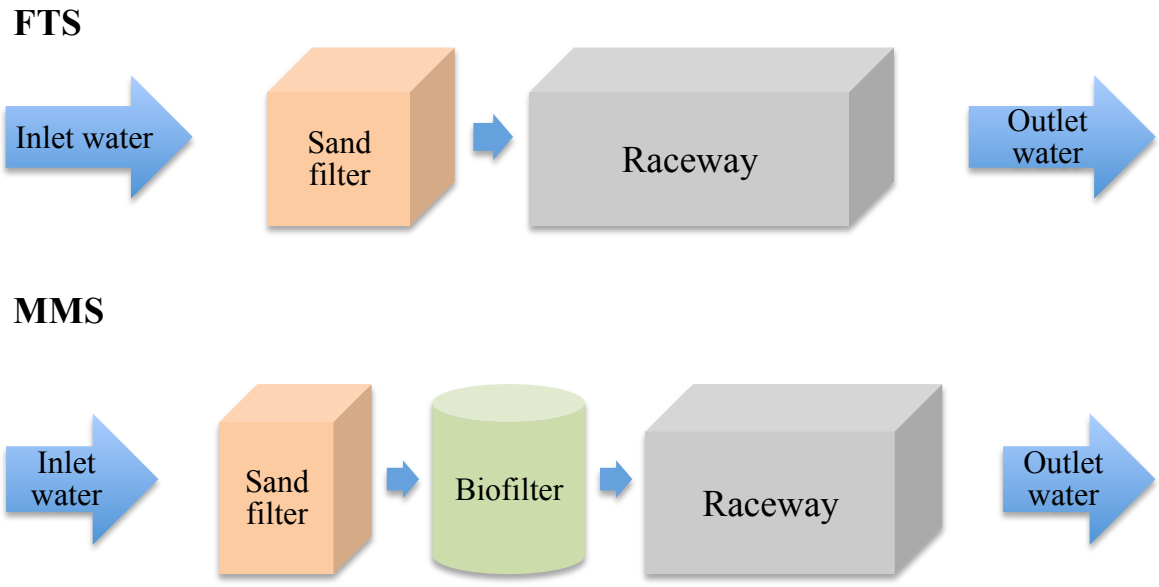


Figure 2.2: Illustration of the flow-through system (FTS) and microbially matured system (MMS) in rearing of European lobster juveniles. Both systems applied a sand filter for seawater filtration, while the MMS had an additional biofilter for microbial maturation of the water upon entry to the rearing raceway.

2.4 Rearing of lobster juveniles

Parameters of water quality were measured by monitoring temperature, dissolved oxygen and salinity to ensure stability. A multiparameter (YSI) was applied to measure temperature and oxygen, while a refractometer was used to measure salinity. The temperature of the rearing water was kept at 14 °C, the dissolved oxygen at 95 – 100 %, and the salinity at 3.5 %. Every two days, ½-year-old lobster juveniles were fed a commercial artemia mixture. In total, 5 grams of artemia was rinsed in fresh water and distributed to the 14 chambers. The 1 and 2-year-old lobsters were fed one frozen shrimp (*Pandalus borealis*) each, which was rinsed in fresh water before distribution. The amount of shrimp added was based on observations that the lobsters took approximately two days to consume one shrimp each. It was not desired to add more feed as this could cause a build up of bacteria and organic matter. The raceways were cleaned regularly using a suction hose and a brush to reduce build-up of organic matter and growth of bacterial biofilm on the surfaces. To euthanize sickly or dying individuals,

liquid nitrogen was applied. The individual was submerged into a container of liquid nitrogen, which resulted in a quick and painless death. The lobsters were kept in a dark environment to reduce stress, and light was only turned on for the minimum amount of time needed to perform necessary tasks. To further reduce the effects of stress, a short, hollow plastic tube was added to each chamber to allow the lobsters to hide.

2.5 Sampling

2.5.1 Experiment 1

Samples of faeces, water and feed were collected over a time period of 3.5 months. In total, 7 samplings were carried out, approximately every 14 days (an overview of the sampling dates is provided in Appendix 4). Sampling procedures are described in 2.5.3.

2.5.2 Experiment 2

Samples were taken approximately every 14 days (an overview of the sampling dates is provided in Appendix 4). After the change in water system, no samplings were carried out for three weeks to allow the lobsters to adapt to the new water system. Before the change of water treatment at experimental day 50, 6 samplings were performed for 1 year-olds while 5 samplings were performed for 2 year-olds. After the change of water treatment system, three samplings were conducted for each of the FTS and MMS raceways. As the samplings were very time consuming, each of the two systems was sampled on separate days. Sampling procedures are described in 2.5.3.

2.5.3 Sampling procedures

Faecal sampling:

Faecal samples were collected approximately 3-6 hours post-feeding. Since the lobsters were kept in a system where water and small objects could freely pass through the individual compartments, the lobsters were transferred to glass (1/2 year-olds) or plastic (1 and 2 year-olds) beakers filled with water from the system to avoid any mixing of faecal samples. After the juveniles had been placed in individual containers, they were fed artemia (1/2 year olds) or shrimps (1 and 2 year-olds) and left in a dark environment to avoid stress for 3-6 hours until excretion of faeces. The exact time of sampling varied between the individuals

depending on factors such as stress from being moved and handled and the time since last feeding. The beakers were checked for excrement every hour to minimize further growth of bacteria from the water on the surface of the excrement. Fresh water from the system was added to the beakers every hour to avoid oxygen depletion and temperature increase. Faecal samples were collected using a pipette and the samples were viewed under a microscope to verify that they were excrement from the lobsters. Each individual sample was rinsed two times in milliQ water, placed in an eppendorf tube, and stored at -20°C. Any excess water was removed from the tube to avoid any damage to the DNA when freezing the sample.

Water sampling:

The water samples were taken from both inlet and outlet water using a 50 mL syringe, and 40 mL of water was then pressed through a 0.22 µl Dynaguard filter tip (Microgen). The filter tips were frozen and stored at -20°C to avoid any further growth of bacteria.

Feed sampling:

Sampling of feed in both experiment 1 and 2 was performed immediately post feeding. In experiment 1, 20 mL of the artemia mixture was collected with a 20 mL syringe and as much as possible was pressed through a 0.22 µm Dynaguard filter tip. Feed samples in experiment 2 were taken by crushing shrimps in a small amount of fresh water and the liquid was collected with a 20 ml syringe. Further, as much liquid as possible was pressed through a 0.22 µm Dynaguard filter tip. All filter tips with samples were stored at -20°C.

2.6 Analytical methodology

To study the microbiota of lobster faeces, rearing water and feed an approach involving DGGE analysis of 16s rDNA amplicons was adopted.

2.6.1 DNA extraction

DNA was extracted from samples of faeces, water and feed by using the Powersoil® DNA Isolation Kit from MO BIO Laboratories, Inc, which is based on a protocol involving bead-beating in the lysis step (Appendix 1).

Extraction of DNA from lobster faecal samples:

The faecal samples collected in experiments 1 and 2 were thawed and the entire load of sample from each individual, about 0.25 g, was transferred to the Powerbead lysis tubes provided by the DNA extraction kit using a pipette. Beyond this point the provided protocol was followed.

Extraction of DNA from water samples:

DNA was extracted from water samples collected in experiments 1 and 2 (as described in section 2.5, water samples were taken using Dynaguard 0.22 µm filter tips). In order to extract DNA from the filter tips, the hollow fibres inside the plastic cartridge was pulled out and transferred to the Powerbead lysis tubes using a needle. Beyond this step the provided protocol was followed.

Extraction of DNA from feed samples:

DNA was extracted from feed samples collected in experiments 1 and 2. The feed samples were collected using a syringe with a Dynaguard 0.22 µm filter tip and the extraction of DNA was performed as described above for water samples.

2.6.2 PCR (Polymerase chain reaction)

The polymerase chain reaction (PCR) was conducted on the samples of extracted DNA in order to amplify the gene region of interest, i.e. the variable 3 region (V3) of the 16S rRNA gene of all bacterial DNA present in the samples. The amplification of the V3 region was conducted by using the forward primer 338F-gc (5′cgcccgccgcgcgcggcgggcggggcgggggcacgggggACTCCTACGGGAGGCAGCAG-3′) and reverse primer 518R (5′-ATTACCGCGGCTGCTGG-3′). A mastermix was made with 0,2 mM of each dNTP, 2 mM MgCl2, 0.3 µM of each primer, reaction buffer solution, milliQ water, and Taq polymerase from Invitrogen. 1 µl of approximately 5 ng/µl isolated DNA was added as template to 24 µl of mastermix, resulting in a total reaction volume of 25µl. The PCR reaction was run for 35 cycles following the PCR temperature cycling program as illustrated in table 2.1. For some samples with low DNA concentrations (all water samples and some faeces samples), the amplification was repeated using 2 µl of DNA template and the number of PCR cycles was increased to 38.

Table 2.1: PCR cycling conditions applied for the amplification of the V3-region of bacterial 16S rDNA. Step 2-4 was repeated 35-38 times depending on the samples.

Step	Temperature (°C)	Time	Reaction
1	95	3 minutes	denaturation
2	95	30 seconds	denaturation
3	50	30 seconds	annealing
4	72	60 seconds	elongation
5	72	10 minutes	elongation
6	10	∞	

2.6.3 Agarose gel electrophoresis

After the DNA had been extracted and amplified by PCR, agarose gel electrophoresis was conducted to examine the quality and quantity of the PCR product. Gel electrophoresis made it possible to detect any unspecific amplification that may have occurred in the previous steps, and the intensity of the resulting bands could be compared to determine the relative amounts of DNA in the samples.

The agarose gel solution was prepared by mixing 1 g of agarose powder with 400 mL of 50 x TAE buffer (Appendix 2). The solution was heated in a microwave until all agarose had dissolved completely. GelRed (5 µl for 400 mL gel solution) was added for subsequent visualization of DNA in the gel. The agarose solution was then poured into a gel mould, a well comb was applied to create the wells, and the gel was left to polymerize for approximately 10 minutes. Loading dye (1 µl per 3 µl PCR product) was added to the PCR product samples (3 µl) and the mixtures were applied to the wells in the gel along with a DNA marker for size comparison and one negative sample using a pipette. The gel was run in a 1x TAE buffer solution at 100-140 volts for 40-45 minutes depending on the size of the gel. A UV-cabinet was applied for visualization of the resulting DNA bands in the gel.

2.6.4 DGGE (Denaturing Gradient Gel Electrophoresis)

The denaturing gradient gel electrophoresis (DGGE) was conducted using the INGENYphorU system (INGENY). Two polished glass plates were assembled in a gel box with a spacer between them, and a comb was applied for the creation of wells. All casted gels were 8 % acrylamide gels with a 35 % - 55 % denaturing gradient, where 100 % denaturing correspond to 7 M urea and 40 % formamide. Two DGGE acrylamide stock solutions with 0 % and 80 % denaturing concentrations were used to produce the 35 % denaturing and 55 % denaturing acrylamide solutions (Table 2.2). Tetramethylenediamine (TEMED) and 10 % ammonium persulphate (APS) were added to start polymerization. The protocol for the production of the stock solutions is shown in Appendix 2. To create a denaturing gradient in the acrylamide gel, a gradient mixer and a pump were used to load the high (55 %) and low (35 %) denaturing acrylamide solutions into the glass plates. A 0 % denaturing acrylamide stacking gel was applied at the top of the gel and this was then left to polymerize for two hours.

A buffer tank was prepared with 17 l of 0.5 x TAE which was preheated to 60°C. The comb was removed from the gel, and the gel box was mounted in the buffer tank. Wells were rinsed with 0.5 x TAE using a syringe, water circulation was applied in the buffer tank, and the voltage was set at 100 Volts. Depending on the concentration of PCR product in each sample (relative amounts visualized by agarose gel electrophoresis), 5,10 or 15 µl sample was added with 2,3 or 4 µl loading dye (Fermentas) and applied to the wells. The aim was to apply the same amount of PCR product to each well despite differences in the success of amplification. The 6 outermost wells were left empty to avoid smiling effects on the gel. The voltage was turned back on, and the system was run for 17-18 hours.

In order to stain the gel, it was transferred to a sheet of plastic foil and covered with a solution containing 30 ml milliQ water, 3 µl SYBR® Gold (Invitrogen) and 600 ul 50 x TAE for 1 hour. After staining the gel was rinsed with milliQ water and then viewed and photographed using a UV-cabinet.

Table 2.2: Denaturation solutions applied for casting of an 8 % acrylamide (AA) DGGE gel with a 35 - 55 % denaturing gradient.

Denaturing %	0 % AA	80 % AA	TEMED + APS	Total volume
35	13.5 ml	10.5 ml	16 ul + 87 µl	24 ml
55	7.5 ml	16.5 ml	16 ul + 87 µl	24 ml
0 (stacking gel)	8 ml	-	10 ul + 40 µl	8 ml

2.6.5 Statistical analysis

DGGE band profiles were converted to histograms using the computer program Gel2k (Svein Norland, Department of Biology, University of Bergen, Norway). The intensities of peaks in the histograms, representing bands on the DGGE gels, were converted to peak area values using Gel2k and exported to Microsoft Excel sheets. The values were normalized for comparisons between samples by dividing the intensity value of each band by the total intensity value of all bands in the same lane (Equation 2.1).

$$p_i = \frac{n_i}{N} \tag{2.1}$$

where p_i = normalized band intensity values, n_i = intensity value of a single band, and N = the sum of intensity values of all bands in the lane. The computer program package PAST was applied for statistical analyses (Hammer et al., 2001).

The diversity indices band richness (K´), Shannons diversity index (H´) and Pielous evenness index (J´) were calculated by using PAST. The band richness is the number of bands in the DGGE profile and is a reflection of species richness in the applied samples. The Shannon diversity index takes both the band richness and evenness into account when calculating microbial community diversity, which increases with increasing band richness and evenness (Equation 2.2) (Harper, 1999).

$$H' = -\sum_{i=1}^{K}(p_i \ln p_i) \quad (2.2)$$

where K = band richness and p_i = normalized band intensity value. H'_{max} is the maximum diversity in a community with band richness K and is given by equation 2.3.

$$H'_{max} = \ln K \quad (2.3)$$

Pielou´s evenness index is a method for calculating diversity by means of how evenly different species are distributed within a sample (Equation 2.4). J´ is calculated from the observed diversity (H´) and the maximum diversity (H'_{max}). The evenness value is a number between 0 and 1, where a high J´ value reflects even distribution of species in the community (Harper, 1999).

$$J' = \frac{H'}{H'_{max}} \quad (2.4)$$

Bray-Curtis similarities were calculated for comparison of community profiles among samples within each DGGE gel. Bray-Curtis similarity matrices were calculated in PAST based on the normalized peak-area values. The Bray-Curtis similarities range between 0 and 1, where 0 is the absence of any common bands and 1 represents identical band profiles (Bray and Curtis, 1957). For comparison of groups of samples, average Bray-Curtis similarities were calculated within or between groups.

Based on Bray-Curtis distances, ordination by principal coordinates analysis (PCoA) was performed for visualization of community profile similarity between samples (and groups of samples). This method of ordination allows visualization of distance between DGGE profiles represented as data points plotted in relation to each other in a coordinate system. Similar profiles are plotted closer in proximity to each other compared to dissimilar profiles, which will be further away from each other in the coordinate system (Hammer et al., 2001)

Differences between groups of samples were investigated by the application of two different statistical tests in PAST: the one-way ANOVA test and the one-way PERMANOVA (Non-Parametric MANOVA) test. The one-way ANOVA (Analysis of Variance) test establish

whether several univariate data sets have the same mean, and was applied to test whether observed differences in diversity indices (including average Bray-Curtis similarities) between groups of samples were significant ($p < 0.05$) (Davis, 1986).

The one-way PERMANOVA test was applied to determine whether there were significant differences in microbial community composition between two or more groups of samples. Distances between groups of samples are used by this permutation based test to determine whether there are significant differences in composition (significant when $p < 0.05$) (Anderson, 2005).

3 Results

3.1 Survival and growth

All 1- and 2-year-old lobster juveniles were weighed regularly and deaths were recorded during the 85-day long experiment (experiment 2, see section 2.2.2) to monitor lobster growth (Fig. 3.1) and survival within age groups and within water treatment systems (Fig. 3.2A and B). From day 0 to 50, 1-year-old lobster juveniles had an average weight increase of approximately 2 grams. After the change in water treatment system for 50 % of the individuals (day 50 – 85), the FTS group had an approximate increase of 1 grams body weight, while the MMS group had an approximate increase of 2 grams. The 2-year-olds had an average weight increase of approximately 2 grams during the first 50 days. From day 50 to 85 (after the change in water treatment), the FTS group displayed an approximate body weight increase of 2 grams, while the increase was 4 grams in the MMS group.

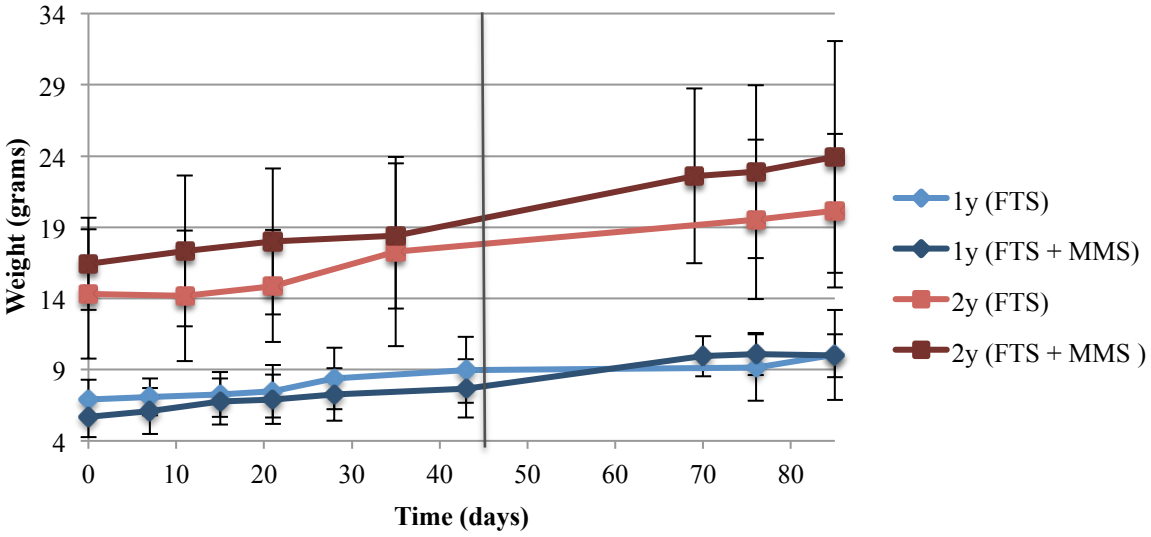


Figure 3.1: Average weight (grams) of 1-year-old (1y) and 2-year-old (2y) lobster juveniles reared in two different water treatment systems over a time span of 85 days. A change in water treatment system from flow-through (FTS) to microbially matured (MMS) was performed for 50 % of the individuals in both age groups at day 50 (indicated by vertical line). Squares illustrate the weight of individuals reared in FTS for 85 days, and triangles illustrate the weight of individuals reared in FTS until day 50 and then MMS until day 85. Error bars indicate standard errors.

When comparing the survival according to water treatment system it was found to be higher for the individuals reared in FTS (Fig. 3.2A). The survival in this group from day 1 to 50 was

95 %, while for the remainder of the individuals it was 90 % from day 50 to 85. In comparison, within the group subsequently moved to an MMS the survival rate was 90 % from day 1 to 50 when the individuals were reared in FTS. When the remaining individuals were reared in MMS from day 50 to 85 the survival rate was approximately 80 %.

The survival percentage was somewhat lower for the 1-year-olds with deaths starting to occur at an earlier point of time compared to the 2-year-olds (Fig. 3.2B). The total survival in 2-year-old juveniles was 86.4 %, while it was 77.3 % in the 1-year-old juveniles.

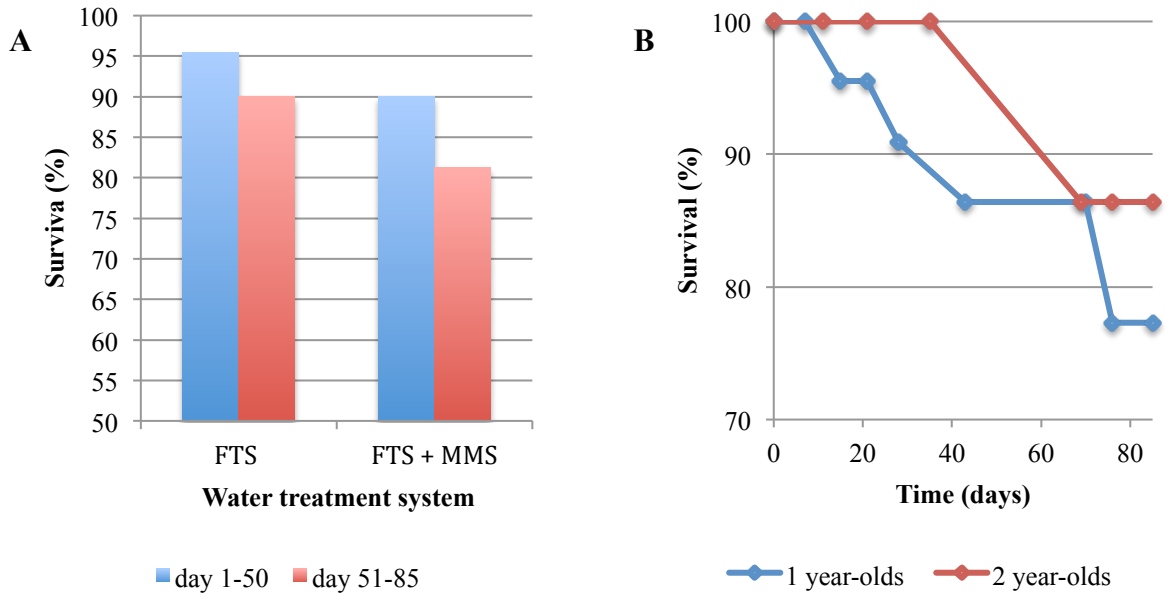


Figure 3.2: A: Survival percentage of 1- and 2-year-old lobster juveniles reared in FTS for 50 days, at which 50 % of the individuals from each age group were moved to a MMS and reared until day 85. Columns FTS represent individuals reared in FTS for 85 days, and columns FTS + MMS represent individuals who were moved to MMS at day 50. **B**: Survival percentage of 1- and 2-year-old lobster juveniles over a time span of 85 days.

3.2 Temporal development of faecal microbiota in lobster juveniles

3.2.1 Comparison of the faecal microbiota in ½-, 1- and 2-year-old lobster juveniles

To compare the microbial community structure between different age groups (1/2-, 1- and 2-year-old lobster juveniles), a DGGE gel was run with faecal samples from three different individuals from each age group at day 0, 30 and 60 (experiment 1 and 2, see 2.2) (Fig. 3.3).

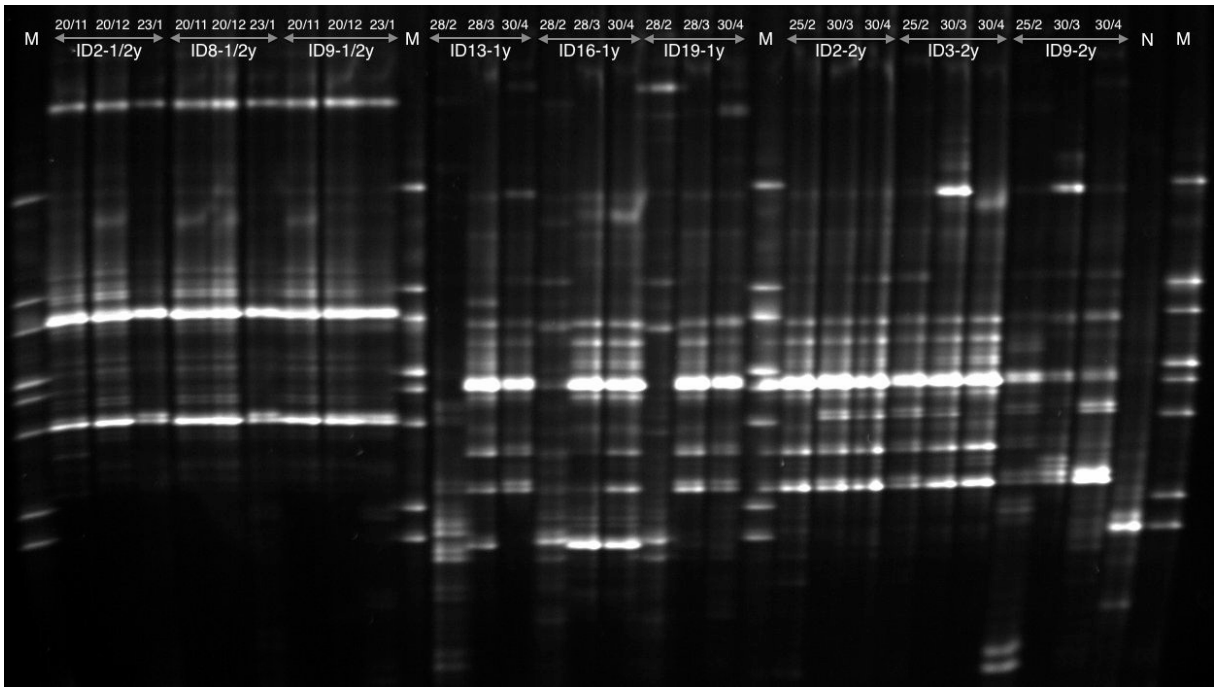


Figure 3.3: DGGE gel with 16S v3 region PCR-products from faecal samples collected from three lobster individuals from three different age groups over time: ½ year, 1 year and 2 years old. Each individual is labelled with a unique ID number, and the sampling dates are given for each well. M = marker, N = negative control; 1/2y = ½-year-old; 1y = 1-year-old; 2y = 2-year-old.

Ordination by PCoA using Bray-Curtis similarities based on the profiles in the DGGE gel indicated that the microbial communities in the gut of the ½-year-old individuals were different from the communities in the 1- and 2-year-old individuals (Fig. 3.4). The plot also indicated that the microbiota was similar in the 1- and 2-year-olds. The plot further suggested that there was more variation between 1-year-old individuals compared to individuals in the other two age groups, and that the least amount of individual variation was within the ½-year-old group.

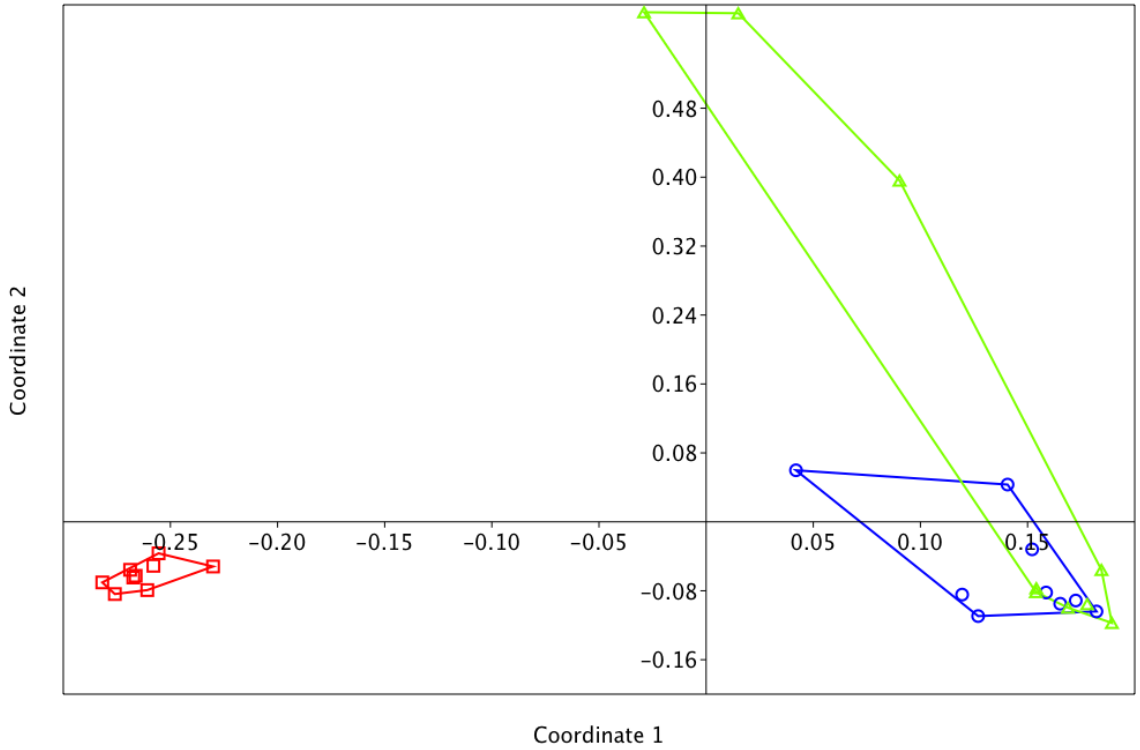


Figure 3.4: PCoA plot based on average Bray-Curtis similarities for comparison of DGGE profiles of faecal microbiota of ½-, 1- and 2-year-old lobster juveniles (□ = 1/2y, Δ = 1y, O = 2y).

Average Bray-Curtis similarities were calculated in order to compare individual lobster DGGE profiles within and between age groups to investigate the inter-individual variation in the microbiota with respect to age (Fig. 3.5). The average Bray-Curtis similarities of the faecal microbiota within the age groups supported that the gut microbiota of ½-year-old lobster juveniles was significantly more similar between individuals compared to the 1- and 2-year-old lobster juveniles (ANOVA, $p \leq 0.0001$). There were no significant differences between individuals within the 1- and 2-year-olds.

The average Bray-Curtis similarities for comparisons of microbiota between the three different age groups showed that there was low similarity between ½-year-olds and the two other age groups (average Bray-Curtis similarities < 0,1) (Fig. 3.5). The 1- and 2-year-olds were more similar to each other (average Bray-Curtis similarity > 0.4) compared to the ½-year-olds. It was confirmed that the ½-year-old lobster faecal microbiota composition was significantly different from the faecal microbiota composition of both the 1 year, and 2 years old lobsters when this was tested statistically (PERMANOVA, $p \leq 0.0006$). There were,

however, no significant differences in the composition of faecal microbiota between 1-year-old, and 2-year-old lobsters (PERMANOVA, p = 0.067).

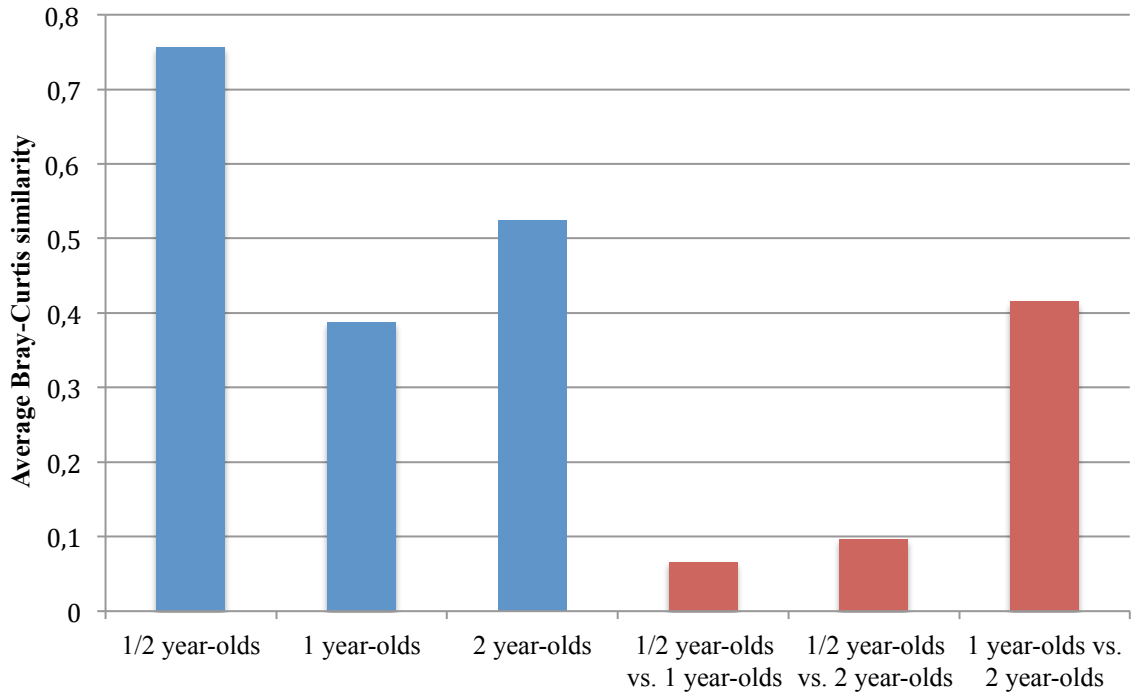


Figure 3.5: Average Bray-Curtis similarities of faecal microbiota within (blue columns) and between (red columns) the three lobster juvenile age groups (1/2-, 1- and 2-year olds).

Average band richness, Shannon´s diversity and evenness were determined within each age group, and the diversity indices were found to be similar between age groups (Table 3.1).

Table 3.1: Average band richness (k), Shannon´s diversity index (H`) and evenness (J`) of faecal microbiota within ½-, 1- and 2-year-old lobster juveniles, including standard errors.

Age group	Band richness (k)	Shannon index (H`)	Evenness index (J`)
½ year-olds	22.78 ± 3.15	2.01 ± 0.21	0.33 ± 0.04
1 year-olds	19.89 ± 2.93	2.02 ± 0.24	0.40 ± 0.12
2 year-olds	21.67 ± 3.39	2.07 ± 0.22	0.36 ± 0.08

Ordination by principal coordinates analysis using Bray-Curtis similarities was performed for each age group based on the profiles in the DGGE gel (Fig. 3.6). Indications were that the ½-year-old faecal microbiota at day 60 was different from that at day 0 and 30 (Fig. 3.6A). For the faecal microbiota of 1-year-olds, individuals were clustered together at day 30 and 60 and differed from day 0 (Fig. 3.6B). In the 2-year-old group there were indications that the faecal

microbiota of the individuals was different at each of the three time points (Fig. 3.6C). These differences in gut microbiota between the age groups over time were however not significant when this was tested statistically (PERMANOVA), suggesting that there was no temporal development of gut microbial community composition within any of the age groups during the 60 experimental days.

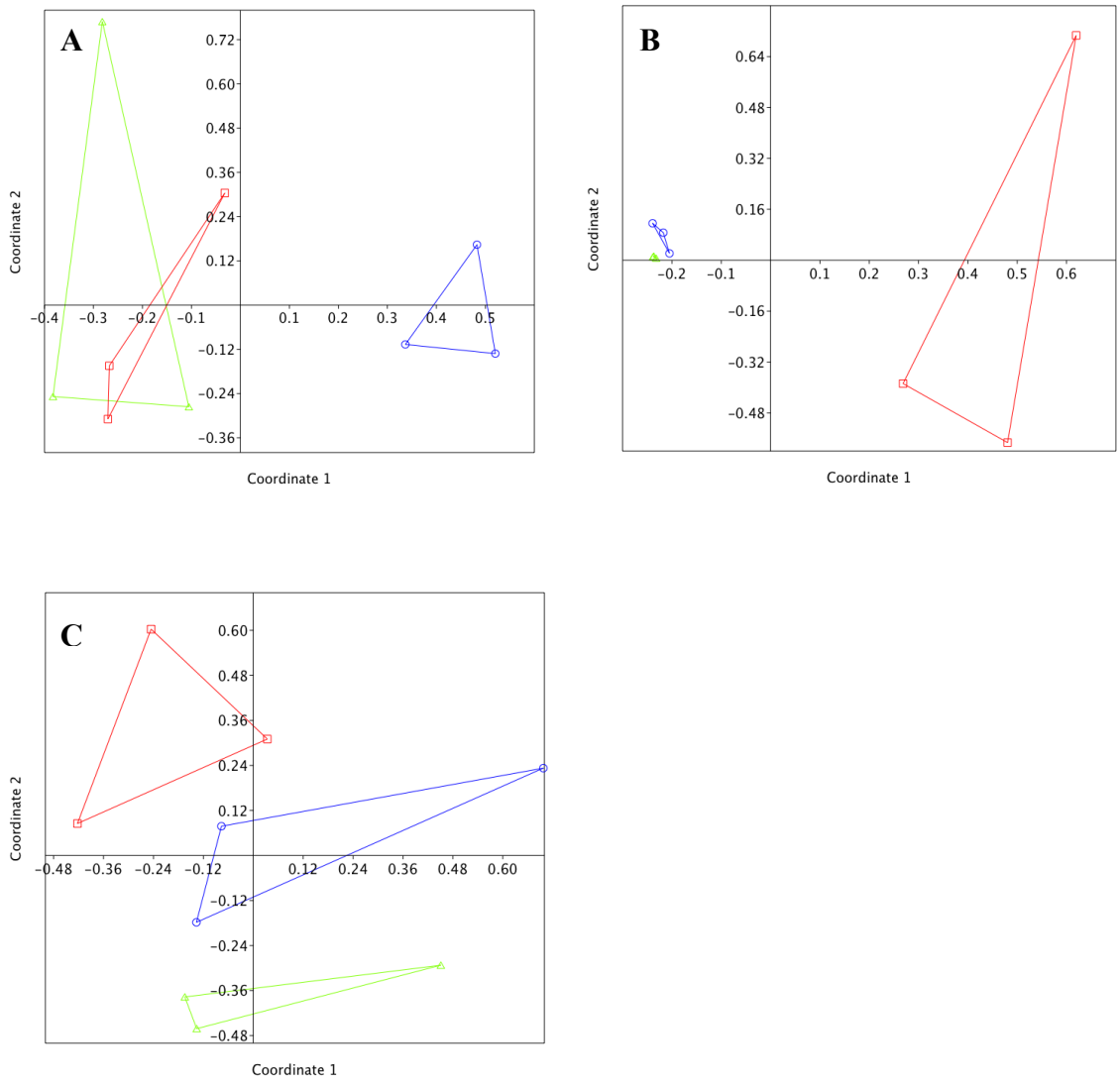


Figure 3.6: PCoA plots based on average Bray-Curtis similarities for comparison of DGGE profiles of faecal samples from ½-, 1- and 2-year-old lobster juveniles at time 0, 30 and 60 days. Plot **A**, **B** and **C** represents ½-, 1- and 2-year-olds respectively. □ = day 0, Δ = day 30, O = day 60.

Average Bray-Curtis similarities were calculated for comparison of individual lobster DGGE profiles within all age groups at 0, 30 and 60 days in order to study the inter-individual variation in the microbiota (Fig. 3.7). It was found that there was no clear change in the amount of inter-individual variation in faecal microbiota in the ½-year-old lobsters during the

sampling period. In the 1-year-old group an increase in average Bray-Curtis similarities was observed, suggesting less variation between individuals at later sampling time points. For the 2-year-old juveniles, a small decrease occurred over time. However, no significant differences in similarity were found, except when comparing day 30 and 60 in the ½ year-old group (ANOVA, $p = 0.035$) and day 0 and 30 in the 1 year-old group (ANOVA, $p = 0.035$). Likewise, no significant differences in faecal microbial community composition were found between individuals at any point of time in any of the age groups (PERMANOVA). These results indicate that there was low inter-individual variation of the microbiota in all age groups over time.

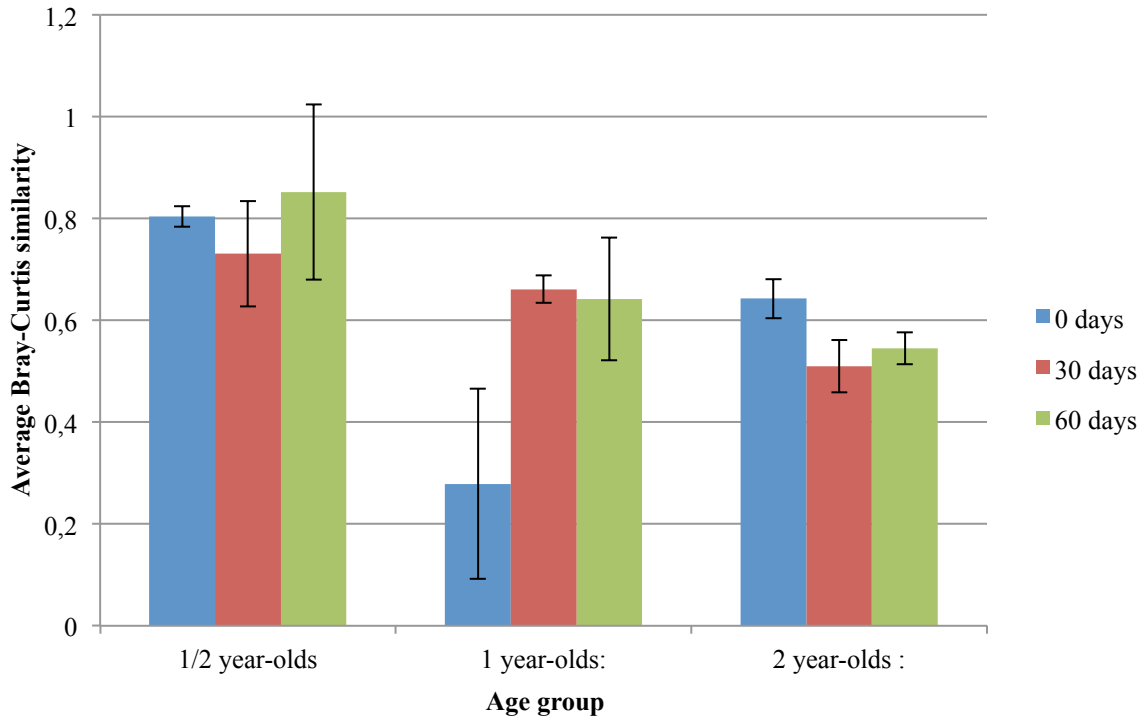


Figure 3.7: Average Bray-Curtis similarities of faecal microbiota within different lobster juvenile age groups (1/2-, 1- and 2 years old) over time (0, 30 and 60 days), including standard errors.

3.2.2 Community dynamics of faecal microbiota in 1-year-old lobster juveniles

To study the community dynamics of the gut microbiota in 1-year-old lobster juveniles, faecal samples from 4 individuals and inlet water samples at 6 points of time (experiment 2, see 2.2.2) were analysed and compared on a DGGE gel (Fig. 3.8).

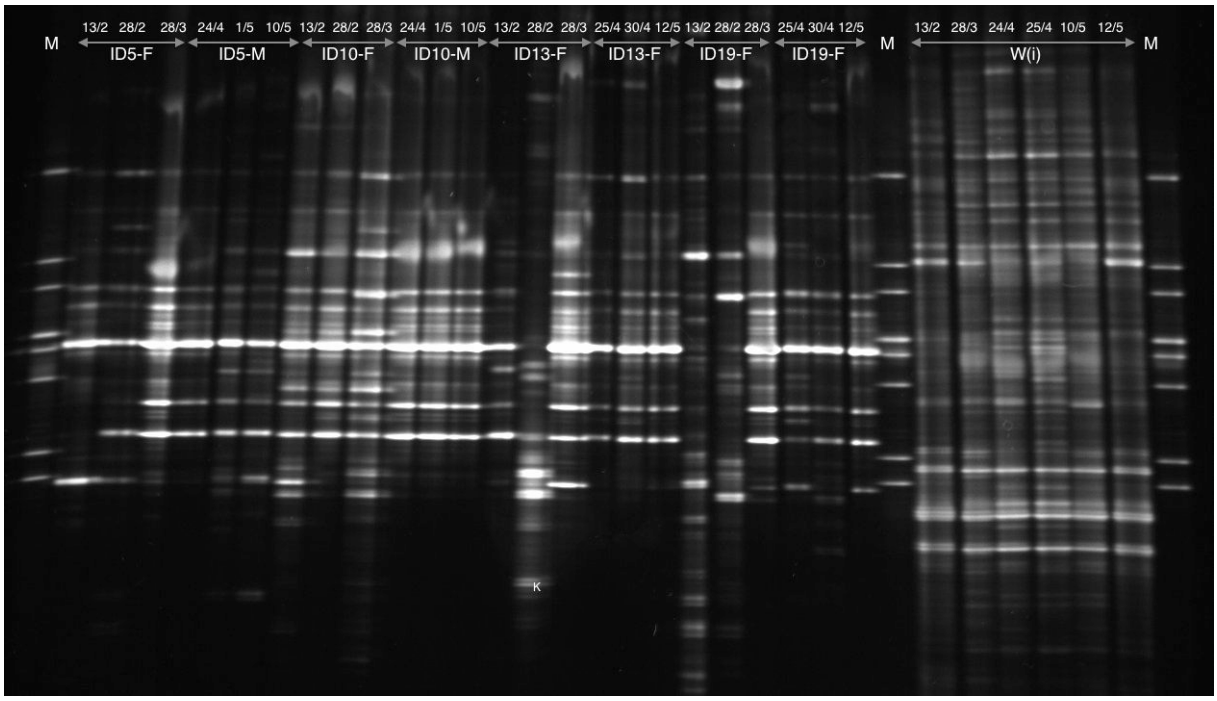


Figure 3.8: DGGE gel with 16S v3 region PCR-products from faecal microbiota samples collected from four 1-year-old lobster juveniles at 6 time points. Each well is marked with the individual number (ID5, ID10, ID13, ID19) and date of sampling. Halfway through the experiment, a change in water treatment system from FTS to MMS was performed for ID5 and ID10. Water treatment systems in rearing are marked by F (FTS) and M (MMS) for each individual. W(i) = inlet water samples; M = markers.

PCoA ordination using Bray-Curtis similarities was performed including all samples on the DGGE gel grouped by similarity between sampling dates (Fig. 3.9A) and individuals (Fig. 3.9B). The PCoA plot indicated that the microbial community profiles of lobster faeces were similar between dates (Fig. 3.9A), and thus no clear change of faecal microbiota for the 1-year-old lobster juveniles during the experiment. However, there appeared to be more variation between individuals within the first half of the experiment, while less variation was apparent in the latter half. For each individual, there was relatively high similarity in faecal community profiles between individuals as there was a high degree of overlap in the PCoA plot (Fig. 3.9 B). The community profiles of the water samples were found to be grouped separately from the faecal samples in both plots, and indicated that the community structure of the water samples was different from the faecal samples. This was further investigated in 3.3.

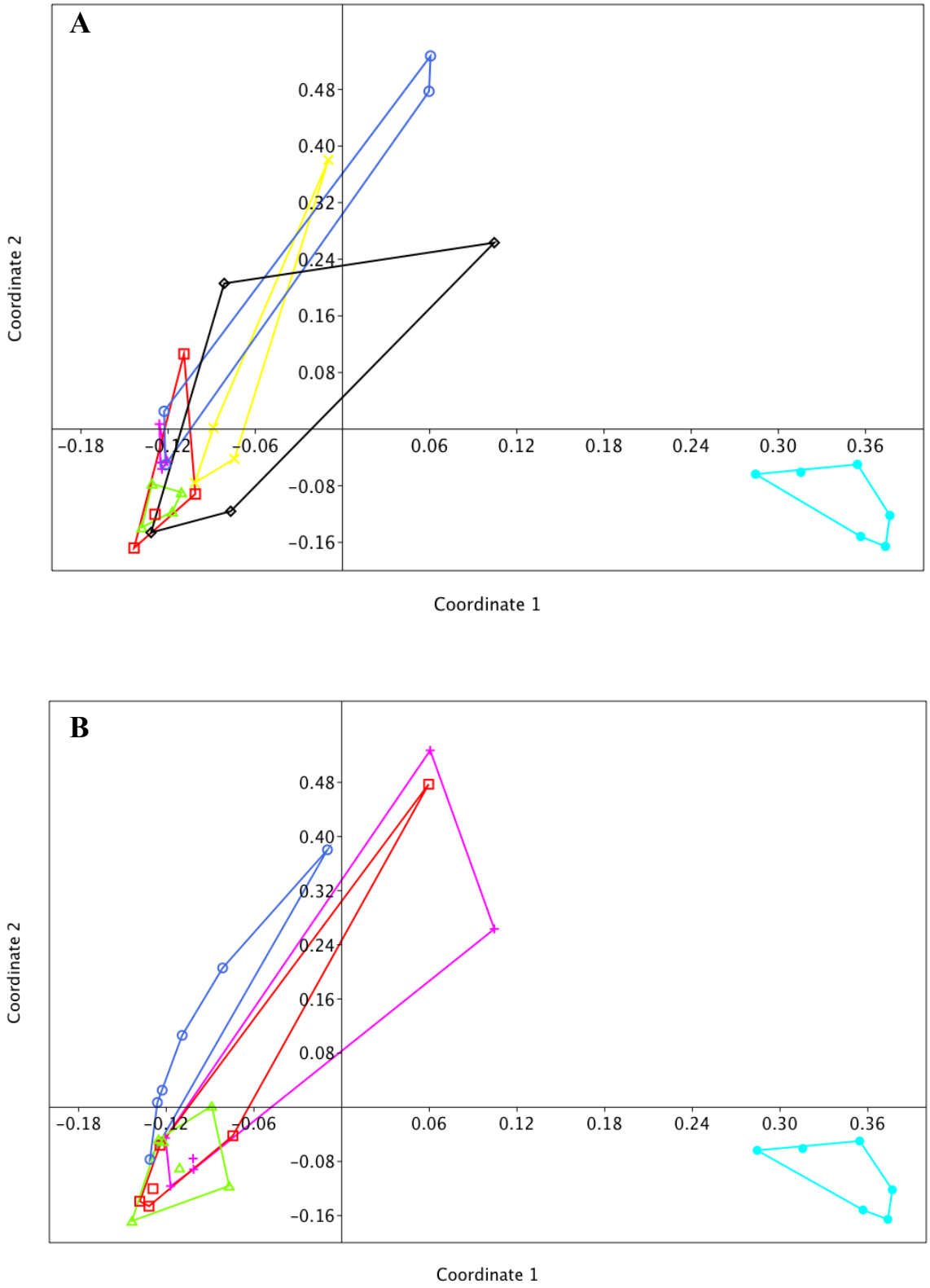


Figure 3.9: PcoA plots based on average Bray-Curtis similarities for comparison of 1-year-old lobster juvenile faecal microbiota, grouped according to sampling dates (A) and individuals (B). **A:** ◊ = 13/2, X = 28/2, O = 28/3, □ = 25/4 + 25/4, + = 30/4 + 1/5, Δ = 10/5 + 12/5, ● = inlet water, **B:** Δ = ID5, O = ID10, □ = ID13, + = ID19, ● = inlet water.

Increasing similarities of the microbial communities was observed between individuals over time. This observation was based on average Bray-Curtis similarities between the microbiota of individual lobster faeces within each sampling date (Fig. 3.10). The one-way ANOVA test confirmed that there were significant differences in average Bray-Curtis similarities of 1-year-old lobster faecal microbiota when the early sampling dates were compared to the late sampling dates (p-values are presented in Appendix 3). This indicates that the gut microbial communities of the lobster juvenile individuals became more similar over time.

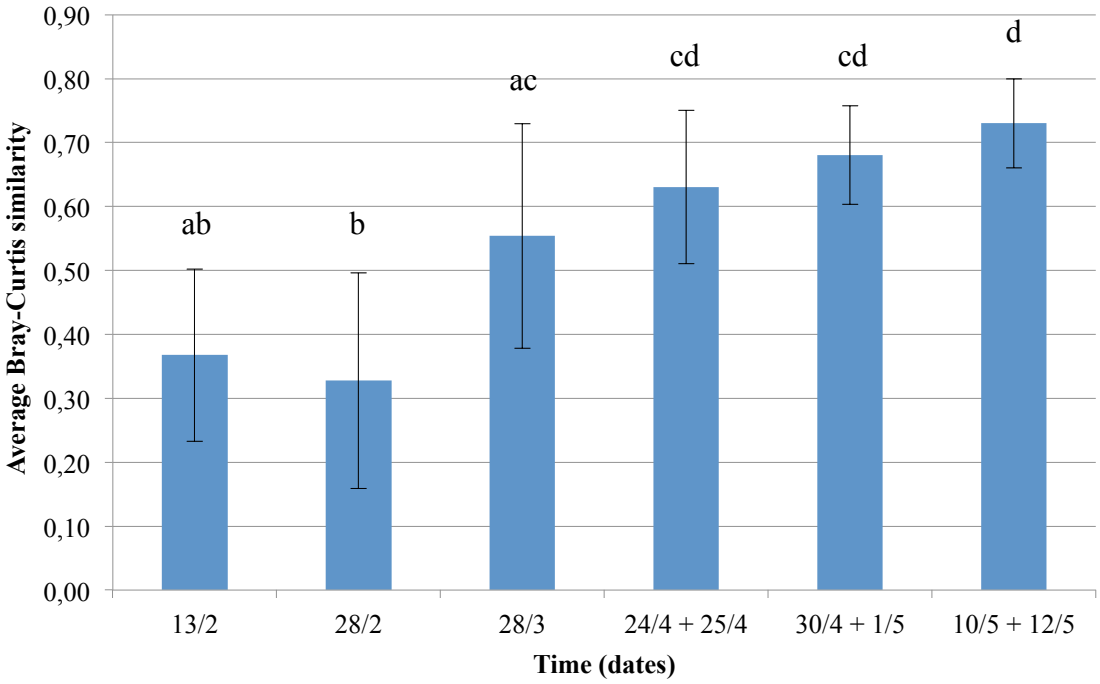


Figure 3.10: Average Bray-Curtis similarities for comparison of 1-year-old lobster DGGE profiles within each sampling date. Error bars indicate standard errors, and different letters indicate significant differences (ANOVA, $p < 0.05$).

No significant differences in microbial community composition were observed in the microbiota of individual lobster faeces between the different sampling dates except when comparing 28/3 and 12/5 (PERMANOVA, $p = 0.028$). These results indicate that there was no clear temporal development of faecal microbiota composition in the 1-year-old lobster juveniles over the time span of three months.

In general, there was a tendency towards higher diversity indices (average band richness, Shannon´s diversity and evenness, Fig. 3.11) in faeces microbiota of 1-year-old lobsters in the first sampling dates (13/2 – 28/3) compared to the later sampling dates.

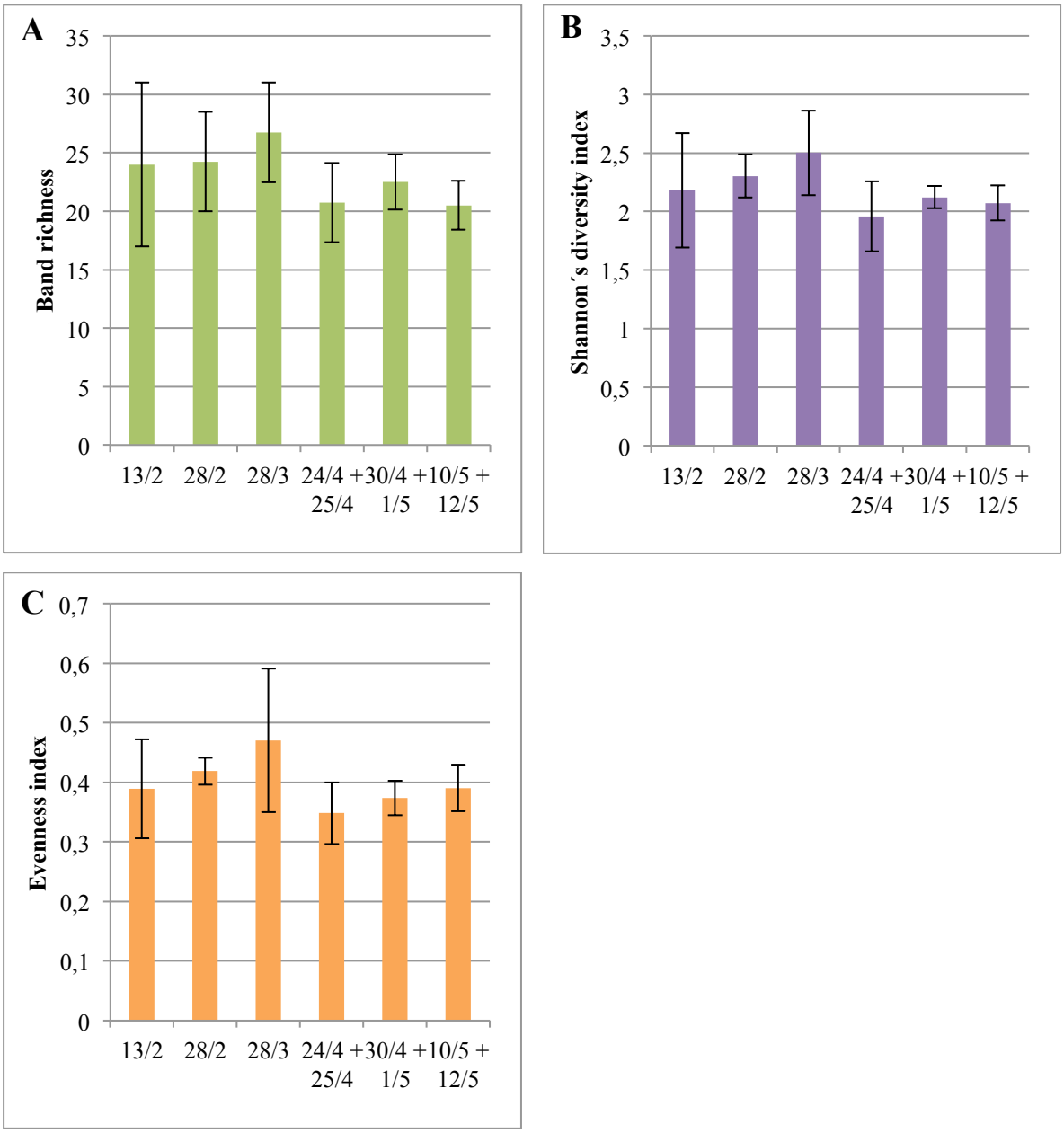


Figure 3.11: **A**: Average band richness (k), **B**: Shannon´s diversity index (H`) and **C**: evenness (J`) calculated within each sampling date for faecal microbial community profiles of 1-year-old lobster juveniles. Error bars indicate standard errors.

The high degree of overlapping profiles between individuals (Fig. 3.9B) indicated that there was a high similarity between individual microbial community profiles, with the exception of between individuals ID5 and ID10. The one-way PERMANOVA test confirmed that there were no significant differences in the microbiota of individual 1-year-old lobster faeces except between individuals ID5 and ID10 ($p = 0.0149$).

3.2.3 Community dynamics of faecal microbiota in 2-year-old lobster juveniles

To study the community dynamics of the gut microbiota in 2-year-old lobster juveniles, faecal samples from 4 individuals and inlet water samples at 6 points of time (experiment 2. See 2.2.2) were analysed and compared on a DGGE gel (Fig. 3.12).

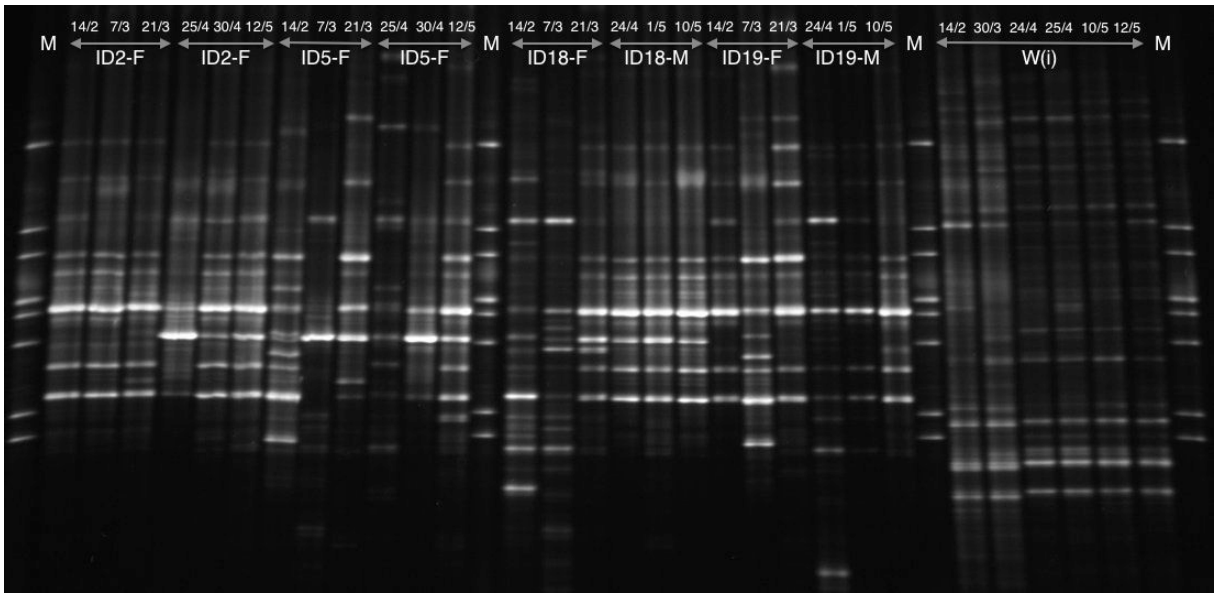


Figure 3.12: DGGE gel with 16S v3 region PCR-products from faecal microbiota samples collected from four 2-year-old lobster juveniles at 6 time points. Each well is marked with the individual number (ID2, ID5, ID18, ID19) and date of sampling. Halfway through the experiment, a change in water treatment system from FTS to MMS was performed for ID18 and ID19. Water treatment systems in rearing are marked by F (FTS) and M (MMS) for each individual. W = inlet water samples; M = markers.

PCoA ordination using Bray-Curtis similarities was performed including all samples on the DGGE gel grouped by similarity between sampling dates (Fig. 3.13A) and individuals (Fig. 3.13B). There were indications in the PCoA plot that community profiles are similar between dates, and hence that no clear change in the faecal microbiota occurred for the 2-year-old juveniles during the experiment. There was further relatively high similarity in faecal community profiles between individuals, and some variation within each individual (Fig. 3.13B). The community profiles of the water samples were separated from the faecal samples in both plots, indicating that the community structure of the water samples was different from that of the faecal samples (Fig. 3.13A and B). This was investigated and elaborated in 3.3.

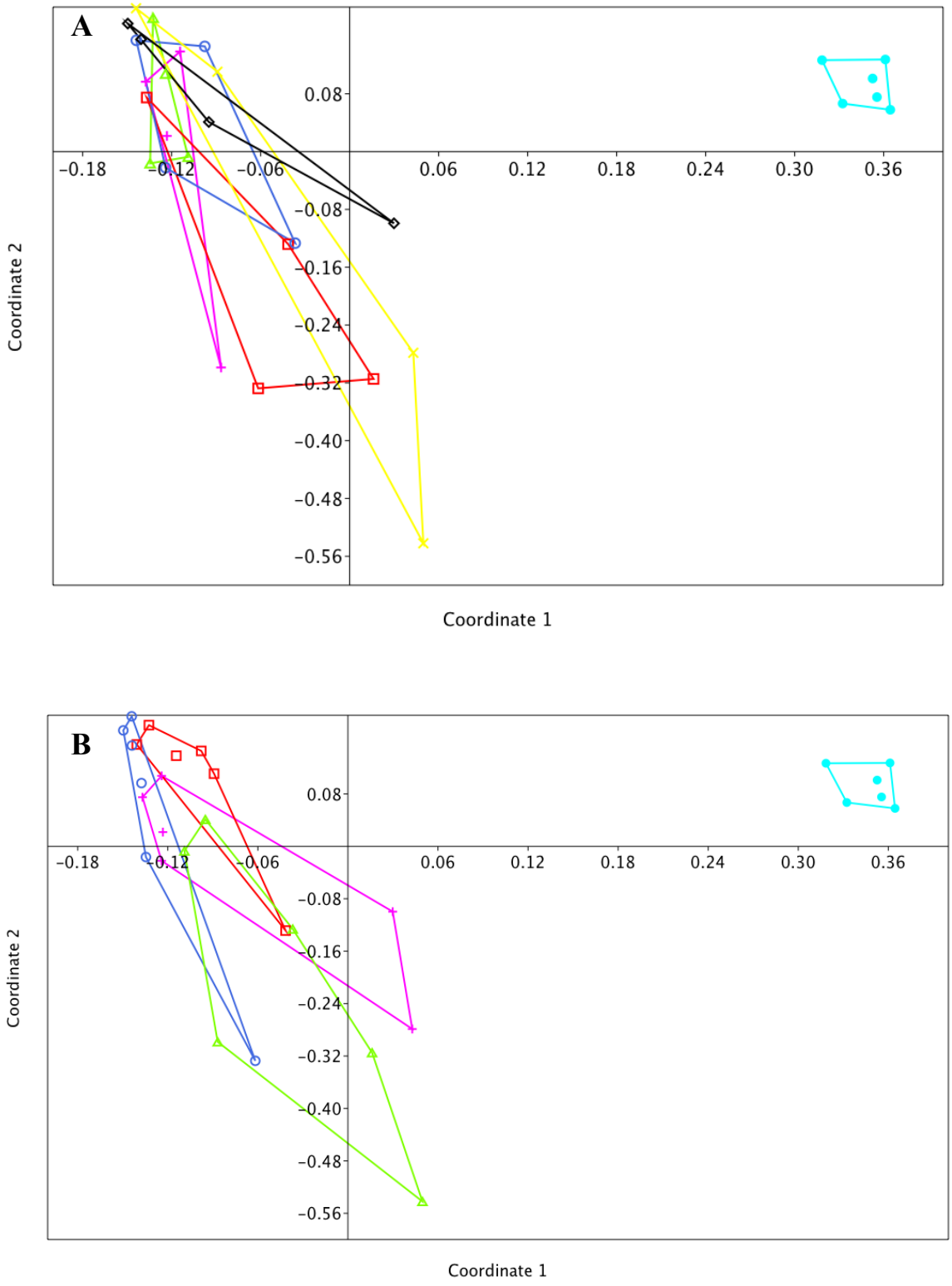


Figure 3.13: PcoA plots based on average Bray-Curtis similarities for comparison of 2-year-old lobster juvenile faecal microbiota, grouped according to sampling dates (**A**) and individuals (**B**). **A:** ◊ = 14/2, X = 7/3, O = 21/3, □ = 25/4 + 25/4, + = 30/4 + 1/5, Δ = 10/5 + 12/5, ● = inlet water), **B:** O = ID2, Δ = ID5, + = ID18, □ = ID19, ● = inlet water.

Average Bray-Curtis similarities were calculated for comparison of individual lobster DGGE profiles within each sampling date in order to investigate the inter-individual variation in the microbiota at each time point (Fig. 3.14). There were fluctuations in average Bray-Curtis similarities between individuals on the different sampling dates. Over time, there was a trend in increasing similarity between the microbial communities in the faeces of the 2-year-old lobster juveniles. There were significant differences when late sampling dates were compared to early sampling dates, especially when the microbial community similarities between individuals at 12/5 were compared to earlier time points (ANOVA, $p < 0.05$, p-values are presented in Appendix 3). This indicated that the microbial communities of the lobster juvenile individuals became more similar between individuals over time.

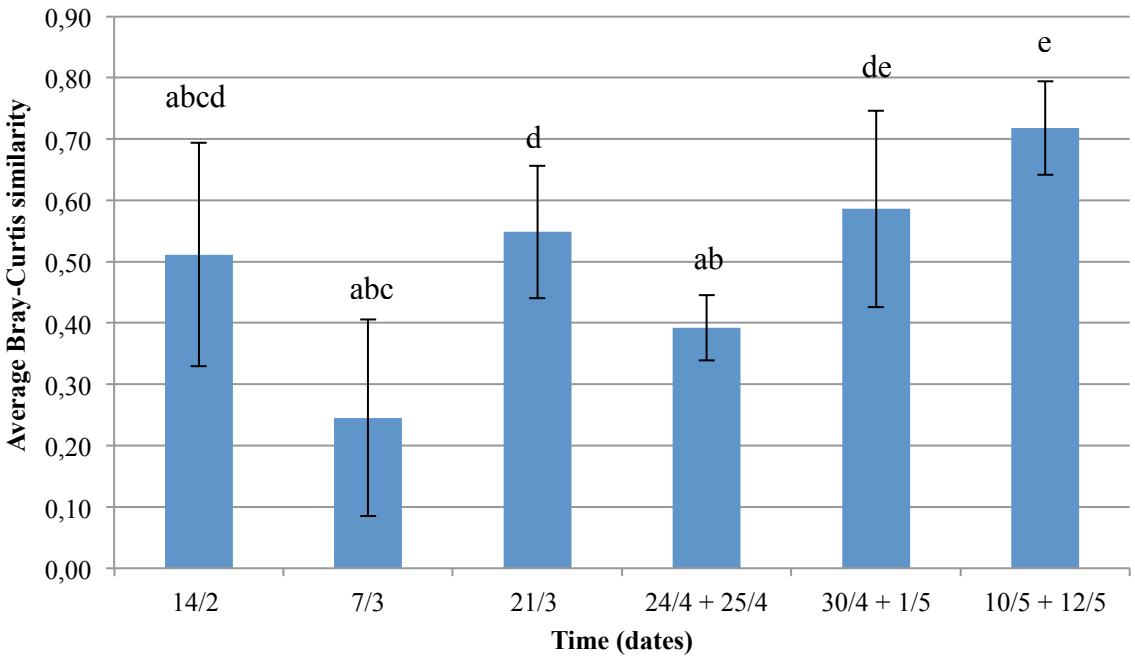


Figure 3.14: Average Bray-Curtis similarities for comparison of faecal microbiota DGGE profiles of 2-year-old lobsters within each sampling date. Error bars indicate standard errors, and different letters indicate significant differences (ANOVA, $p < 0.05$).

There were no significant differences in microbial community composition of 2-year-old lobster faeces between any of the different dates (PERMANOVA). These results indicated that there was no temporal development of faecal microbiota composition in the 2-year-old lobster juveniles over the timespan of three months.

In general there were fluctuations in diversity indices (average band richness, Shannon´s diversity and evenness, Fig. 3.15) for the faecal microbiota of 2-year-old lobsters, and no clear trends were observed.

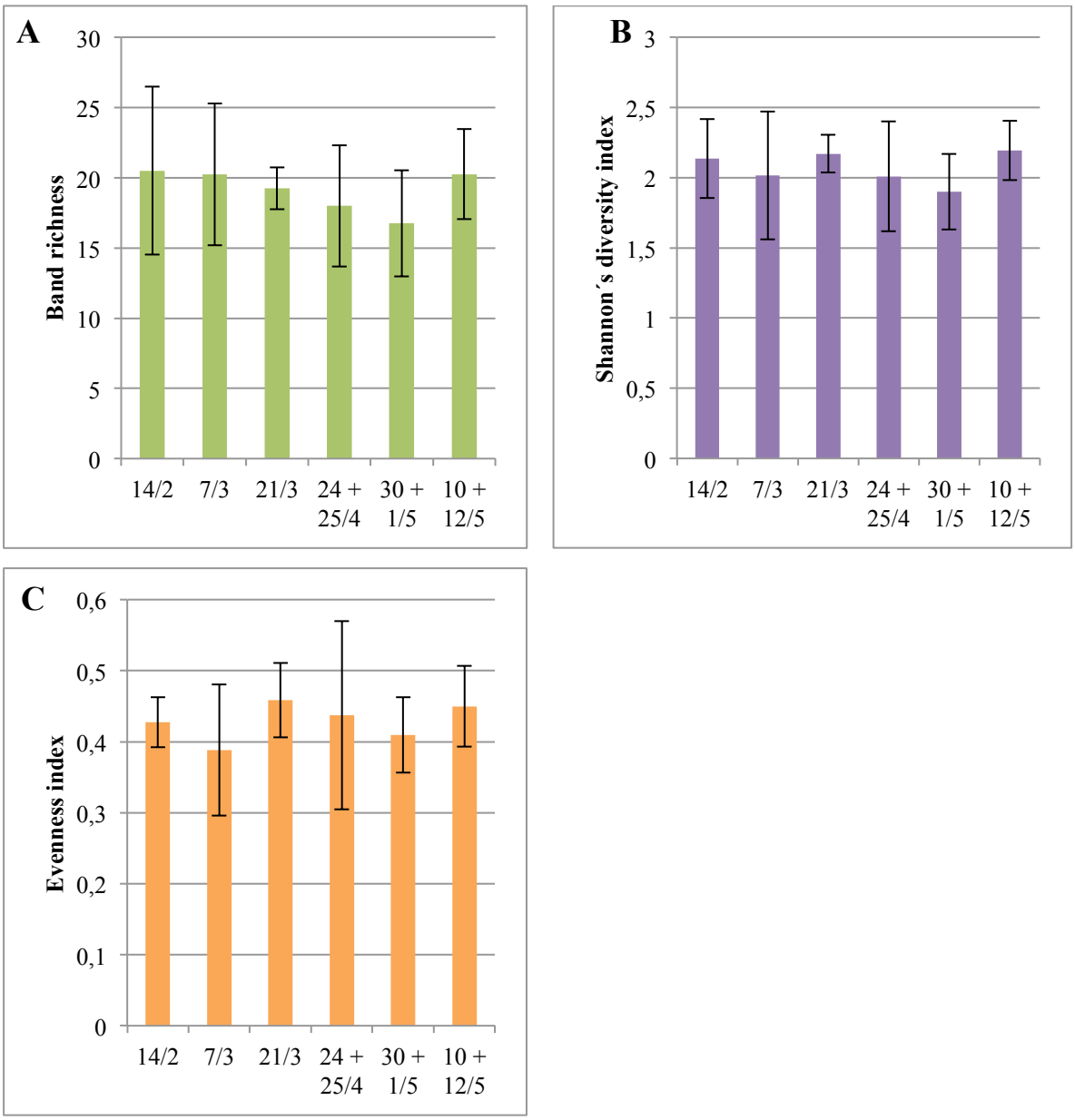


Figure 3.15: A: Average band richness (k), **B**: Shannon´s diversity index (H`) and **C**: evenness (J`) calculated within each sampling date for faecal microbial community profiles of 2-year-old lobster juveniles. Error bars indicate standard errors.

The similarity in faecal microbiota observed between individuals (Fig. 3.13B) was confirmed statistically (PERMANOVA, Table 3.2). Significant differences were mostly absent, except when comparing ID5 to ID19, and ID5 to ID2. This could also be observed in the PCoA plot,

where the overlap of profiles between ID5 and ID19 in addition to ID5 and ID2 was lower compared to other individual overlap comparisons (Fig. 3.13B).

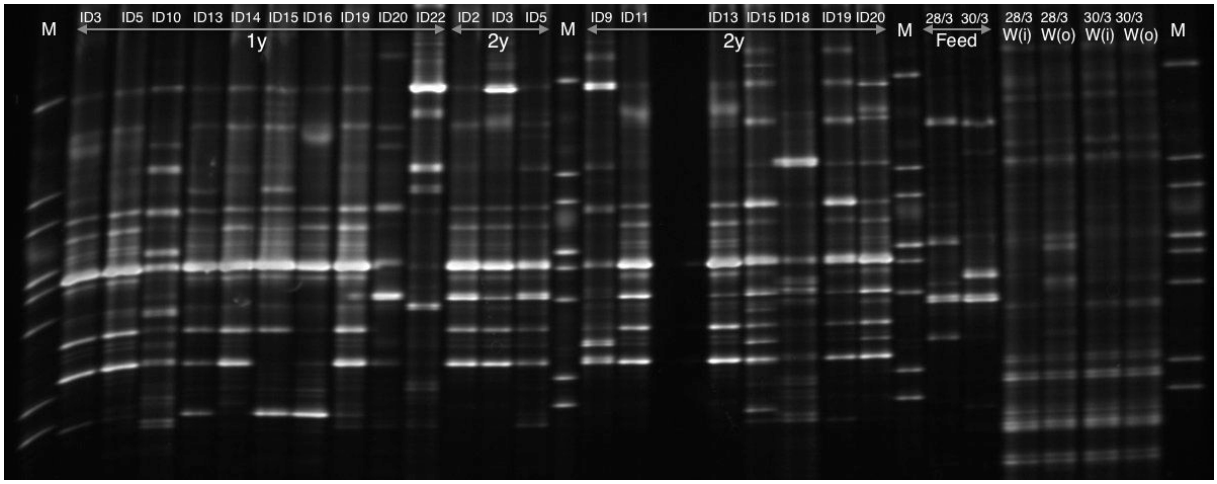
Table 3.2: Uncorrected p-values for one-way PERMANOVA testing the hypothesis that there are no differences in faecal microbiota composition between 2-year-old lobster juvenile individuals (ID2, ID5, ID18, ID19). Significant p-value are highlighted in red.

	ID19	**ID18**	**ID5**	**ID2**
ID19				
ID18	0.201			
ID5	0.0056	0.1217		
ID2	0.206	0.2363	0.034	

3.3 Effects of water treatment on faecal microbiota in lobster juveniles

To study the effect of water treatment on gut microbiota in 1- and 2-year-old lobster juveniles, faecal samples from individuals reared in firstly, separate raceways and, secondly, different water treatment systems (experiment 2, see 2.2) were analysed on three DGGE gels (Fig. 3.16A-C). Each DGGE gel represented one time point, and included faecal samples from lobster juveniles (1- and 2 years old), inlet and outlet water, and feed. As described in 2.2.2, all of the lobster juveniles were kept in a FTS for the first 50 days, and then 50 % of the individuals from each age group were then transferred to a MMS.

A



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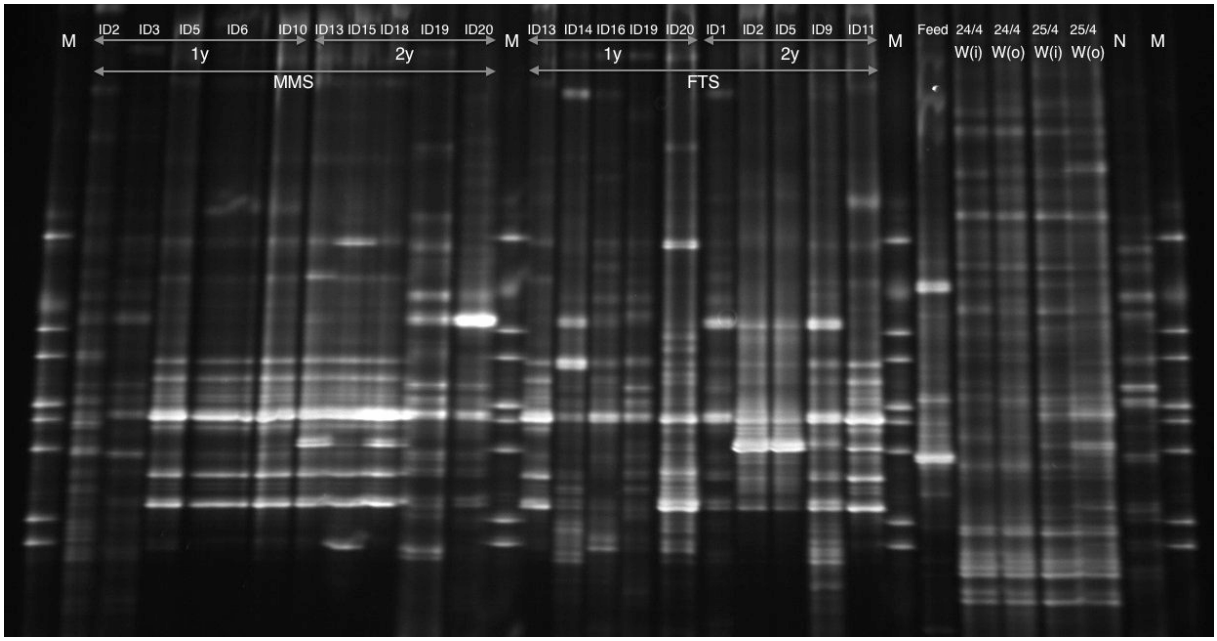

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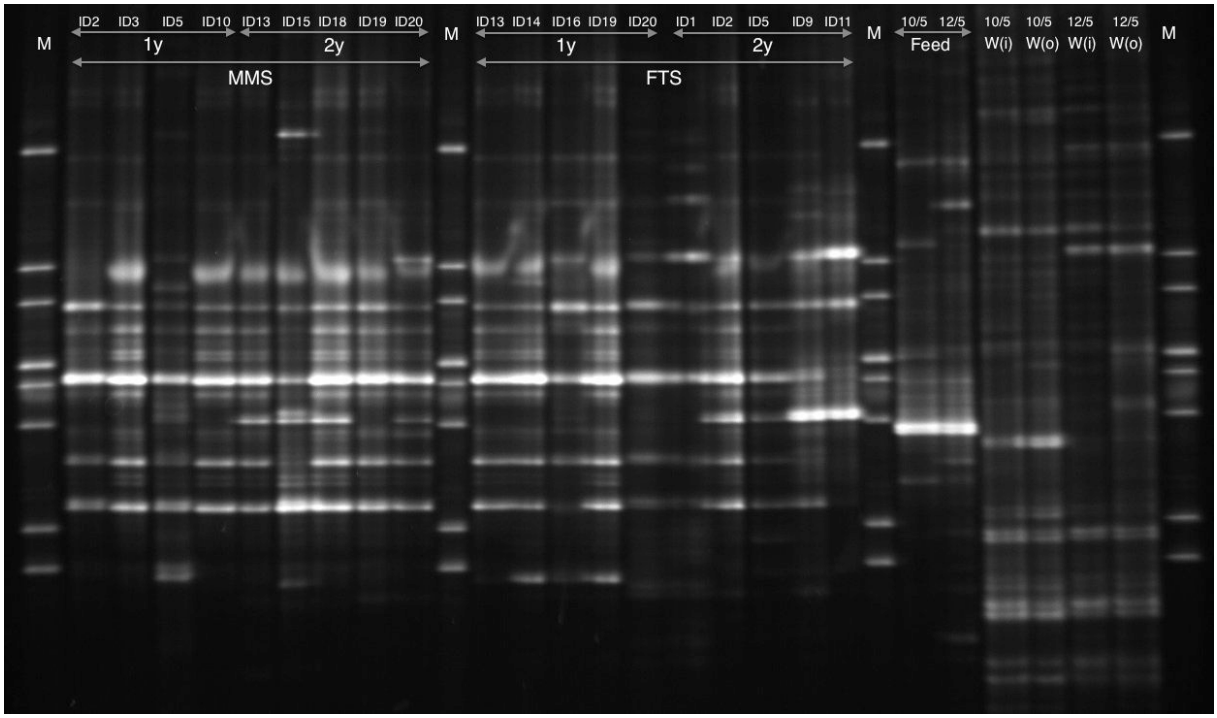
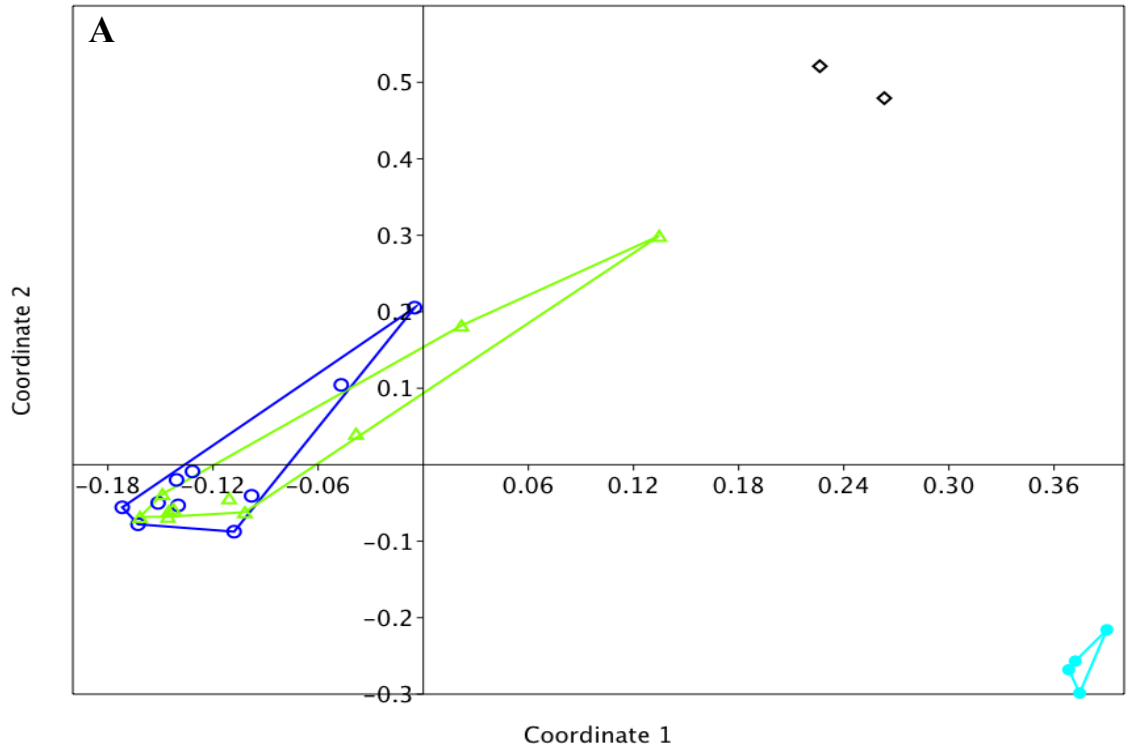


Figure 3.16: DGGE gels with 16S v3 region PCR-products from faecal samples collected from 10 1-year-old and 10 2-year-old lobster juveniles, samples of inlet and outlet water (Wi and Wo) and feed. **A:** Samples collected at 28/3 and 30/3, when all individuals were reared with FTS; **B:** Samples collected at 24/4 + 25/4, when water treatment system had been changed to MMS for 50% of the individuals; and **C**: Samples collected at 10/5 + 12/5, where 50 % of the individuals were reared with FTS and 50% with MMS. M = marker; W(i) = inlet water; W(o) = outlet water; 1y = 1-year-old; 2y = 2-year-old; N = negative control.

PCoA ordination using Bray-Curtis similarities was performed for each gel (Fig. 3.17). The PCoA plot representing the first sampling date indicated that there was high similarity in the microbiota between the two raceways (R1: 1-year-olds, R2: 2-year-olds) (Fig. 3.17A), thus suggesting that neither age nor rearing in separate raceways affected the faecal microbiota. At the first point of time after a change in water treatment system from FTS to MMS for 50 % of individuals, no obvious clustering according to raceway or water treatment system was observed, indicating that the juvenile gut microbiota was similar between rearing systems (Fig. 3.17B). Similarly, no clear differences in microbiota according to raceway or water treatment system could be observed at the third point of time (Fig. 3.17C). All plots, however, indicated a clear separation between the juvenile gut microbiota and the microbial communities associated with water and feed samples.



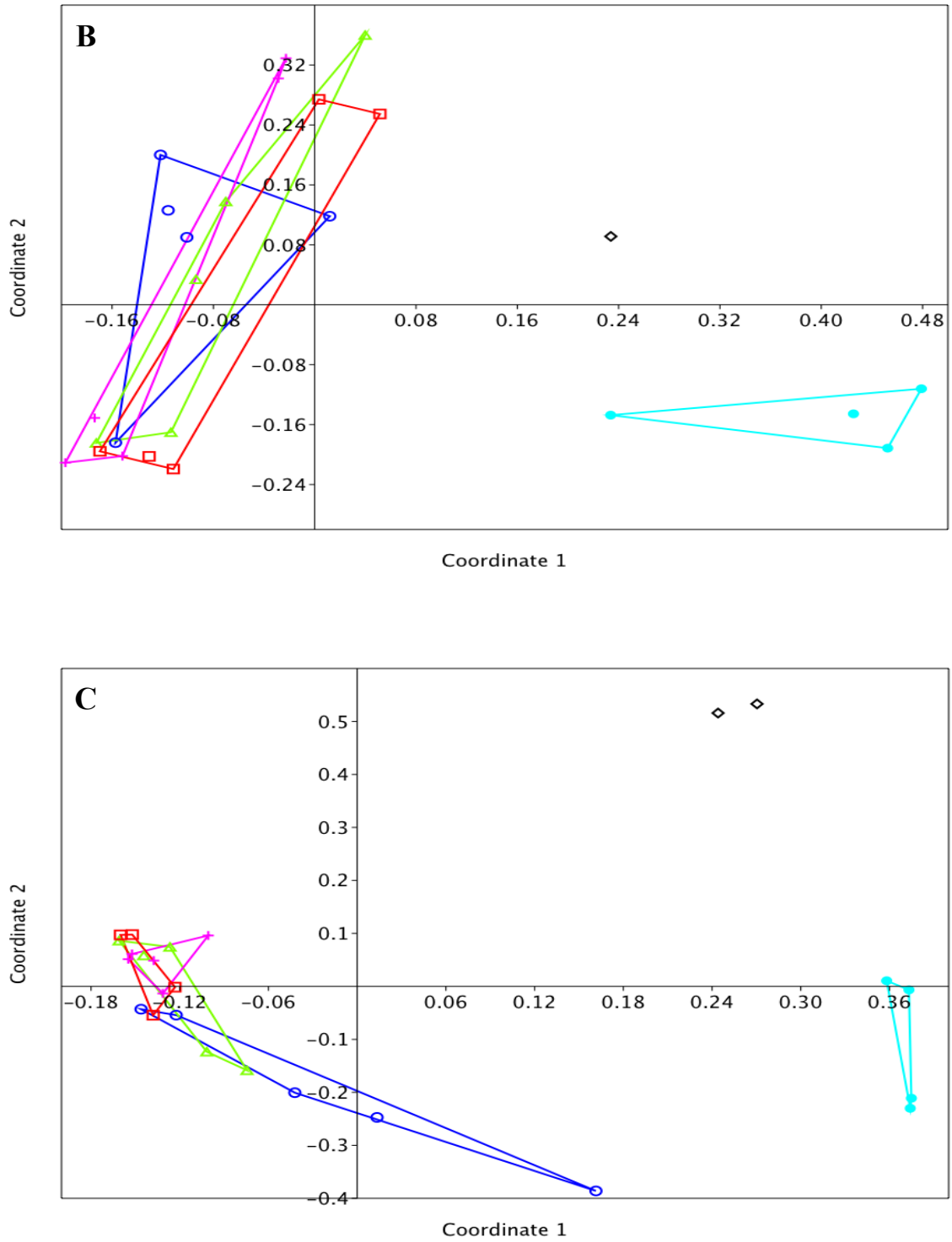


Figure 3.17: PCoA plots based on average Bray-Curtis similarities for comparison of DGGE profiles of faecal samples from 1- and 2-year-old lobster juveniles reared in FTS and MMS. **A:** Samples collected 28/3 and 30/3, when all individuals were reared with FTS (O = 2-year-olds, Δ = 1-year-olds, ● = water, ◊ = feed); **B:** Samples collected 24/4 + 25/4, when water treatment system had been changed to MMS for 50% of the individuals; and **C**: Samples collected at 10/5 + 12/5 where 50% of the individuals were reared with FTS and 50% with MMS (Δ = 1y-FTS, □ = 1y-MMS, + = 2y-FTS, O = 2y-MMS, ● = water, ◊ = feed).

One-way PERMANOVA was performed to investigate whether there were significant differences in microbial community composition between the two raceways at each of the three time points. The test revealed that there were no significant differences in gut microbial community composition between lobsters in R1 and R2 (R1: 1-year-olds; R2: 2-year-olds) at time point 1, prior to the change in water treatment system. At time point 2, which was 25 days and the first time point after the change from FTS to MMS in R2, there were no significant differences in microbial community composition between individuals reared in MMS and FTS. However, at time point three, the second point of time and 35 days after the change of water treatment system in R2, there were significant differences in gut microbiota between individuals reared in the two systems (PERMANOVA, $p = 0.01$). These results indicate that the water treatment system had an effect on the microbial community composition in the gut of lobster juveniles

Average Bray-Curtis similarities were calculated for comparisons of gut microbial community profiles within and between individuals reared in R1 and R2, and FTS and MMS, at the three different time points (Fig. 3.18A-C). At the first point of time the average Bray-Curtis similarity among individuals within R1 was 0.46, while within R2 it was 0.58 (Fig. 3.18A). The similarities indicate that there was more variation between individuals in R1 compared to R2. One-way ANOVA confirmed that there were significant differences in variation between the two groups ($p = 0.009$). At the second point of time, which was the first sampling after the change in water treatment system, the average Bray-Curtis similarity between individuals reared in MMS and FTS was found to be 0.47. In comparison, the average Bray-Curtis similarity within FTS was found to be 0.45, and within MMS it was 0.49 (Fig. 3.18B). This indicates that the microbiota was similar between the two systems. At time point 3, the second point of time after the change in water treatment systems, the average Bray-Curtis similarity between individuals in FTS and MMS was found to be 0.57. In comparison, the average Bray-Curtis similarity was calculated to 0.54 within the FTS and 0.70 within the MMS (Fig. 3.18C). The significantly higher similarity within MMS (ANOVA, $p = 0.0002$) suggests that there were differences in lobster gut microbiota similarity between individuals within the two systems. However, the similarity within FTS was lower compared to the similarity between FTS and MMS.

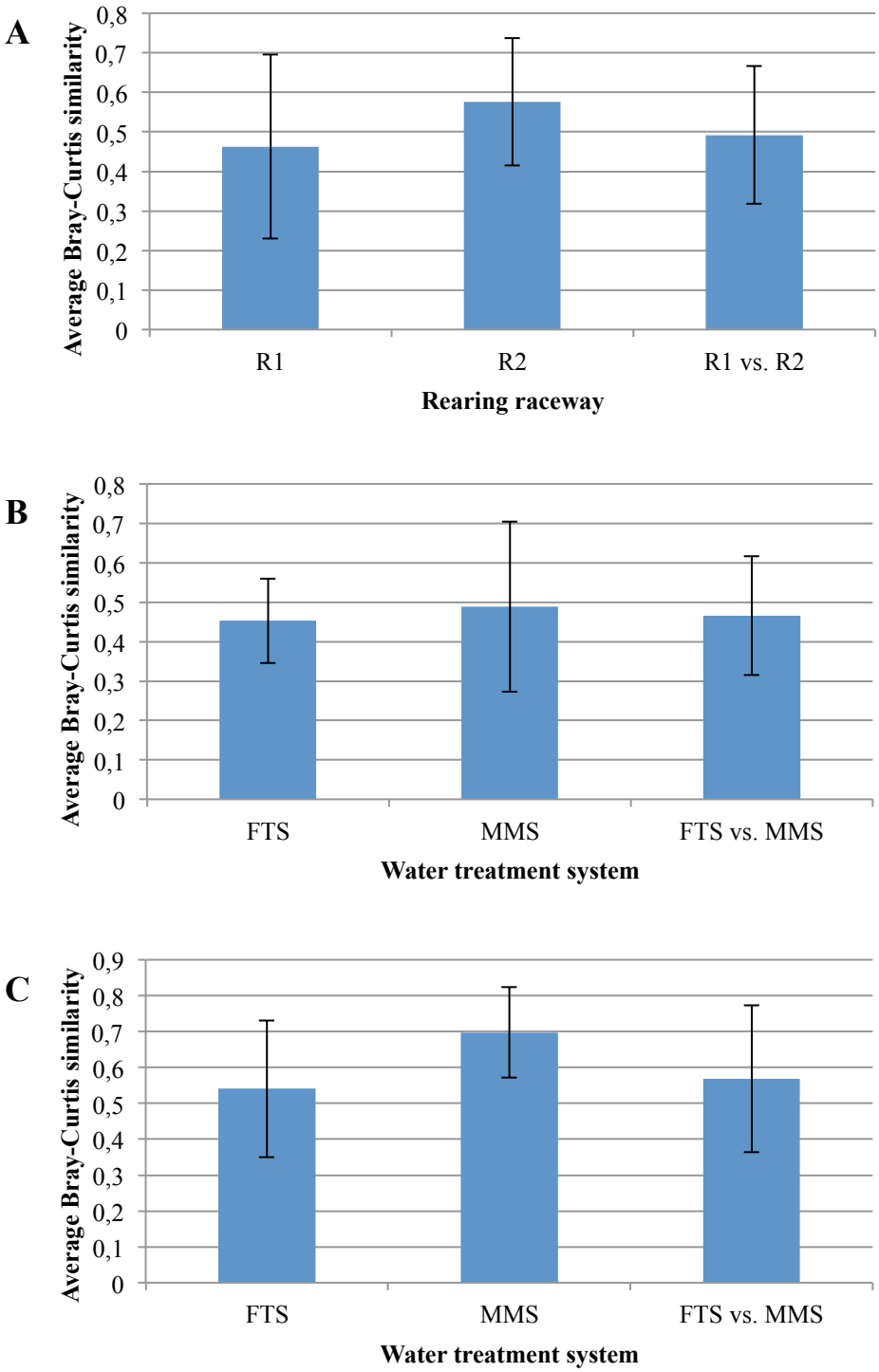


Figure 3.18: Average Bray-Curtis similarities for comparison of DGGE profiles of faecal microbiota in 1- and 2-year-old lobster juveniles reared in FTS and MMS, calculated within and between rearing raceways **A:** Similarities at 28/3 and 30/3, when all individuals were reared with FTS (R1 and R2); **B:** Similarities at 24/4 + 25/4, when water treatment system had been changed to MMS for 50% of the individuals and 50% remained in FTS; and **C**: Similarities at 10/5 + 12/5, where 50 % of the individuals were reared with FTS and 50% with MMS.

Average diversity indices were calculated within individuals in each of the two raceways at time point 1 (28/3 + 30/4), and each water treatment system at time point 2 and 3 (24/4 + 25/4 and 10/5 + 12/5) to study the microbial community diversity between raceways and water treatment systems (Table 3.3). The diversity indices were strikingly similar between the raceways at the first point of time, when all individuals were reared in FTS. Furthermore, there were no significant differences in diversity between individuals reared in FTS and MMS at the second point of time. However, differences in species richness and evenness were found between individuals reared in FTS and MMS at the third point of time. Within individuals in MMS, the band richness was significantly higher compared to individuals in FTS (ANOVA, $p = 0.006$). This indicated that lobster juveniles reared in MMS had more community members in their gut microbiota compared to when reared in FTS, and were hence more diverse. The evenness was also significantly higher within individuals reared in FTS compared to MMS (ANOVA, $p = 0.002$).

Table 3.3: Average band richness (k), Shannon´s diversity index (H`) and evenness (J`) of lobster juvenile faecal microbiota, calculated within each raceway (R1 and R2; time point 1), and water treatment system (FTS and MMS; time point 2 and 3). Standard errors are included.

Time (date)	Water treatment system	Band richness (k)	Shannon index (H`)	Evenness index (J`)
28/3 + 30/4 (Time point 1)	FTS (R1)	22 ± 3.7	2.23 ± 0.22	0.43 ± 0.07
	FTS (R2)	21.9 ± 2.0	2.19 ± 0.16	0.42 ± 0.06
24/4 + 25/4 (Time point 2)	FTS (R1)	23.6 ± 4.1	2.28 ± 0.24	0.43 ± 0.08
	MMS (R2)	22.6 ± 2.6	2.28 ± 0.25	0.44 ± 0.09
10/5 + 12/5 (Time point 3)	FTS (R1)	19.8 ± 3,8	2.02 ± 0.11	0.39 ± 0.06
	MMS (R2)	24.7 ± 2.8	2.03 ± 0.15	0.31 ± 0.03

3.4 Comparison of lobster faecal microbiota with water and feed microbiota

Based on the three DGGE gels and PCoA plots representing samples collected at three different time points in experiment 2 (Fig. 3.17A-C and Fig. 3.17A-C), average Bray-Curtis similarities were calculated for comparisons between faecal and water microbiota and between faecal and feed microbiota (Fig. 3.19). This analysis was carried out to investigate whether the microbiota of feed and water was similar to the gut microbiota of the lobster juveniles, and whether there were differences in similarity between faeces and feed, and faeces and water. The average Bray-Curtis similarities between both feed and faeces, and water and faeces were very low at all three time points (> 0.15), indicating that the bacterial communities of the faeces were highly dissimilar from bacterial communities associated with water and feed. One-way PERMANOVA was used to investigate whether these observed dissimilarities were significant (Table 3.4). The resulting p-values showed that the microbial communities of the juveniles were significantly different from both feed and water at all three points of time. These results suggest that the microbial community composition of the lobster juvenile gut is not a reflection of the community composition of water and feed microbiota. However, average Bray-Curtis similarities between faeces and water microbiota appeared to be slightly higher compared to similarities between faeces and feed microbiota. One-way ANOVA showed that these differences in similarity were not significant.

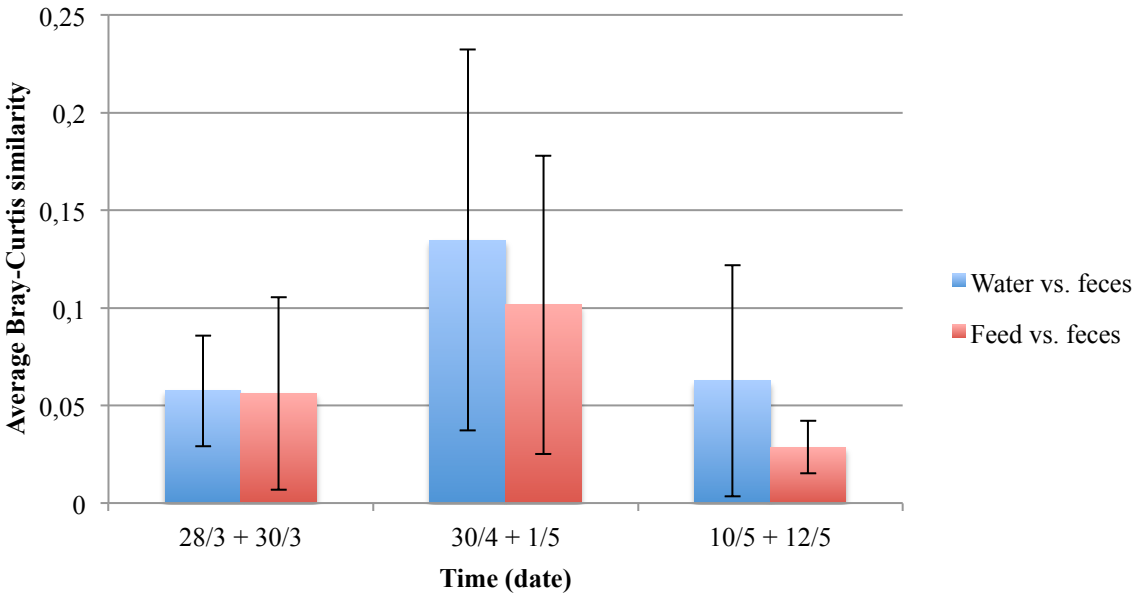


Figure 3.19: Average Bray-Curtis similarities between the microbiota of faecal and rearing water samples, and the microbiota of faecal and feed samples at three different time points for 1- and 2-year-old lobster juveniles in cultivation. Error bars indicate standard errors.

Table 3.4: p-values obtained from one-way PERMANOVA, testing the hypothesis that there are no differences in microbial community composition of lobster juveniles faecal microbiota compared to feed and rearing water microbiota. Significant values are highlighted in red.

	28/3 + 30/3	**24/4 + 25/4**	**10/5 + 12/5**
Faecal vs. water	0.0002	0.0003	0.0002
Faecal vs. feed	0.0042	0.046	0.043

Average Bray-Curtis similarities between feed and faeces microbiota and between water and faeces microbiota in each of the two age groups (1- and 2-year-olds) were calculated to investigate whether there were any age related variance in similarity (Table 3.5). There were indications that the similarity between faeces and feed microbiota was higher in the 1-year-olds compared to the 2-year-olds. However, this variance in similarity between the two age groups was not significant for any of the three sampling times (one-way ANOVA). Similarly, no significant variance in faeces similarity to water was observed between the two age groups. These results suggest that the observed dissimilarity between the microbiota of faeces compared to water and feed was equal in both 1- and 2-year-old lobster juveniles.

Table 3.5: Average Bray-Curtis similarities between faecal microbiota and feed and rearing water microbiota in 1- and 2-year-old lobster juveniles in cultivation.

	28/3 + 30/3	**24/4 + 25/4**	**10/5 + 12/5**
1-year-olds vs feed:	0.07	0.13	0.08
2-year-olds vs. feed	0.05	0.07	0.03
1-year-olds vs. water	0.06	0.15	0.06
2-year-olds vs. water	0.05	0.14	0.10

4 Discussion

4.1 Evaluation of methods

To investigate the community dynamics of lobster juvenile gut microbiota, faecal samples were chosen as the focus of study. Analysing faecal samples rather than the gut tissue itself drastically reduced the number of lobster individuals needed to study temporal development of the gut microbiota, as analysing tissue would have required lobsters to be euthanized at every sampling. The use of faecal samples also enables studies of single individuals over time. The downside to analysing faecal samples, however, is that it excludes the possibility of analysing different sections of the GI tract and, furthermore, it does not provide an exact image of the communities actually colonizing the gut. Nevertheless, the microbial communities present in the faecal samples provided an indication of the communities present in the gut.

Samplings from each of the two experiments were conducted over a maximum time period of 3 months. Studies based on culture-dependent techniques have indicated that the gut microbiota of marine species develops at a high rate in larvae before becoming relatively stable after metamorphosis (Olafsen, 2001, Eddy and Jones, 2002). As lobster juveniles were chosen for this study, it is possible that the microbiota of the juveniles had become relatively stable, and that the individuals should have been studied for a longer period of time to detect potential changes. An extended period of sampling could have provided more information on the effects of water microbiota, feed microbiota, water treatment systems and time on lobster juvenile gut microbiota.

The actual sampling of the lobster faeces required handling the lobsters, as they had to be moved from the raceways to individual containers, and were subsequently weighed. This handling provoked stress responses in the lobsters, which was discovered by increased activity, aggression and a loss of appetite. Stress has been shown to affect the community structure in the gut of mice (Bailey et al., 2011), and it is a possibility that the stress had effects on the lobster gut microbiota in some individuals.

To study the bacterial communities in the lobster gut, a PCR/DGGE strategy was chosen because it is relatively inexpensive and not very time consuming. Compared to cultivation techniques, PCR/DGGE was better suited for the study of microbial communities because the problem with non-cultivable bacterial species was eliminated. Furthermore, PCR/DGGE allowed the analysis of the v3 region of the bacterial 16S rRNA gene of multiple samples simultaneously, enabling the comparison of bacterial community dynamics in different samples. However, there are drawbacks to PCR/DGGE. Due to the difficulty of creating the exact same running conditions and denaturing gradient for every gel, the reproduction of band patterns between gels is inaccurate (Muyzer and Smalla, 1998). This limits the number of samples that can be compared because comparisons of community profiles between gels should be avoided, which might subsequently affect the strength of statistical analyses. Furthermore, DNA from multiple bacterial species may denature at the same localization in the denaturing gradient, and is hence visualized as only one band (Muyzer and Smalla, 1998, Ranjard et al., 2000). DNA from one bacterial strain may also result in multiple bands on the gel, which causes an overestimation of species richness (Muyzer and Smalla, 1998). Another bias to PCR/DGGE is the potential amplification of bacterial DNA present in the Taq polymerase, which may result in bands on the DGGE gel that are interpreted as bacterial DNA from the applied samples (Maiwald et al., 1994). Negative controls were however applied in order to detect large amounts of bacterial DNA from the polymerase.

Regarding resolution, DGGE is an effective method for comparing the effects of external cues on microbial community composition but is less suited for specific taxonomical characterization (Malik et al., 2008, Sanz and Köchling, 2007). High throughput sequencing would have provided high-resolution taxonomical information, compared to medium-resolution with DGGE, but it is also more expensive. For this study, however, the comparison of microbial community compositions between samples was the objective and DGGE was considered to be a suitable method.

The number of tools for DGGE gel computer analysis are limited, and the program Gel2k applied in this study involve a high level of manual processing. The high richness of bands in the produced gels caused Gel2k to make mistakes when determining which bands were identical and, furthermore, multiple bands were not detected by the program. These errors were corrected manually, and subjective decisions had to be made in several cases when it was unclear whether observed bands were identical. However, these potential mistakes were

considered as less important for the general characterization of microbial communities between samples.

Regarding the one-way PERMANOVA test, it was determined to not to apply correction to p-values when comparing multiple groups. This may have led to false positive results (type I error). Bonferroni correction was considered, however, this method is very conservative and would have increased the risk of making a type II error (false negative) and hence differences between groups would potentially not have been detected (Armstrong, 2014).

4.2 Survival and growth

The survival and growth of marine species in rearing provides an indication of the overall experimental conditions and success of the rearing process. In this study, the growth of the lobster juveniles was continuous throughout the experiment, indicating satisfactory rearing conditions. Both within the 1- and 2-year-olds, the weight increase was higher within individuals reared in MMS compared to in FTS. However, the standard deviations were very large and hence this difference in growth was likely not significant. Few studies have been published on the survival rates of lobster juveniles in rearing systems, but survival rates ranging from 80 % - 90 % have been found in 1-year-olds (Tveite and Grimsen, 1995). A study on the survival of European lobster larvae found survival rates ranging from 30 % to 65 % during a time span of approximately 20 days (Øien, 2014). However, the survival rates of larvae are generally lower compared to older individuals. The survival rate of the lobster juveniles in this study ranged from 80 % to 95 % and indicated that the rearing of the lobsters had been successful. A slight difference in survival was detected between the FTS and MMS systems but as the number of individuals was relatively low this was assumed to be random. The overall survival and growth in this study was relatively satisfactory, and it is therefore reasonable to assume that the general rearing conditions did not have a negative impact on the subsequent analyses in this study.

4.3 Temporal development of faecal microbiota in lobster juveniles

When comparing ½-, 1- and 2-year-old lobster juvenile gut microbiota, there were significant differences between ½- year olds and the two other age groups. There were no differences in microbial community structure between the 1- and 2-year-olds. The gut microbiota of marine

species has been shown to change the most at the larval stage, especially during metamorphosis as the digestive tract develops and there often is a shift in diet (Olafsen, 2001, Eddy and Jones, 2002). Other studies have provided indications that the microbiota of the gut in fish develops during first-feeding, after which it remains relatively stable (Romero and Navarrete, 2006). It is assumed that the structure of GI microbial communities changes during the development of the GI tract due to changes in selection pressure during this process, and subsequently becomes relatively stable when the development of the gut is complete (Kohl et al., 2013, Palmer et al., 2007). The results in this study may be explained by that the gut microbiota of ½-year-old, developing lobsters has not yet stabilized, while it is relatively stable by the time lobsters have become 1 year of age.

However, it should be taken into consideration that environmental factors could have affected the gut microbiota. The ½-year-old lobster juveniles had been reared in the experiment FTS since the larval stage, as opposed to the 1- and 2-year-old lobster juveniles, which were transferred from a separate rearing facility. The implications of this differential treatment are that the gut microbiota of the ½-year-olds had more time to develop in response to local external cues compared to those of the other two age groups. It is possible that the gut microbiota of the 1- and 2-year-olds were established based on the environment in the system in which they developed, and hence were fundamentally different from the microbiota of the ½-year-olds. Furthermore, the ½-year-olds were fed a commercial artemia mixture, while the older juveniles were fed shrimp. This difference in feed might have contributed to the difference in faecal microbiota. It is also a possibility that there were differences in tank water microbiota between the rearing of the ½-year-olds and the 1- and 2-year-olds, which further could have led to dissimilar effects on the gut microbiota of the juveniles. Finally, the ½-year-old lobster individuals were all descendants of the same mother, while it is not known whether the 1- and 2-year-olds had different mothers originating from different locations. Genetic differences in the host may cause inter-individual variation in the gut microbiota (Smith et al., 2015), and this may be the explanation for why the gut microbiota of the 1- and 2-year-olds were different from that of the ½-year-olds.

Within both the 1-year-old and 2-year-old age groups, no significant differences in gut microbial community composition were found between sampling dates. These results show that there was no clear temporal development of community composition during the 3-month long experiment. These findings coincide with the findings when ½- 1- and 2-year-olds were

compared, which showed no differences in gut microbiota between the 1- and 2-year-olds. The absence of change in gut microbiota could be due to a stabile, commensal gut microbiota in lobsters that are 1 and 2 years old. No studies have been conducted on temporal development of gut microbiota in lobster juveniles, and hence it is not known when a potential stabilization occurs.

However, there was an increase in inter-individual similarity over time in both age groups, showing that the gut microbiota of the 1- and 2-year-old individuals became more similar between individuals over the 3 months. Diversity indices indicated a slightly higher diversity of 1 year-old individual gut microbiota within early sampling dates compared to later sampling dates. As the lobsters were retrieved from a different rearing system, the gut microbiota could have been affected by the move, causing variation in the microbiota depending on each individual at early sampling dates. Another possible explanation is that the gut microbiota of the 1-year-old lobsters became less varied between individuals over time as it stabilized and adapted to the new external conditions. In the 2-year-old age group, no trends of change in diversity indices were found. The difference between the 1- and 2-year-olds might reflect a more stable gut microbiota in the 2-year-olds, and also that this group was less affected by being moved to a new rearing facility.

4.4 Effects of water treatment on faecal microbiota in lobster juveniles

When comparing the individual gut microbiota composition between raceway 1 and 2, 35 days prior to the change to MMS, no significant differences were observed. This excludes the possibility of tank effect on the gut microbiota. As 1-year-olds were reared in raceway 1, and 2-year-olds in raceway 2, these results also support previous findings in this study of there being no differences in gut microbiota between the two age groups.

Likewise, there were no significant differences in microbial community composition in the gut of lobster juveniles reared in FTS compared to MMS 25 days after introduction to MMS. However, there were significant differences between FTS and MMS individuals 35 days post MMS introduction. These results suggest that the water treatment affected the microbial community structure in the gut of the lobster juveniles. Microbial maturation of rearing water has shown increased performance of marine larvae, for example in the European lobster, the Atlantic halibut, cod and turbot (Øien, 2014, Skjermo et al., 1997, Attramadal et al., 2012,

Salvesen et al., 1999). This high performance is thought to be due to the high level of microbial stability in microbial maturation systems, which reduces the level of *r*-strategic, opportunistic and potentially pathogenic bacteria in the rearing water (Skjermo et al., 1997). No studies have been published on the effect of microbial matured rearing water on lobster juvenile performance or gut microbiota. However, it is possible that the differences in gut microbiota between individuals reared in MMS and FTS were due to effects of the microbial stability in the rearing water of each system. Furthermore, it appeared as if the effects of the water treatment systems required some time to become evident, as differences were not observed 25 days post introduction to MMS but was observed after 35 days.

Average Bray-Curtis similarities showed that the variation among individuals reared within MMS compared to FTS was similar at the first point of time, 25 days after the change in water treatment system. However, at the second point of time, 35 days after the change the similarity among individuals was higher in the MMS system. This provides indications that the MMS created a more stable environment for the juveniles, and reduced the variation between individuals. These results support that a development in response to the water treatment system occurred. However, more research on the effects of water treatment systems and microbial stability on lobster gut microbiota must be performed to verify the results found in this study, and further to understand the mechanisms of the effects.

4.5 Comparison of lobster faecal microbiota with water and feed microbiota

There were substantial and significant differences in microbial community composition when comparing gut microbiota with both feed and water microbiota. To date there have been no studies published on this particular topic regarding lobsters or other crustaceans but similar studies have been conducted on other marine species. It has generally been assumed that the gut microbial communities at larval stages are a reflection of communities present in the environment, including the microbiota of feed and water (Nayak, 2010, Llewellyn et al., 2014, Austin, 2002). However, recent studies performed on marine species such as cod, orange-spotted grouper, zebrafish and tiger shrimp have found that the gut microbiota is highly dissimilar to the microbiota of both culture water and feed, and coincides with the findings in this study (Bakke et al., 2015, Sun et al., 2013, Yan et al., 2012, McIntosh et al., 2008, Chaiyapechara et al., 2012, Sullam et al., 2012). These results indicate that the gut microbiota composition is a result of selective pressure posed by the host or the commensal

gut microbiota, and is not a passive reflection of microbial communities present in external factors such as feed and water (Sullam et al., 2012). The results obtained in this study strongly suggest that this is also the case for lobster juveniles.

Notwithstanding the foregoing, it has been found that the composition of microbial communities in the gut of fish is affected by the properties of the diet (Ingerslev et al., 2014, Miyake et al., 2015). Specific nutrients available in different prey organisms may provide more favourable conditions for some bacteria compared to others and hence may affect the microbiota composition. This does not necessarily mean that the microbiota of the faeces is similar to the feed microbiota. As the lobster juveniles received the same feed during the entire experiment, it was not possible to determine whether the feed itself affected the gut microbiota of the lobsters. In summary, it is possible that the microbial communities in the feed affected the gut microbiota of the lobster juveniles but the community structures were highly dissimilar when feed and faeces were compared. Hence, there are indications that the majority of bacteria in the feed do not become established in the lobster gut, most likely due to selective pressure posed by the commensal microbiota and the host (Sullam et al., 2012).

The gut microbiota of the lobster juveniles were similarly different from both water and feed microbiota. Some fluctuations in similarity was observed, however these were not significant. In contrast to these findings, research conducted on cod found that the microbial communities in the gut were more similar to the communities associated with water compared to feed associated communities (Bakke et al., 2013). A possible explanation for these opposing results is that cod larvae were used in the experiment by Bakke et. al. while significantly older lobster juveniles were studied in this experiment. Hence, the differences might be age related. Secondly, taxonomic differences could be the cause of the differences in results (Li et al., 2012). No differences in gut community similarity to water and feed communities were observed when comparing the 1- and 2-year-olds. No studies on the effects of water and feed microbiota on lobster gut microbiota at different life stages have been published, but several studies on other species previously mentioned have revealed little similarity between feed/water communities and gut communities of both larvae and juveniles. However, the number of water and feed samples in this study were very limited, the average Bray-Curtis similarities were based on few comparisons, and the standard deviations were large. These factors make it difficult to detect differences between water/gut and feed/gut similarities.

Despite the substantial difference between water microbiota and lobster juvenile gut microbiota, the different water treatments did result in a significant difference in gut microbiota. This suggests that the water microbiota does have an effect on the bacterial community structure in the gut of the lobster, even though this did not involve high similarity between water and gut microbiota. The application of 16S-amplicon sequencing could have contributed to an increased understanding of how the bacterial communities in the rearing water affected the microbiota in the lobster gut.

4.6 Future work

In this thesis, lobster juveniles that were ½-, 1- and 2 years old were studied. Hence, all lobsters were post-metamorphosis. For future studies, lobsters at pre-metamorphosis larval stages should be included to provide more information on the dynamics of the gut microbiota at different life stages. The study of lobster larvae may also provide information on the establishment of the gut microbiota, which is a scientific area where knowledge is scarce.

It was shown in this study that water treatment had an effect on the lobster juvenile gut microbiota. However, the number of water samples included in this experiment was low, which limited the possibility to perform statistical analyses. Studies including a higher number of water samples would allow more thorough statistical testing, and may contribute to the understanding of how the rearing water, and the microbial stability in the rearing water, affects the gut microbiota of the lobster. Regarding water treatment, it would also be interesting to include RAS in future experiments as it provides even more benefits than the MMS in terms of microbial stability. In this study, the water treatment system was changed approximately halfway through the experiment. In future studies on the effect of different water treatment systems on lobster survival, growth and gut microbiota the lobsters should be reared in the respective systems from the beginning of the experiment and for a longer period of time. This could contribute to confirming the effect of water treatment, as was observed in this study, and determining how strong the effects are. As larvae are more easily influenced by external factors compared to juveniles, the study of larvae could be an approach to understanding the effects of water treatment.

The microbiota of the feed and lobster gut was compared in this study, and was found to be highly dissimilar. However, the feed may still have had an effect on the lobster juvenile gut

microbiota. Conducting a study where juvenile lobsters are reared in separate systems and fed different feed could provide indications on whether the feed affects the gut microbiota.

Regarding future studies on European lobster gut microbiota, Illumina sequencing should be applied as an approach to retrieving more, high-resolution taxonomic information and knowledge on the microbial communities, their composition, development and effects on the lobster juvenile. More knowledge on the establishment and development of the gut microbiota in the lobster may contribute to reducing the high mortality rates observed in aquaculture of lobsters at early life stages, and could further enable more efficient and cost-effective rearing of the European lobster.

5 Conclusions

- The microbial community composition in the gut of ½-year-old lobster juveniles was significantly different from community composition in 1- and 2-year-old lobster juveniles. The gut microbiota was similar in 1- and 2-year-olds, indicating a stable gut microbiota when lobsters have become 1 years of age.

- The gut microbiota of 1- and 2-year-old lobster juveniles did not change during a time period of two months, which support the finding of a stable gut microbiota in lobsters from 1 years of age.

- Water treatment system affected the composition of the lobster juvenile gut microbiota.

- The microbial community composition of feed and rearing water was highly dissimilar to the gut community composition of both 1- and 2-year-old lobster juveniles, indicating a highly selective gut environment.

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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 Recipes for DGGE solutions

0 % denaturing acrylamide solution:

8% acrylamide in 0,5 x TAE (per 250 ml):

- 50 ml 40% acrylamide solution (BioRad)
- 2,5 ml 50 x TAE
- Must be stored at 4 °C, protected from light

80 % denaturing acrylamide solution:

8% acrylamide, 5.6M urea, 32% formamide in 0,5 x TAE (per 250 ml):

- 50 ml 40% acrylamide solution (BioRad)
- 2,5 ml 50 x TAE
- 84 g urea
- 80 ml Deionized formamide
- Stir to dissolve urea
- Must be stored at 4℃, protected from light
- Solution must be filtered before use

50 x TAE-buffer

Per litre:

- 242 g tris base
- 57.1 ml glacial acetic acid
- 100 ml 0.5 M EDTA (pH 8.0)

Autoclave the buffer

Deionized formamide:

- 200 ml formamide
- 7.5 g DOWEX RESIN AG 501X8
- Stirr for 1 hour at room temperature.

Appendix 3 p-values from one-way ANOVA

Table A: p-values resulting from a series of one-way ANOVA tests comparing the average Bray-Curtis similarities of faecal microbiota between 2-year-old lobster individuals for all sampling dates.

	14/2	**7/3**	**21/3**	**25/4**	**30/4**	**12/5**
14/2						
7/3	0,0231					
21/3	0,675	0,0033				
25/4	0,1561	0,0596	0,0099			
30/4	0,4682	0,0043	0,6464	0,0185		
12/5	0,0283	0,0003	0,0107	0,0001	0,0980	

Table B: p-values resulting from a series of one-way ANOVA tests comparing the average Bray-Curtis similarities of faecal microbiota in 1-year-old lobster juveniles between sampling dates.

	13/2	**28/2**	**28/3**	**25/4**	**30/4**	**12/5**
13/2						
28/2	0,66					
28/3	0,066	0,046				
25/4	0,005	0,005	0,399			
30/4	0,0006	0,00089	0,137	0,410		
12/5	0,00016	0,00045	0,046	0,110	0,272	

Appendix 4 Sampling regimes

Table A: Sampling dates of faecal, water and feed samples from ½-year-old lobster juveniles

½-year-olds (FTS)							
Sampling dates:	07.11.13	20.11.13	05.12.13	20.12.13	10.01.14	23.01. 14	20.02. 14

Table B: Sampling dates of faecal, water and feed samples from 1- and 2-year-old lobster juveniles

1-year-olds (FTS, R1)						
Sampling dates:	13.02.14	20.02.14	28.02.14	06.03.14	13.03.14	28.03.14
2-year-olds (FTS, R2)						
Sampling dates:	14.02.14	25.02.14	07.03.14	21.03.14	30.03.14	
Introduction to MMS for 50 % of individuals from each age group						
MMS (R2)						
Sampling dates:	24.04.14		01.05.14		10.05.14	
FTS (R1)						
Sampling dates:	25.04.14		30.04.14		12.05.14	