Line Moen

Nitrification in marine RAS

Nitrifying capacity and nitrifying communities of biofilters in RAS for production of Atlantic cod and Atlantic salmon.

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Master's thesis in Biotechnology
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Abstract

In this master thesis, the nitrifying capacity, salinity tolerance, and microbial biofilm community composition of biofilters operated at high salinities were investigated. Lab-scale batch experiments were conducted with biofilm carriers from two commercial RAS: one cultivating Atlantic Cod at 35 ppt and one cultivating post-smolt at 15 ppt. Furthermore, the microbial community compositions of biofilm samples from three different commercial RAS operated at high salinities were investigated. Havlandet has a RAS operated at 35 ppt for the production of Atlantic cod and Erko has a RAS operated at 15 ppt for the production of post-smolt of Atlantic salmon, both using moving-bed bioreactors. Lerøy Belsvik has a RAS operated at 22 ppt for the production of post-smolt, using a fixed-bed bioreactor.

Illumina sequencing showed that *Nitrospira* and *Nitrosomonas* were the most important NOB and AOBs, with *Nitrospira* dominating the bacterial nitrifying communities, and ASVs representing *Nitrospira* were the most significant contributors to the difference in the biofilm community composition between the facilities. The biofilm carriers from Erko showed superior nitrification capacity with a max capacity of 0.12 mg L^{-1} min^{-1} and had both a three times higher nitrifying capacity and relative abundance of nitrifiers than the biofilm carriers from Havlandet. The capacity of the Erko carriers was maintained both in 31 ppt salinity and in freshwater. The nitrifying capacity was reduced by 50% in freshwater for biofilm carriers from Havlandet, compared to brackish and seawater.

A low abundance of AOB was found in all biofilm samples, indicating that AOAs might contribute to ammonia oxidation. The archaeal communities in the biofilm samples from Havlandet and Erko were entirely dominated by one ASV representing *Nitrosopumilus*, known to oxidize ammonia. Another ASV, also representing *Nitrosopumilus*, dominated the biofilm communities in samples from Belsvik. Sanger sequencing and qPCR confirmed the presence of an *amoA* gene sequence in the samples from Havlandet and Erko that were identical to a previously reported for *Nitrosopumilus oxyclinae*. The results from Sanger sequencing indicated the presence of multiple *amoA* gene sequences in samples from Belsvik.

Sammendrag

I denne masteroppgaven ble nitrifiseringskapasitet, sensitivitet for salinitet og de mikrobielle biofilmsamfunn i biofiltre operert ved høye saliniteter undersøkt. Lab-skala batch-eksperimenter ble utført med biofilm bærere fra to kommersielle RAS: en for produksjon av atlantisk torsk ved 35 ppt salinitet og en som for produksjon post-smolt av Atlantisk laks ved 15 ppt. Videre ble sammensetningen av de mikrobielle samfunnene i biofilmprøver fra tre ulike kommersielle RAS med høy salinitet undersøkt. Havlandet har et RAS som produserer atlantisk torsk ved 35 ppt, og Erko har et RAS som produserer post-smolt ved 15 ppt, hvor begge bruker moving-bed biofilm reaktorer. Lerøy Belsvik har et RAS som produserer post-smolt ved 22 ppt, hvor de bruker en fixed-bed biofilm reaktor.

Illumina-sekvenseringen viste at *Nitrospira* og *Nitrosomonas* var de viktigste nitritt- og ammoniumoksiderende bakteriene, hvor *Nitrospira* dominerte de bakterielle nitrifiserende samfunnene, og ASVer som representerte *Nitrospira* var de viktigste bidragsyterne til forskjellen i sammensetningen av biofilmsamfunnene mellom anleggene. Biofilm bærerne fra Erko viste overlegen nitrifiseringskapasitet, med en makskapasitet på 0.12 mg L^{-1} min^{-1} ,og hadde både tre ganger så høy nitrifiseringskapasitet og relativ mengde nitrifiserende bakterier enn biofilm bærerne fra Havlandet. Kapasiteten til bærerne fra Erko ble opprettholdt både ved 31 ppt og i ferskvann. Nitrifiseringskapasiteten ble redusert med 50% i ferskvann for biofilm bærerne fra Havlandet, sammenlignet med brakk- og sjøvann.

En liten mengde ammoniumoksiderende bakterier ble funnet i biofilm-prøvene, noe som indikerte at ammoniumoksiderende arker kan bidra til oksidering av ammonium. Arkesamfunnene i biofilm-prøvene fra Havlandet og Erko var fullstendig dominert av en ASV som representerte *Nitrosopumilus*, en arke kjent for å oksidere ammonium. En annen ASV, som også representerte *Nitrosopumilus*, dominerte arkesamfunnet i biofilmprøvene fra Belsvik. Sanger-sekvensering og qPCR bekreftet tilstedeværelsen av en *amoA*-gensekvens i prøvene fra Havlandet og Erko som var identisk den en tidligere rapportert *Nitrosopumilus oxyclinae*. Resultatene indikerte også tilstedeværelsen av flere amoA-gensekvenser i prøvene fra Belsvik.

Acknowledgement

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List of abbreviations

Abbreviations	Meaning
AmoA	Ammonia monooxygenase
AOA	Analysis of cariance
AOB	Ammonia-oxidizing archaea
ASV	Ammonia-oxidizing bacteria
CMS	Cardiomyopathy Syndrome
CN	Copy number
Comammox	Complete ammonia oxidation
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
FBBR	Fixed-bed bioreactor
FEAP	The Federation of European Aquaculture Producers
FTS	Flow-through system
H_PS	Havlandet during the production of post-smolt
H_Cod	Havlandet during the production of cod
MBBR	Moving-bed bioreactor
MT	Metric tonnes (1,000 kilogram)
NOB	Nitrite-oxidizing bacteria
NSC	Norwegian Sequencing Centre
NTC	Non-template control
NXR	Nitrite oxidoreductase
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariant analysis of variance
ppt	parts per thousand
qPCR	real-time polymerase chain reaction
RAS	Recirculating aquaculture system
RDP	Ribosomal database project
rRNA	Ribosomal ribonucleic acid
SIMPER	Similarity percentage
TAN	Total ammonia nitrogen
Tukey HSD	Tukey's honest significant difference

1   Introduction

The ocean which was considered an unlimited source of fishery products is now estimated to have reached, or will soon reach, the limit for the sustainable yield of many species. The fast-growing population require new and improved technology for more efficient production of fish [1]. The annual Fish Health Report (Fiskehelserapport) from the Norwegian Veterinary Institute (Veterinærinstituttet) [2] reported high mortality numbers for both salmon and lumpfish in 2021. A total of 54 million salmon died following their transfer to sea cages, representing 15,5% of the total fish population. There are several different causes of high mortality. Trauma linked to mechanical removal of lice and cardiomyopathy syndrome, CMS, constitute the most common causes. Even though a lot of resources have been put into making the production more sustainable, and many good results have come from it, the fish mortality in the industry is still too high. The biggest challenge in the salmon industry is the problem with salmon lice in sea cages. Delousing is both an expensive treatment and to some degree harmful for the fish. A high prevalence of salmon lice can have a negative impact on wild salmon. If salmon were kept in land-based systems for a more extended period after smoltification, where rearing conditions are highly controlled, they would be bigger and more robust, decreasing the lice issue, and decreasing the problems linked to the sea cages due to spending less time there [2]. The increasing demand for food further drives the industry to advance, and in the context of fish production, the discovery and development in the cultivation of new species can be revolutionary.

1.1   Aquaculture in Norway

According to the annual report from the Federation of European aquaculture producers, FEAP, from 2022, Norway was the main producer of fish in Europe, accounting for 58% of the total supply [3]. The industry had a breakthrough in 1970 after a successful release of salmon to seawater. Since then, there has been strong development and growth in the industry. Atlantic salmon is the main species produced in Norway. Salmon is an anadromous species, meaning they inhibit both freshwater and saltwater. Their lifecycle starts with spawning and juvenile rearing in rivers, followed by migration to seawater to feed, grow and mature. Finally, they return to freshwater to spawn [4]. Salmon is mainly cultivated in land-based facilities during the freshwater phase and later put out in sea cages until fully grown. New technology has enabled also the sea phase to be on land in marine facilities.

In addition to salmon, there is increased production of trout, cod, halibut and several more species. During the 2000s, intensive attempts at cod farming were made in Norway, Iceland, the Faroe Islands and Canada. However, Norway's attempt was unsuccessful due to insufficient knowledge about cod biology and an economic breakdown in Europe. The Atlantic cod population is known to be unpredictable, and there have been reports of flocculation in quotas over the years. Moreover, the traditional fishery in Norway is highly seasonal, posing further challenges. To overcome these issues, various strategies have been proposed, including stock enhancement, capture-based aquaculture, and land-based cod farms [5].

In 2020, 1.5 million tonnes of Norwegian fish were produced and sold. In comparison, the number of sold farmed fish in 1994 was just above 200,000 tonnes. That is an increase of 59 billion kroner in 26 years [6]. One of the key factors driving the growth of aquaculture in Norway is the development of new farming techniques, such as closed-containment systems and land-based facilities. These methods allow for greater control over the production environment, enabling farmers to optimize feeding, water quality, and other factors that affect fish health and growth. The growth of land-based salmon farming in Norway has also led to innovations in breeding and genetics, resulting in the development of faster-growing, more disease-resistant salmon strains. This has helped to improve the overall efficiency and sustainability of the industry [7].

1.2 Recirculating aquaculture systems

Recirculating aquaculture system (RAS) is a type of land-based aquaculture system where the water is re-used, in comparison to a flow-through system (FTS) where the water is used only once (Figure 1.1). FTS was previously the most used system for land-based production, but due to the advantages of RAS, FTS is becoming less common [1]. RAS gives a unique opportunity for control and stability of the system, with optimal temperatures throughout the year and management of the rearing conditions (i.e. oxygen, carbon dioxide, pH, alkalinity and total ammonia nitrogen) which again creates an optimal environment for the fish. Due to the recirculation of the water, RAS use significantly less water than a FTS. A disadvantage of RAS is that the water needs to be treated before being reused. This requires advanced technology in addition to being an expensive process. Compared to FTS, RAS requires higher operational complexity, more capital expenditure, land area and higher energy demand. RAS can in theory be placed anywhere in close proximity to a water source, while a FTS needs to be by a coastline. This gives RAS the advantage to be placed closer to the market, which will reduce emissions and costs linked to transport [8]. There is a shift from ocean-based production to more intensive land-based production. RAS is an environmentally friendly technology which can provide

the world per capita needs for aquatic species [1]. Due to the increasing global water crisis, the aquaculture industry is facing an increasing access limitation to freshwater. Technologies and methods to decrease water use are more important than ever [9].
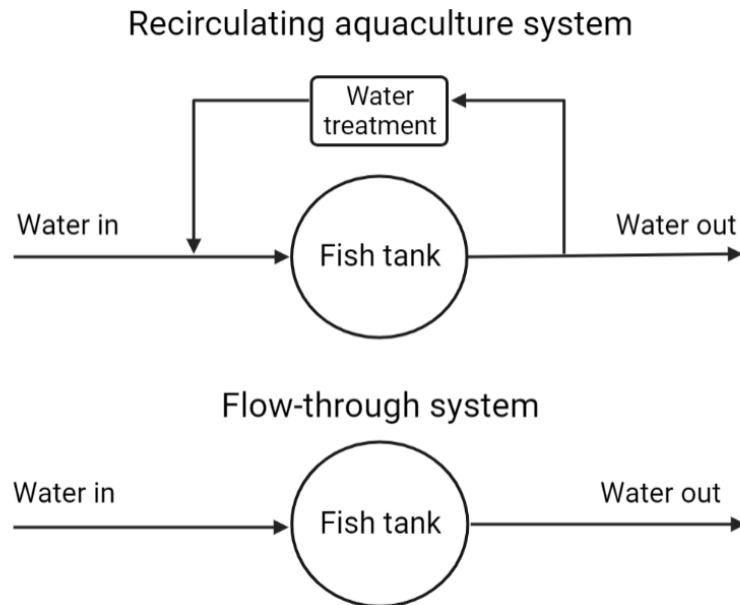
Figure 1.1: Illustration comparing a recirculating aquaculture system (top) and a flow-through system (bottom).

1.3   Water treatment in RAS

Due to the reuse of water in RAS, the circulating water needs proper treatment before being returned to the system. The treatment loop in a RAS usually consists of a mechanical filter, biofilter, degasser and oxygen control [8]. The mechanical filter can be e.g. a drum filter or a belt filter and is responsible for the removal of particles in the recirculating water. Particles originate from feed, faeces and bacteria from biofilters and biofilm in the system[10]. Accumulation of particles will affect both the fish and the bacteria in the system. The biofilter removes toxic ammonia and nitrite. A biofilter can be either emerged or submerged. A trickling filter, where wastewater cascades over the biofilm media, is an example of an emerged biofilter, while moving-bed bioreactor, MBBR, and fixed-bed bioreactor, FBBR, are examples of submerged biofilters. In a MBBR, the biofilm carriers remain in suspension while the wastewater run through the biofilter. In a FBBR, the biofilm grows on surfaces with a fixed position in the system[10]. The degasser removes CO_2 in the water. CO_2 accumulates in the system and is toxic for the fish [11]. Before the water is recirculated into the fish tank again, oxygen is added [10].

Fish excrete ammonia mainly in the form of unionized ammonia through their gills [12]. Without water treatment for the recirculating water, the ammonia will accumulate and be toxic to the fish. In RAS, biological water treatment is used to convert toxic ammonia to less toxic nitrate. Ammonia, nitrite and nitrate have high solubility in water. Ammonia exists in two forms: ionized, NH_4^+(ammonium) and unionized NH_3(ammonia), and the sum of ammonia and ammonium is total ammonia nitrogen, TAN. Both ammonium and ammonia are toxic at high concentrations, but ammonia is much more toxic [13]. The proportion of ammonia and ammonium is determined mainly by pH. At pH below 9, most ammonia is in the form of NH_4^+. Nitrification is the microbial process of converting TAN, NH_4^+/NH_3 to nitrate, NO_3^-. This is a two-step process, where ammonia-oxidizing bacteria or ammonia-oxidizing archaea, AOB or AOA, convert ammonium to nitrite, NO_2^-, and nitrite-oxidizing bacteria, NOB, convert nitrite to nitrate, as shown in Equation 1.1 [14].

$$NH_{4\,(toxic)}^+ \xrightarrow{AOB} NO_{2\,(toxic)}^- \xrightarrow{NOB} NO_{3\,(non-toxic)}^- \tag{1.1}$$
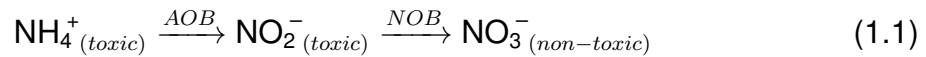
AOB oxidize ammonium as shown in Equation 1.2. *Nitrosomonas* and *Nitrococcus* are examples of AOBs, whilst *Nitrosopumilus* is a common AOA. Nitrite is then oxidized by NOB as shown in Equation 1.3. Examples of NOBs are *Nitrobacter, Nitrospira, Nitrospina* and *Nitrococcus.* The entire nitrification process can be summarized as shown in Equation 1.4 [14].

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \tag{1.2}$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \tag{1.3}$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \tag{1.4}$$
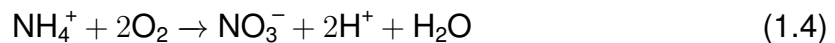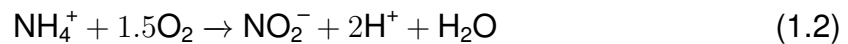
It has recently been discovered that nitrifying microorganisms can oxidize both ammonium and nitrite to nitrate. This process is called complete ammonia oxidation, comammox. All comammox discovered per 2022 are identified as *Nitrospira* strains. A low AOB:NOB ratio has been discovered in freshwater RAS, which can indicate the presence of comammox organisms [15].

1.4 Parameters affecting the biofilter

Autotrophic bacteria, like ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), use oxygen as the oxidizing agent. In the biofilter, the bacteria grow in a biofilm. Biofilm carriers can be made from various materials like plastic or lightweight expanded clay aggregate (LECA), delivering a high surface area. To assure optimal bacteria growth in the biofilters, several factors are important, such as substrate and oxygen concentration, temperature, organic matter, pH and salinity[10].

In nitrification, the presence of sufficient substrate is critical, and a low concentration of substrate can limit nitrification. The threshold of TAN and nitrite is 2 mg/L and 0.1 mg/L respectively, and concentrations below this can give a reduction in nitrification rate. On the other hand, a high concentration of TAN or nitrite can inhibit nitrification. In RAS, the substrate concentration is usually low, and therefore, the limitation of the substrate is a bigger issue than the inhibition by substrate[13].

Heterotrophic bacteria, which use organic matter as a carbon source, have a faster growth rate than nitrifying bacteria and coexist with nitrifying bacteria in the biofilter. High concentrations of dissolved and particulate organic matter can cause heterotrophic bacteria to outcompete nitrifying bacteria for oxygen and space in the biofilter, inhibiting nitrification. A low concentration of oxygen will therefore inhibit nitrification [13].

The efficiency of the biofilter is significantly affected by the pH. The optimal pH for *Nitrosomonas* is 7.2-7.8 and for *Nitrobacter* 7.2-8.2. Nitrification has a relatively wide optimum pH range of 7-9, due to the bacterial adaptation to the rearing conditions. In RAS, it is advised to keep the pH at the lower range to minimize ammonia stress on the cultivated species. Rapid changes in pH, up to one unit during a short time span can stress the biofilter and time to adapt to the new conditions is required. Nitrification releases H^+, causing a reduction in pH levels. Carbonate is therefore much used as a buffer to keep pH at a neutral level [10].

The temperature also affects the biofilter, with a wide range of optimum temperatures for nitrification. For nitrifying bacteria, the optimal temperature range is typically between 25°C to 30°C, although some species can tolerate temperatures up to 40°C or down to 5°C. AOB and NOB have different optimal temperature ranges, with AOB being more sensitive to low temperatures and NOB being more sensitive to high temperatures [10]. Nitrifying bacteria can adapt to a broad range of temperatures, and the optimum temperature for the cultivated species determines the temperature at which the system is operated at [16].

Salinity can have a significant impact on the performance of a biofilter, particularly on nitrification. The presence of salt in the water can inhibit the activity of nitrifying bacteria, which can negatively affect the efficiency of the biofilter [17]. A change in salinity can change the osmotic balance in a microorganism. Hyperosmotic shock can occur when the salinity is increased rapidly. This causes inhibition of cell growth and nutrient uptake due to dehydration and plasmolysis [17]. Some microorganisms are adapted to high salinity and are able to tolerate high-saline environments. These are called halophiles. On the other hand, a decrease in salinity also has a negative effect on microorganisms. Navada et al. [18] reported from a study looking into strategies for increasing salinities in RAS that the nitrification rate can be reduced in marine water compared to freshwater. However, nitrifying bacteria can adapt to different salinity levels given enough time. It takes significantly longer for a biofilter to acclimate to saltwater than freshwater. An increase in salinity can also have a greater effect on nitrite oxidation than ammonia oxidation [18].

1.5 Marine recirculating aquaculture systems

RAS is widely used for the production of freshwater species like salmon, trout and bass. Now, there is an increasing interest in producing marine stages of salmon in RAS, in addition to other marine species. The marine RAS industry in Norway has seen significant growth and developments in recent years. One of the key players in this is Erko Seafood, who built the first marine RAS facility in Sagvåg at Stord in 2015. The facility produced post-smolt that was moved to sea cages at a later stage than smolt [19]. Another major contributor to the marine RAS industry in Norway is Fredrikstad Seafood, which operated the country's first large-scale land-based salmon farm. Since its establishment in May 2019, the facility has produced Atlantic Salmon of 3.5 to 6.0 kilos, with an annual production capacity of 1500 MT [20]. Lerøy Seafood As was established in 1899 and is today the second biggest producer of Atlantic salmon and trout in Norway. In early 2022, their new post-smolt facility was completed, with a planned production capacity of 5000 MT [21]. Havlandet Marin Yngel received in May 2020 the first concession for a cod hatchery in Norway which was ready in 2022. They also had a goal to establish a facility producing cod up to slaughter, and the first production cycle was expected to be completed in the summer of 2023 [22]. After an incident of H_2S poisoning at Havlandet during Christmas 2022 almost all the fish died. Despite this, the company remains optimistic to establish a successful cod production facility [23]. Kristina Sigurdsdottir Hansen, the State Secretary to the Minister for Fisheries and Ocean Policy said in April 2022 that the Government wish that cod farming might be an even bigger adventure than salmon farming in Norway [24].

1.6 Nitrifying biofilter in marine RAS

Nitrification is an important pathway in the nitrogen cycle and naturally occurs in different environments like soil, ocean water, freshwater lakes and wastewater. Nitrification is a part of the ocean's nitrogen cycle that produces the greenhouse gas nitrous oxide and oxidizes nitrogen in forms that phytoplankton and other microorganisms in the sea can utilize [25]. One of the difficulties linked to marine RAS is the problem to establish a well-functioning biofilter. The start-up time for a biofilter in freshwater is significantly shorter than the start-up time in seawater [26]. Attempts to adapt well-functioning biofilters from freshwater systems to seawater have been made, which turned out to be challenging in establishing marine biofilters. More knowledge about the microbial communities in the biofilm of well-functioning biofilters is needed, which can improve the process of establishing and adapting biofilters in RAS to high salinities.

A recent study by Navada et al. [26] showed that biofilms from brackish water were more robust to increase in salinity than freshwater biofilms. Starting up a biofilter in brackish water had a significantly shorter start-up period than starting up in seawater. This indicated that a start-up in brackish water can be a strategy for improving salinity acclimation in biofilms. There is today many RAS for salmon post-smolt with a salinity of 15-22 ppt. For starting up biofilters for use in seawater systems, using biofilters from brackish water systems as inoculums is an efficient strategy [26]. This knowledge can enable land-based production of more species, like Atlantic cod, in addition to facilities for producing post-smolt salmon on land.

A recent study by Tesdal from 2021[27] investigated two different start-up strategies of a nitrifying MBBR biofilter at high salinity, 25 ppt, at Havlandet. The RAS at the pilot-scale was for the production of grow-out Atlantic salmon. The first strategy was to use liquid, commercial inoculums and clean biofilm carriers. When this strategy did not give results of a successful nitrifying biofilter, the second strategy was used. The biofilter was inoculated with 1,5 m^3 biofilm carriers from a marine RAS from Erko Seafood operated at 15 ppt. The biofilter at Havlandet was at this point at 15 ppt and later increased to 25 ppt. Successful nitrification was achieved just a few days after the biofilm inoculum was mixed with clean biofilm carriers. One single ASV representing *Nitrospira* was found with a relative abundance of 51% in the biofilm inoculums from Erko, relating to *Nitrospira salsa*. The strain was highly abundant in the biofilm on new carriers after a few days. The study also showed a 77% abundance of nitrifiers in the marine biofilter [27]. Previous studies show a range of 0-20% relative abundance of nitrifiers in RAS biofilters [28].

Nitrospira are obligate chemolithotrophic bacteria, meaning they use inorganic compounds as a source of energy. The species thrive and grow in high nitrite environments, the opposite of *Nitrobacter* which are less tolerant of high nitrite concentrations, making them well suited for use in biofilters. *Nitrospira* as the dominant NOB in freshwater aquaria was first identified by Hovanec et al. [29] and Juretschko et al.[30] This genus was later found in marine aquaculture [31]. To understand the bacterial community in RAS, bacterial diversity in marine biofilters has been investigated using 16S rRNA amplicon sequencing. It was found in a study by Lee et al. [28] from a marine RAS that all nitrifying bacteria were associated with *Nitrosomonas, Nitrospira* and *Nitrospina*. Nitrifying bacteria represented up to 16% of the bacterial communities [28]. Kumar et al.[32] found that the most important organism performing ammonia conversion in marine RAS was the AOBs *Nitrosomonas* sp. and *Nitrococcus* sp, and for nitrite conversion, the NOBs *Nitrospira* sp. and *Nitrobacter* sp. A study found that while in freshwater, the NOB guild was composed of species within *Nitrobacter* and *Nitrospira*, in saltwater *Nitrospira marina* was found [33].

A low relative abundance of ammonia-oxidizing bacteria was found by Fossmark et al. [15], indicating the possible involvement of comammox *Nitrospira* or AOAs in ammonia oxidation in marine biofilters. Per 2022, no marine comammox species have been characterised. Comammox *Nitrospira* has previously been found in freshwater RAS [34], so it is possible that it can be found in marine biofilters too [27], as evidenced by its reported importance as an oxidizer in salt marshes along the southern coastline of China [35]. AOAs have previously been found in biofilters in RAS with a higher abundance than AOBs [27]. Little is known about the role AOAs have in RAS biofilters and to what extent they play an important role in nitrification [36].

The microbial communities on biofilters are highly complex and diverse, composed of numerous species that interact with each other and with the surrounding environment in intricate ways. Despite extensive research, much remains unclear about the structure and function of these communities. The study of biofilter microbial communities is thus an active area of research, with the potential to yield insights into fundamental ecological and metabolic processes. In this context, understanding the complexity of biofilter microbial communities is crucial for optimizing their performance and developing new approaches for sustainable water treatment.

1.7   Studying microbial communities

Microorganisms live in complex microbial communities, where they interact with each other, the surrounding environment and other organisms[37]. Zuckandl and Pauling in the 1960s, suggested that the evolutionary relationship between organisms could be studied to find information about their macromolecules, in particular, nucleic acids and proteins. If two organisms were related, the sequence of the in-

dividual units in a macromolecule would be more similar than if the organisms were not related [38]. The studies used protein sequencing. In the 1970s, Carl Woese pioneered the utilization of ribosomal RNA, rRNA, providing a better view of phylogenetic diversity. Three domains were identified by Woese, Bacteria and Archaea have prokaryotic cell structures, and Eukarya with more complex eukaryotic cell structures [38].

Two major breakthroughs in studying microbial communities have been made, starting with the use of PCR targeting the 16S rRNA gene [39]. 16S rRNA is a marker gene for microbial diversity. It is universally distributed and present in all prokaryotes. It consists of conserved and nine variable regions (v1-v9), allowing the use of a universal PCR primer. The variable region of the 16S rRNA gene is unique for every species and is therefore fit to use for taxonomic identification, diversity and phylogenetic analysis [40]. Many bacterias are only known through the sequencing of the 16S rRNA gene. Previous methods to identify sequence diversity of PCR products were gel-based or based on Sanger sequencing. Sanger sequencing could only sequence 800 nucleotides per reaction, and larger molecules had to be cut into smaller pieces [37]. The second big breakthrough was high-throughput sequencing technology, HTS, that enabled the sequencing of several different DNA sequences at once. Illumina sequencing is a high-throughput sequencing technology and is the most common sequencing technique used today, enabling the processing of millions of sequence reads at a time [41].

Nitrification is catalyzed in part by ammonia monooxygenase, AMO. AMO is encoded by the *amo* operon, consisting of three genes: enzyme active site, *amo*A, enzyme subunit, *amo*B and membrane protein, *amo*C [38]. The *amoA* gene can be used as a marker gene for studying the diversity and abundance of these nitrifying microorganisms in microbial communities. PCR amplification of the *amoA* gene, followed by sequencing and phylogenetic analysis, provides information on the taxonomic diversity of AOB and AOA in a given environment. Real-time PCR (qPCR) can be used to estimate the abundance of AOB and AOA in a sample [37]. Nitrite oxidoreductase (NXR) is a key enzyme of nitrite oxidation and is the best candidate to become a specific functional marker for NOB, as AMO is for AOB [42].

1.8 Aims

There is currently limited knowledge about the microbial communities responsible for nitrification in well-functioning biofilters in marine and brackish RAS. Gaining knowledge within the field can contribute to making start-up strategies more efficient, and optimizing how marine biofilters should be operated. The overall aim of this project is to improve the knowledge about nitrifying microorganisms in RAS biofilters operated at high salinities.

This thesis aims to achieve the following objectives:

1. Determine and compare nitrification capacity and tolerance for varying salinity for biofilm carriers from two commercial RAS: one RAS cultivating Atlantic Cod at 35 ppt and one RAS cultivating post-smolt at 15 ppt, in small lab-scale batch reactors.

2. Characterise the bacterial and archaeal communities of the nitrifying biofilm from three different commercial RAS by amplicon sequencing of the 16S rRNA gene.

3. Identification and quantification of the ammonia-oxidizing archaeal populations by qPCR and Sanger sequencing targeting the *amoA* gene.

2 Method

To gain more knowledge about marine biofilters and the microbial community composition in these biofilters, biofilm carriers from different well-functioning biofilters operated at different salinities were investigated. Both the 16S rRNA and *amoA* gene in bacteria and archaea were investigated to get a bigger understanding of the nitrifying community in marine biofilters. The differences in the microbial community between facilities, in addition to differences within the same facility over time and following a change in cultivating specie, were studied. This knowledge could be used to evolve how marine biofilters should be started up and operated.

2.1 Sample collection

Samples used for analysis in this thesis were collected from three RAS facilities, Havlandet, Erko and Lerøy Belsvik. Erko has a marine RAS for the cultivation of Atlantic salmon with a salinity of 15 ppt. During the start-up of this biofilter in 2017, the system had a salinity of 25 ppt, before it later was reduced to 15 ppt. On October 14th, 1 litre of biofilm carriers for use in a batch experiment (see Section 2.2) and for microbial community analyses were collected and shipped on ice overnight. Three biofilm carriers were sampled and stored at -20°C until microbial community analysis. The batch experiment was started the same day as the biofilm carriers arrived, and how the carriers were handled is described further in Section 2.2. The samples from Erko used in this project originated from the same biofilter that previously had been used as inoculum for the biofilter of the pilot-scale RAS at Havlandet.

The marine RAS at Havlandet AS was second in Norway in producing slaughter-ready salmon in RAS. In June 2022 the production of salmon was terminated. The salinity in the system was at this point 25 ppt. In August 2022, the pilot RAS was used for the production of Atlantic cod. Prior to the cod production, from June to August, the salinity was increased gradually to 35 ppt, and the biofilter was fed with ammonia. For studying microbial communities, biofilm carriers were collected on April 26th and May 10th, when salmon was cultivated. After switching the production from salmon to cod, carriers were collected on September 2nd, 8th and 26th, October 12th and November 16th and 27th. Between three and six carriers were sampled at each sampling date. The samples were stored at -20 °C at Havlandet until all samples had been collected. The samples were collectively shipped on ice overnight and stored at -20 °C until microbial community analysis. On September 26th, 1 litre of biofilm carriers were collected for the use in a batch experiment and shipped on ice overnight. The batch experiment started the same as the biofilm carriers arrived, and how the carriers were further handled upon arrival is described in Section 2.2.

Lerøy Belsvik (referred to as Belsvik) has a marine RAS for post-smolt production at 22 ppt, which uses a fixed-bed biofilter rather than a moving-bed biofilter like at Havlandet and Erko. Swab samples from the biofilter and sludge samples collected during the back-washing of the biofilter were collected on November 28th. Swab samples were directly stored at -20 °C upon arrival while the sludge samples were centrifuged and the pellet was stored at -20 °C until microbial community analysis. An overview of the samples in the study of the microbial community composition and samples used in the batch experiment is given in Appendix A.

2.2 Batch experiments for assessment of nitrifying capacity and salinity sensitivity

The batch experiments based on biofilm carriers from Havlandet and Erko were performed to investigate the change in TAN, nitrite and nitrate concentrations over time and determine the biofilm carriers' nitrifying capacity and salinity sensitivity. Details about the carriers are given in Table 2.1. The experiment was conducted in bioreactors with oxygen supply and magnetic stirring to assure adequate circulation and oxygen availability. Each bioreactor was filled with 350 mL biofilm carriers and media to a total volume of 600 mL. For each facility, three batches were run in parallel and three different medium was made with different salinity, 31 ppt, 15 ppt and 0 ppt. The medium used in this experiment was made with a TAN concentration of 10 mg L^{-1}. For the composition of the medium, see appendix B. The duration of the batch experiment was three days, with the first two days dedicated to biofilm carrier adaptation, followed by the actual experiment on the third day.

Table 2.1: **Specifics on the biofilm carriers used in the batch experiment**. Quantity, pieces and specific surface area are used to calculate capacity per surface area.

Biofilter	Salinity in biofilter of origin [ppt]	Modell	Quantity used in experiment [pcs]	Pieces per m^3	Specific surface area [m^2/m^3]
Havlandet	35 ppt	RK BioElements	80	255 000	750
Erko	15 ppt	BWT15	200	640 000	800

Before the batch experiment was started, the biofilm carriers were adapted as follows: First, upon arrival, the carriers were incubated overnight at the salinity from which they originated, in media with a TAN concentration of 10 mg L^{-1}. Next, 24 hours prior to the experiments, the carriers were divided into three equal portions and incubated overnight in media of three different salinities, 31 ppt, 15 ppt, and 0 ppt. A magnetic stirrer ensured proper movement of the biofilter during the incubation time. Both the incubation and the experiment were conducted at room temperature, 21 °C. On day three, the batch experiment was started, and each bioreactor was filled with 350 mL biofilm carriers and media to a total volume of 600 mL. An overview of the experimental design and timeline for the sampling points is shown in Figure 2.1.
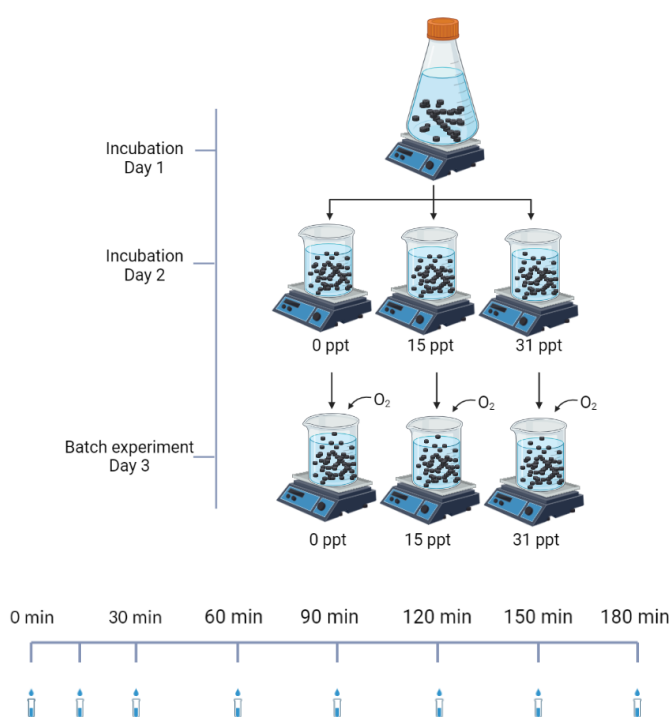
Figure 2.1: Overview of the experimental setup of the batch experiment showing conditions for incubation time and experiment. The timeline shows the duration of the experiment where each sampling point is marked with a water sample.

During the 3-hour long batch experiment, water samples were collected every 15 to 30 minutes, each sampling point shown in Figure 2.1. The water samples were filtered through a 0,2 μm-filter before chloride-elimination kits were used to avoid interference from the salt during spectrophotometric analyses. Chlorid-elimination was performed as described in Appendix C.4. The samples were stored on ice during the experiment and at - 20 °C until concentrations of ammonia, nitrite and nitrate were measured using Hach-Lange kits measured on DR3900 Laboratory Spectrophotometer for water analysis (HACH®), as described in Appendix C. Concentrations of TAN, NO$_2$ and NO$_3$ measured in the water samples are collected in Appendix D.1 and D.2, and were used to calculate the nitrifying capacity. This was done by linear

regression from the measurements where the reduction in TAN was linear.

The biofilm carriers from Havlandet and Erko had different specific surface areas, and different quantities of biofilm carriers were used in each batch experiment. This gave a difference in total surface area. For the possibility to compare nitrifying capacity between the different biofilters, nitrifying capacity per surface area was calculated.

2.3 Characterization of microbial communities of nitrifying biofilms by 16S rDNA amplicon sequencing

In order to characterise the microbial communities in the marine biofilters, an 16S rDNA amplicon library was prepared. The amplicon library included samples from the post-smolt and cod production in the pilot scale RAS at Havlandet, the Erko RAS for post-smolt production and the Belsvik RAS for post-smolt production. Details are given in Appendix A. Three parallels for each sampling date from Havlandet and Erko and a total of five samples from Belsvik gave a total of 32 samples. PCR was conducted using two different primer sets amplifying variable regions 3 and 4 of the 16S rRNA gene to target both bacterial and archaeal communities, resulting in a total of 68 amplicons for sequencing.

2.3.1 DNA extraction

To extract DNA from the biofilm carriers, the MagAttract® Powersoil® Pro DNA kit (Thermo Fisher Scientific) with Thermo Scientific Kingfisher® Flex Purification System was used. The biofilm carriers were cut into smaller pieces with scissors and approximately ¼ of the carriers were used for each DNA extraction. For the swab samples, the tip of the cotton stick was used as input for the DNA extraction. For the sludge samples, the pellet was used for DNA extraction. To prevent DNA contamination, the biofilm carriers were cut on a clean petri dish and the equipment was disinfected with ethanol between each biofilm carrier and the Eppendorf tubes were UV-radiated. The protocol for the DNA extraction kit was followed (Appendix E.1) except for steps 3 and 5. In step 3, instead of using the Tissuelyser II, Precelly 24 Tissue Homogenizer was used with 5500 rpm and 2x30 seconds. In step 5, the supernatant was collected in Eppendorf tubes instead of collection microtube racks. There were a total of 32 DNA extracts in addition to positive and negative DNA-extraction kit control. The positive kit control was a bacteria culture sample where bacteria were known to be present.

2.3.2 PCR

The 16S rRNA gene in bacterial and archaeal DNA was amplified by polymerase chain reaction, PCR. Separate primer pairs targeting bacteria and archaea were used to amplify regions v3 and v4. To amplify bacterial 16S rDNA, primers Ill 341F_KI and Ill 805R were used. To amplify archaeal 16S rDNA, primers Ill-A-340F and Ill-A-760R were used. Primer sequences are given in Table 2.2. A master mix was made with the final concentrations of 1x Phusion HS buffer, 200 μM of each dNTP, 0.3 μM of each forward and reverse primer, and 0.02 units μL^{-1} Phusion Hot Start DNA polymerase. As a template for the PCR reactions, 1 μL of DNA extract was used. The total volume for the PCR reaction was 25 μL. For some samples that were not successfully amplified, the DNA extract was diluted 1:10 and used as a template in a new PCR with the same conditions. Temperature cycles were performed on BioRad Thermal Cycler T100 with the program shown in Table 2.3.

Table 2.2: Primer pairs targeting 16S rDNA gene in bacterial and archaeal DNA. The target sequences are shown in bold.

Primer name	Sequence (5'-3')
Ill 341F_KI	5'-TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGNNNN**CCTACGGGNGGCWGCAG**-3'
Ill 805R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGNNNN**GACTACNVGGGTATCTAAKCC**-3'
Ill-A-340F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGNNNN**CCCTAYGGGGYGCASCAG**-3'
Ill-A-760r	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGNNNN**GGACTACCSGGGTATCTAATCC**-3'

Table 2.3: Temperature and cycling conditions used in the PCR reaction when amplifying the 16S rDNA and when indexing PCR amplification products.

Step	Temperature [℃]	Time	Cycles PCR	Cycles indexing PCR
Denaturation	98	2 min		
Denaturation	98	15 sec		
Annealing	55	20 sec	x35	x10
Elongation	72	20 sec		
Final elongation	72	5 min		

2.3.3 Agarose Gel Electrophoresis

To evaluate the quality and quantity of the PCR products, an agarose gel electrophoresis was performed. A 1% agarose gel was made by dissolving agarose powder in 1 x TAE buffer. The solution was heated to boil in the microwave. The solution was cooled to 65 °C and GelRed was added to a final concentration of 50mM. The solution was poured into a gel chamber with a gel comb and settled into a gel. The PCR products (5 μL) were mixed with 1 μL of 6x Loading dye (Thermo Fisher Scientific), applied to the gel and run at 105V for 1 hour or until sufficient separation. GeneRuler™1 kb Plus DNA ladder (Thermo Fisher Scientific) was used as a size marker. The gel was visualized using a UV chamber.

2.3.4 Preparation of amplicon library

The PCR products were normalized and purified using Sequal Normalization Prep Plate Kit and performed according to the protocol (Appendix E.2). Next, indexing PCR was performed for all 68 amplicons, where each amplicon was marked with a unique combination of two indexes. A master mix with a final concentration of 1x Phusion HS buffer, 200 μM of each dNTP, and 0.02 units μL^{-1} Phusion Hot Start DNA polymerase were mixed with a unique combination of two indexes (2.5 μL of each) from Nextera XT DNA Library Preparation Kit. As a template, 2.5 μL of normalized PCR product was used. The temperature cycle step was performed on BioRad Thermal Cycler T100 as shown in Table 2.3

After indexing PCR, an agarose gel electrophoresis was performed to evaluate the indexing PCR results. Another round of normalization was performed on Sequal Normalization Kit as previously described. All samples were then pooled and afterwards concentrated using an AmiconUltra 0.5 centrifugal filter device, performed according to the protocol (Appendix E.3), except for step 4, where the tube was centrifuged at 14,000 g for 10 minutes. A gel electrophoresis was performed as previously described, and an image of the gel was included when the sample was shipped to the Norwegian sequencing centre (NSC), University of Oslo, and sequenced in one MiSeq run (Illumina, San Diego, CA) with v4 reagents (Illumina).

2.4 Processing of sequencing data for microbial community analysis

Ingrid Bakke processed the sequencing data by using the Usearch pipeline v.11 [43] as follows: The fastq_mergepairs command was used to merge sequence pairs, trim off primer sequences, and filter merged sequences shorter than 380 bp. The fastq_filter command was used for quality-filtering with the default value of 1 for the expected error threshold. Amplicon sequencing variants (ASVs) were generated using the Unoise3 command [44]. The recommended minimum abundance threshold of 8 reads (in the whole data set) was used. The sintax command [45] was used to assign taxonomy to the ASVs [45] with the Ribosomal Database Project (RDP) rdp 16S rRNA training set v18 as reference data. The resulting ASV table was manually inspected. All ASVs classified as chloroplast were removed from the datasets. All ASVs classified as archaea were removed from the ASV table representing bacteria, and vice versa.

A PCR product was obtained for the negative control for the DNA extraction and probably represented contaminating DNA from the DNA extraction or the PCR. Moreover, the positive control for DNA extraction was sequenced. All ASVs that were present in higher abundance in the negative control than in the samples, were removed from the ASV table. Furthermore, the positive DNA extraction protocol had a community profile that was very distinct from the biofilm samples. It appeared that a few samples had been contaminated by the positive kit control during DNA extraction, and therefore, ASVs representing the positive control were also removed from the ASV table. A total of 74 ASVs were removed, resulting in a total of 5557 ASVs in the ASV table representing the bacterial communities and 97 ASVs in the ASV table representing the archaeal communities. The ASV table representing the bacterial communities was normalized to 48,000 reads per sample. The ASV table representing the archeal communities was normalized to 20,000 reads per sample. The normalized ASV tables were used in all further analyses.

2.5 Statistical analyses

PAST (version 4.12b) was used for statistical analyses of the community data [46]. A Bray-Curtis similarity matrix comparing community profiles between samples was created and exported to Microsoft Excel for further analysis. Alfa diversity summarizes the structure of the ecological community. It is typically defined as the number of species or ASVs in a specific sample or as a measure of the richness and evenness of the community. Species evenness describes the equitability of the species abundance. Shannon's diversity index takes both species richness and evenness to account. A high Shannon's diversity indicated a highly diverse community. The

Bray-Curtis similarity index is a measure of beta-diversity and measurement of similarity between two communities. Bray-Curtis similarity is given in values between 0 to 1, where 0 represents complete dissimilarity and 1 represents identical communities. These are two parameters commonly used to evaluate and describe microbial diversity [47].

To visualize similarities and dissimilarities between the community profiles in the biofilm samples, Principal Coordinate Analysis Ordinations, PCoA, based on Bray-Curtis similarities were created. These PCoA plots are based on the Bray-Curtis matrix. The distance matrix is transformed into a lower-dimensional space, typically three-dimensional, with distances between samples indicating their level of Bray-Curtis similarity. Coordinate 1 and coordinate 2 are the dimensions that capture the most variation in the data. Each sample is represented as a point in the plot, and samples that are more similar in terms of pairwise distances are plotted closer together [46].

To assess whether there were any significant differences in the microbial community profiles between sample groups, a one-way permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis similarities was conducted. The significance threshold was set to a p-value below 0.05. When more than two groups were being compared, one-way PERMANOVA with Bonferroni-corrected p-values were used. Similarity Percentage (SIMPER) is a method to assess which taxa are responsible for the difference between groups. Bray-Curtis similarity measure was used to conduct both pairwise comparison and multi-group SIMPER [46].

In Microsoft Excel, an ANOVA test was used to assess whether or not it was a significant difference between groups ($p<0.05$). If the ANOVA test confirmed a significant difference, a Tukey HSD test was used to establish between which groups the difference was significant ($p<0.5$).

2.6   Identification and quantification of archaeal *amoA* gene in the biofilm communities

Results from Illumina sequencing indicated that one ASV classified as *Nitrosopumilus* dominated the archaeal communities. Members of this genus are known to oxidize ammonia (AOA). PCR and qPCR were performed to investigate the presence and abundance of archaea *amoA* genes in the biofilm samples.

2.6.1 Quantification of the *amo*A gene using qPCR.

qPCR is a method used to quantify the amount of DNA in a sample. The DNA is amplified in a PCR and continuously labelled with SybrGreen, a fluorescent dye binding double-stranded DNA. The amount of fluorescence correlates with the amount of PCR product produced, and the PCR product can be detected in "real-time" during the amplification. The cycle threshold, C_T, is defined as the number of cycles needed for the fluorescent signal to cross the threshold. The C_T-value correlates to the amount of target DNA in the sample. Using standard curves where the correlation between the concentration of target-DNA and C_T-value is known makes it possible to determine the amount of target-DNA a given C_T-value represents.

Two sets of qPCR primers were designed by PhD Fernando Fernando as explained in Appendix (F) to target the *Nitrosopumilus amoA* gene sequence, Table 2.4. A qPCR was performed to identify the optimal annealing temperature for the two primer sets, AmoANSPA and AmoANSPB. A master mix with a final concentration of 1X SYBR™Green master mix (ThermoFIsher Scientific) and 250nM each of forward and reverse primer was made. The DNA extract used was diluted 1:100 and 4 μL was used as a template, making a total volume of 20 μL for each qPCR reaction. qPCR was performed on QuantStudio™ 5 real-time PCR System. The qPCR was set up with an annealing temperature gradient across the plate for the two different sets of primers, 57℃, 59℃ and 61℃. One sample from each facility, H_27.11_A, Erko_A and Belsvik_swab_A, in duplicates, was used for the test run (detailed samples names in Appendix A).

Table 2.4: Primers used in qPCR, targeting the *Nitrosopumilus amoA* gene. F=forward primer, R=reverse primer

Primer name	Sequence (5'-3')	Target
*amo*ANSPA-F	GTAGTACGTTGCTGTGCCT	*Nitrosopumilus*
*amo*ANSPA-R	CTTGGACTTCGTACACGGT	*amoA* gene
*amo*ANSPB-F	TGGTCTGGTTAAGACGATGTAC	*Nitrosopumilus*
*amo*ANSPB-R	CAAGCCCAGTCAGTGTAGAAG	*amoA* gen

To be able to correlate the C_T-values obtained in the qPCR with copy numbers of DNA molecules, a standard curve was generated using a specific target sequence with known length and concentration as the template for each of the primer sets. As a template, synthetic oligonucleotides were used. Their DNA sequence was identical to representative *Nitrosopumilus amoA* gene sequences retrieved from Genebank. These were used to create a dilution series with concentrations from 0.1 ng μL^{-1} to 0.00000001 ng μL^{-1}, and used as templates in qPCR. Each dilution was run as a triplicate reaction. The log copy number for each standard dilution was plotted against the C_T-values. The copy number (CN) was calculated using Equa-

tion 2.1, with a DNA length of 107 bp for primer amoANSPA and 112 bp for primer amoANSPB. Linear regression was used to find the slope. The slope was used to calculate the amplification efficiency (Equation 2.2).

$$CN\left(\frac{molecules}{\mu L}\right) = \frac{DNA_{conc}\left(\frac{g}{\mu L}\right) * 6.022 * 10^{23}\left(\frac{molecules}{mol}\right)}{DNA_{length(bp)} * 660\left(\frac{g}{mol}\right)} \qquad (2.1)$$

$$E = \left(10^{\left(-\frac{1}{slope}\right)} - 1\right) * 100\% \qquad (2.2)$$

qPCR was performed, with the following samples: H_Cod_27.11_A and H_Cod_27.11_B from Havlandet, Erko_PS_A and Erko_PS_B and Belsvik_Swab_A and Belsvik_Swab_B (detailed sample overview in Appendix A). The DNA extracts for the samples were diluted 1:100 and used as the templates. The annealing temperature was set to 61 ℃, otherwise, the qPCR conditions were as described previously. The CN for each sample was determined according to the C_T-values based on the standard curve.

2.6.2 PCR amplification and Sanger Sequencing of the *amo*A gene.

Illumina sequencing showed that for biofilm samples from Havlandet and Erko, there was one ASV classified as *Nitrosopumilus* dominating the archaeal community profiles. The end-point PCR was performed to amplify larger regions of the *amoA* gene for the same samples as applied in the qPCR describes previously. Two different primer pairs were designed by phD Fernando Fernando as described in Appendix F(Table 2.5). Primer set AmoArch targeted the broader groups of archaeal *amoA* gene sequences. The expected length of the PCR products amplified with amoAArch was 546 bp. Primer set EPPCRNSP targeted the *Nitrosopumilus amoA* gene. The expected length of the PCR product was 536 bp. For both primer sets, an annealing temperature of 60 ℃ was applied, and for the rest, the PCR was performed as described in Section 2.3.2.

Table 2.5: Primers used in PCR targeting the archaeal *amo*A gene and the *Nitrosopumilus amoA* gene. F=forward primer, R=reverse primer.

Primer name	Sequence (5'-3')	Target
amoAArch-F	TCTACACTGACTGGGCTTGGACWTC	Archaea
amoAArch-R	ACCAAGCGGCCATCCATCT	*amoA* gene
EPPCRNSP-F	TACACTGACTGGGCTTGGA	*Nitrosopumilus*
EPPCRNSP-R	GCGGCCATCCATCTGTA	*amoA* gene

The PCR with two different primer pairs targeting the archaeal and *Nitrosopumilus amoA* gene resulted in two PCR products for each of the samples. QIAquick ® PCR

purification Kit (Qiagen) was used to purify the PCR product by following the protocol (Appendix E.4). For each of the PCR products, two sequencing reactions were set up; one where the forward primer was used as the sequencing primer, and one where the reverse primer was used as the sequencing primer. The purified PCR products (5 μL) and 5 μL of a 5μM primer solution were mixed before being shipped to Eurofins Genomics for Sanger sequencing. That resulted in 12 sequencing reactions.

3 Results

The aim of this thesis was to obtain more knowledge about the microbial communities in biofilm from marine biofilters. Batch experiments were performed with biofilm carriers from Havlandet and Erko, to determine the nitrifying capacity and salinity tolerance of these two different marine biofilters. Furthermore, the microbial communities present in the biofilm samples from Havlandet, Erko, and Lerøy Belsvik were analysed. The focus was to identify and compare the microbial community composition and abundance of ammonia- and nitrite-oxidizing microorganisms in the different biofilters.

3.1 Nitrification capacity from marine biofilm carriers as determined in batch experiments

3.1.1 Havlandet biofilm carriers

The biofilm carriers originated from a seawater RAS at Havlandet cultivating Atlantic Cod at 35 ppt. The aim was to assess the nitrifying capacity of the biofilm under varying salinities, 31 ppt, 15 ppt, and 0 ppt. The concentrations of ammonia, nitrite and nitrate were determined throughout the experiment, collected in Appendix D.1.

A decrease in TAN concentrations was observed in all three reactors, with a faster reduction in seawater and brackish water reactors than in freshwater (Figure 3.1). The TAN concentration reached 0 mg L^{-1} for seawater and brackish water reactors after 180 minutes, while in the freshwater reactor, the TAN concentrations reached half of the initial concentration before the experiment ended. Nitrite concentrations remained below 1 mg L^{-1} throughout the experiments, indicating efficient nitrite oxidation in all reactors. Despite efficient TAN- og nitrite-oxidation, at least at 31 ppt and 15 ppt (Figure 3.1a and 3.1b), a corresponding increase in nitrate concentration was not observed. Only in the freshwater reactor did the nitrate concentration correspond to the decrease of TAN (Figure 3.1c). The results showed a decrease in total nitrogen in seawater and brackish water reactors while total nitrogen concentration remained stable in the freshwater reactor.

The nitrification capacity was calculated using the linear area of the TAN reduction in the graph in Figure 3.1. Nitrification capacities of 0.038 mg L^{-1} min^{-1}, 0.043 mg L^{-1} min^{-1} and 0.020 mg L^{-1} min^{-1} were determined for seawater, brackish water and freshwater, respectively. This experiment showed that the biofilm carriers from Havlandet had the highest nitrifying capacity in seawater and brackish water, and it was reduced by 50% in freshwater.
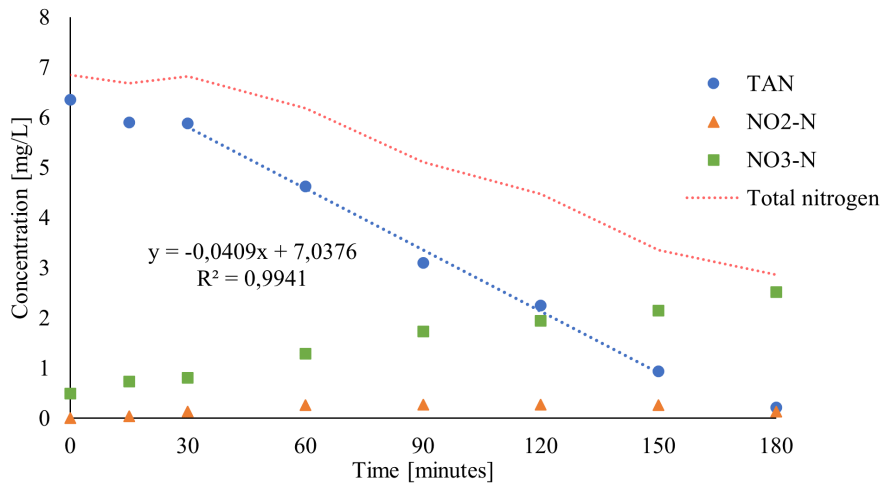
(a)
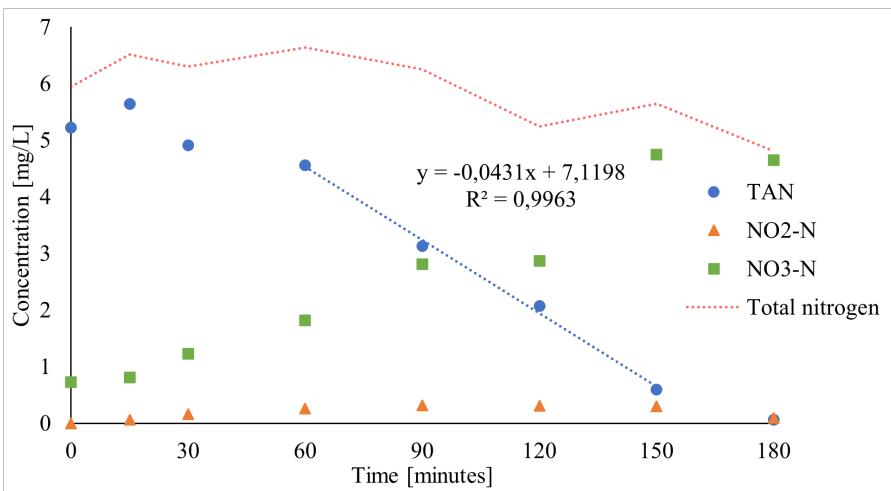
(b)
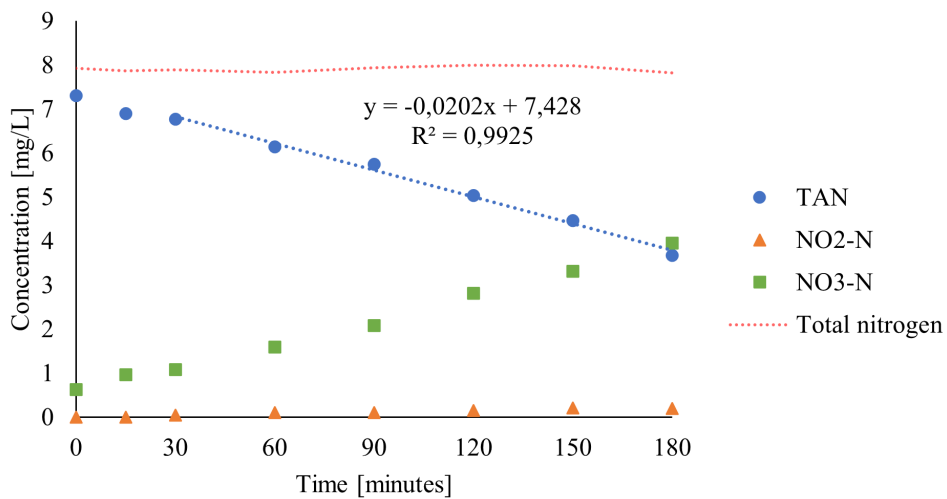
(c)

Figure 3.1: Change in concentration of ammonium, nitrite and nitrate observed during a 3-hour batch experiment in (a) seawater (31 ppt), (b) brackish water (15 ppt) and (c) freshwater (0 ppt), all using biofilm carriers from the same biofilter from a marine RAS at Havlandet originally operated at 35 ppt salinity. Media was added to 350 mL of biofilm carriers to a total volume of 600 mL. The nitrification capacity was estimated by performing a linear regression of the measured TAN concentrations.

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3.1.2 Erko

An equivalent batch experiment was done with biofilm carriers from Erko. These biofilm carriers originated from a biofilter used in a RAS cultivating post-smolt at 15 ppt. The ammonia, nitrite and nitrate concentrations throughout the experiment are collected in Appendix D.2.

A decrease in TAN concentrations was observed in all three reactors(Figure 3.2) and the TAN concentration reached 0 mg L^{-1} after 90 minutes in all three reactors. Nitrite concentrations remained below 1 mg L^{-1} throughout the experiments, indicating efficient nitrite oxidation in all reactors. A corresponding increase in nitrate concentration was not observed in the seawater and brackish water reactor, despite sufficient oxidation of TAN and nitrite (Figure 3.2a and 3.2b). The increase in nitrate concentration corresponded to the decrease of TAN in the freshwater reactor (Figure 3.2c). The results showed a decrease in total nitrogen in seawater and brackish water reactors while total nitrogen concentration remained stable in the freshwater reactor.

Nitrification capacities were found to be 0.10 mg L^{-1} min^{-1}, 0.11 mg L^{-1} min^{-1} and 0.12 mg L^{-1} min^{-1} for seawater, brackish water, and freshwater, respectively. The similar nitrifying capacity indicated that the biofilm carriers from Erko was capable of nitrification regardless of salinity.
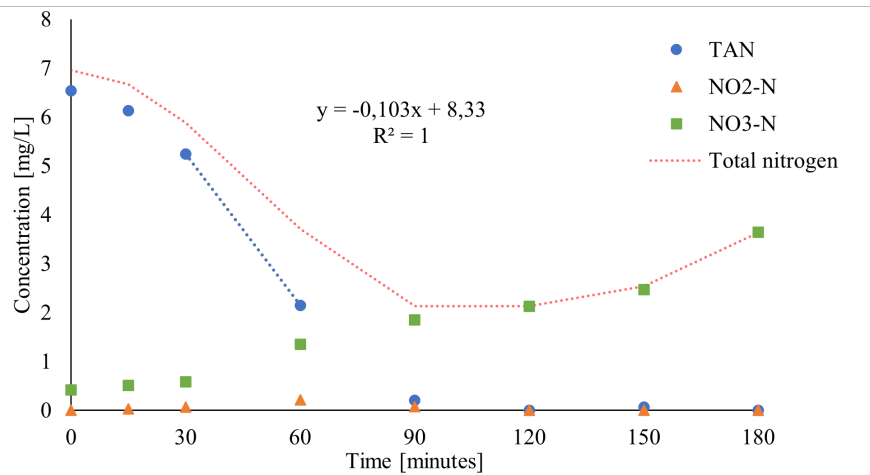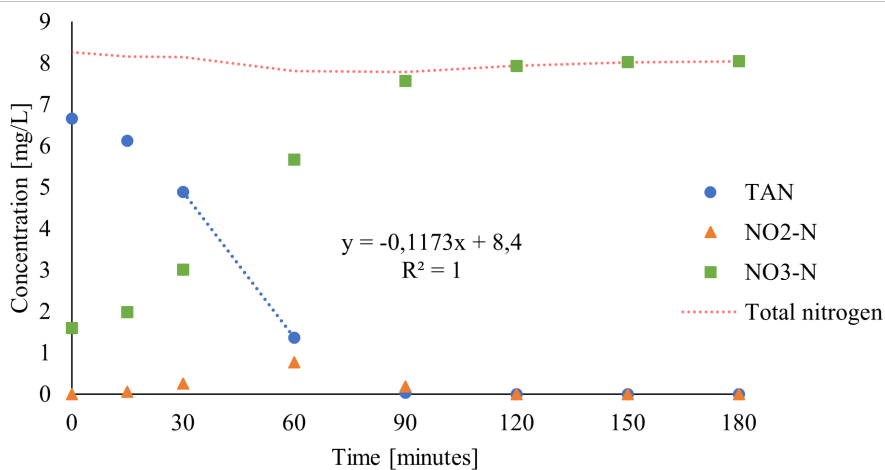
Figure 3.2: Change in concentration of ammonium, nitrite and nitrate observed during a 3-hour batch experiment in (a) seawater (31 ppt), (b) brackishwater (15 ppt) and (c) freshwater (0 ppt), all using biofilm carriers from the same biofilter from a marine RAS at Erko originally operated at 15 ppt salinity. Media was added to 350 mL of biofilm carriers to a total volume of 600 mL. The nitrification capacity was estimated by performing a linear regression of the measured TAN concentrations.
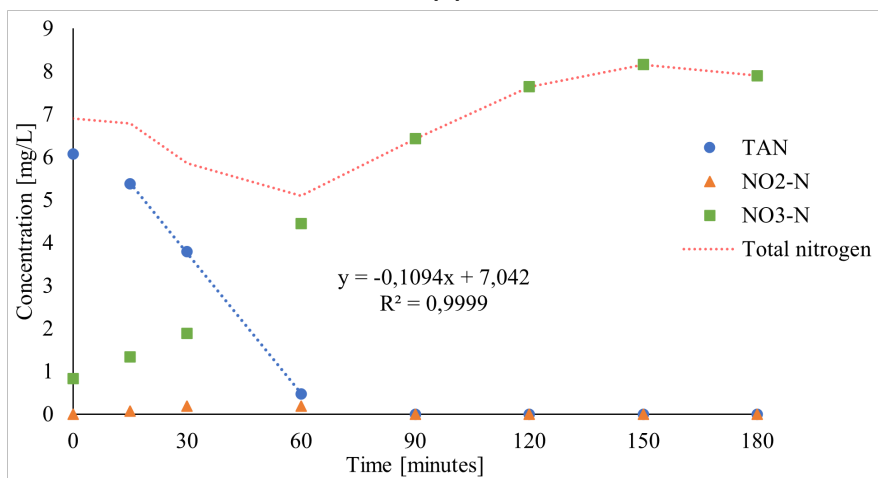
3.1.3 Nitrification capacity

The two sets of biofilm carriers used in the experiments, from Havlandet and Erko, differed in design, specific surface area, and size. To compare their nitrifying capacity, the nitrifying capacity per unit surface area was determined (Table 3.1). The results showed that the biofilm carriers from Erko had, on average, a three times higher nitrifying capacity, compared to those from Havlandet (Figure 3.3). Additionally, in freshwater, the biofilm carriers from Erko exhibited over five times higher nitrification capacity compared to those from Havlandet.

Table 3.1: The nitrifying capacity per surface area calculated for the two biofilm carriers from Havlandet and Erko at 0 ppt, 15 ppt, and 31 ppt.

Biofilter	Salinity	Capacity per surface area [mg N L^{-1} min^{-1} m$^{2-1}$]
Havlandet	0 ppt	0.086
	15 ppt	0.183
	31 ppt	0.174
Erko	0 ppt	0.470
	15 ppt	0.438
	31 ppt	0.412
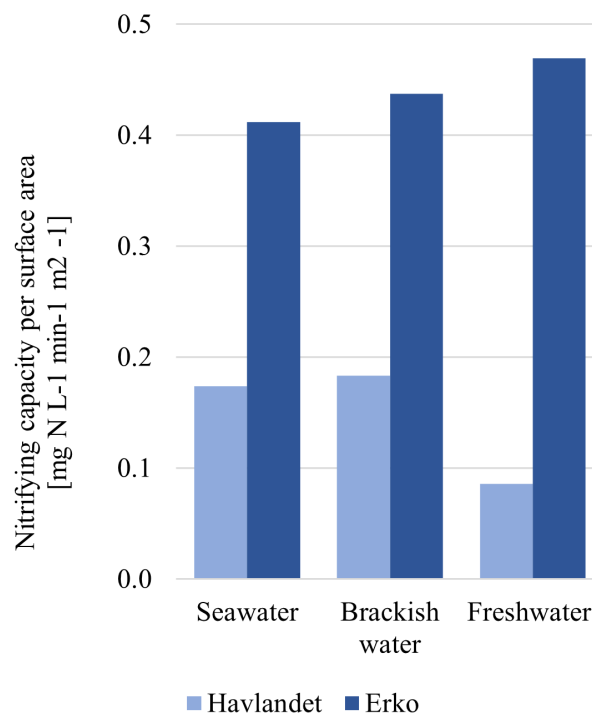
Figure 3.3: The nitrifying capacity per surface area at different salinities for biofilm carriers originating from biofilters at Havlandet and Erko. Seawater= 31 ppt, brackish water=15 ppt, and freshwater=0 ppt.

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3.2 Characterizing communities of the biofilm carriers.

3.2.1 ASV richness and diversity

The biofilm communities were characterized using 16S rDNA amplicon sequencing. A total of 5557 ASVs were identified in the ASV-table representing bacterial communities. The archaeal dataset consisted of 97 ASVs. The ASV table was normalized to 48,000 reads per sample for bacteria, and 20,000 reads per sample for archaea.

ASV richness and exponential Shannon's index, which considers both richness and evenness, were used to evaluate the alpha diversity of the microbial community compositions. For the bacterial biofilm communities, a comparison of the observed and estimated ASV richness (Chao-1) gave an average coverage of $74.9 \pm 9.0\%$ of the richness at the present sequencing depth (Figure 3.4a). The bacterial biofilm communities in the biofilm samples from the biofilter at Belsvik had a lower alpha diversity than the other biofilm samples, both in terms of ASV richness (Figure 3.4a) and exponential Shannon's index (Figure 3.4b).

The bacterial ASV richness was relatively similar for the sample groups, however, a significant difference between the groups was found (ANOVA, $p < 0.05$). Tukey HSD confirmed a significant difference between the biofilm samples for Havlandet during cod production and Belsvik($p < 0.05$). The exponential Shannon's diversity was low compared to ASV richness, (Figure 3.4b). Considering a relatively high ASV richness, the low Shannon diversity pointed to a low evenness in the communities. Tukey HSD confirmed a significant difference in Shannon's diversity between samples from Havlandet during cod production compared with Erko and Belsvik, and between samples from Havlandet during post-smolt production and Belsvik ($p < 0.05$).
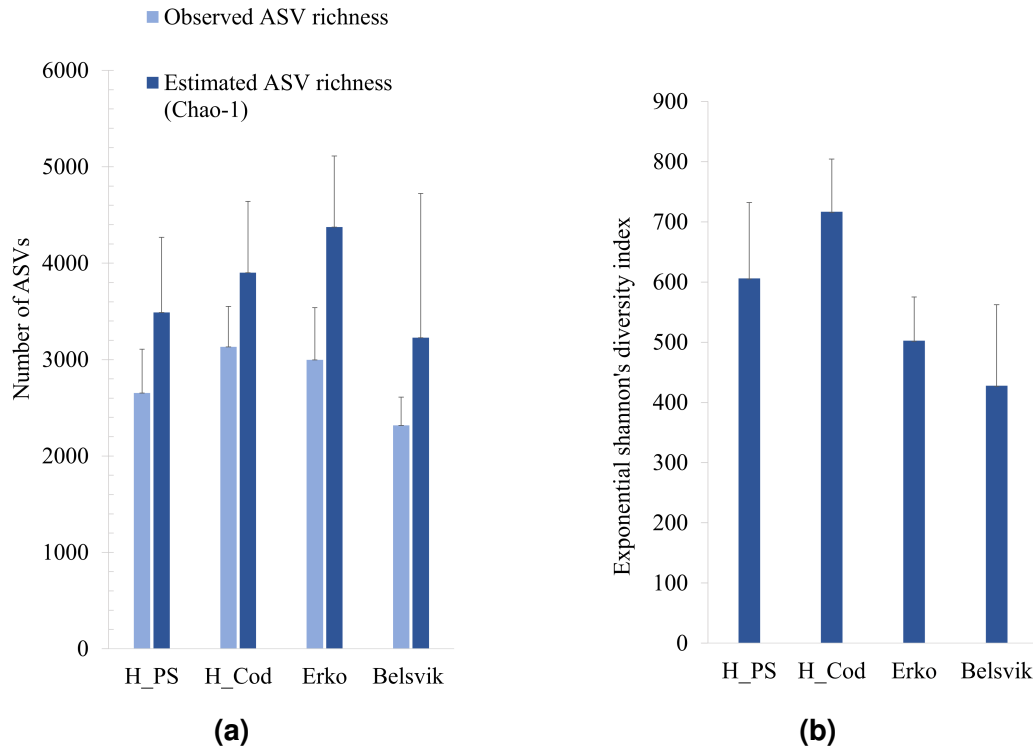
Figure 3.4: Alfa diversity indices for the bacterial biofilm communities for the samples from the Havlandet, Erko, and Belsvik biofilters. (a) Observed and estimated (Chao-1) ASV richness, (b) exponential Shannon's diversity. The alpha diversity indices were based on the normalized ASV-table (normalized to 48,000 reads per sample) All indices were calculated as the means and error bars indicating standard deviation. H_PS= biofilm samples from Havlandet biofilter from the period with post-smolt production at 25 ppt (six samples), H_Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 ppt (18 samples), Erko= biofilm samples from Erko biofilter at 15 ppt (three samples), and Belsvik= biofilm samples including swab and back-washing sludge from biofilter operated at 22 ppt (five samples).

For the archaeal communities, comparing observed and estimated ASV richness gave an average coverage of $77.8 \pm 12.0\%$ of the richness (Figure 3.5a). For the samples from Havlandet and Erko, the exponential Shannon was extremely low with a value of about 1, indicating that the communities were dominated by only one ASV (Figure 3.5b). The observed richness for the samples from Belsvik was approximately four times higher than the observed richness in the samples from Havlandet during post-smolt production. The lowest alpha diversity was found for the samples from Havlandet during post-smolt production, both in terms of ASV richness and exponential Shannon's diversity. A significant difference in observed ASV richness and Shannon's diversity was found for the four groups (ANOVA, $p < 0.05$). A Tukey HSD test confirmed a significant difference in the observed ASV richness between all groups except for Erko compared to Havlandet during cod production and Belsvik ($p < 0.05$). For Shannon's diversity, no significant difference was found between the samples from Havlandet during post-smolt and cod production, and Erko (Tukey HSD, $p < 0.05$).
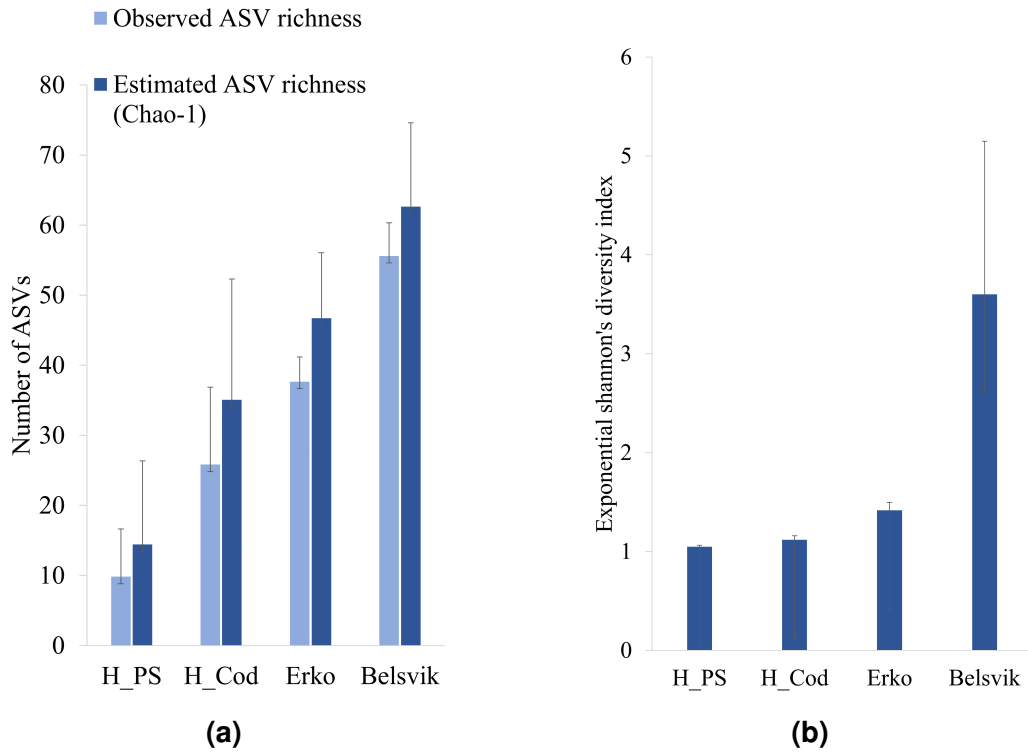
Figure 3.5: Alfa diversity indices for the archaeal biofilm communities for the samples from the Havlandet, Erko, and Belsvik biofilters. (a) Observed and estimated (Chao-1) ASV richness, (b) exponential Shannon's diversity. The alpha diversity indices were based on the normalized ASV-table (normalized to 20,000 reads per sample) All indices were calculated as the means and error bars indicating standard deviation. H_PS= biofilm samples from Havlandet biofilter from the period with salmon production at 25 ppt (six samples), H_Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 ppt (18 samples), Erko= biofilm samples from Erko biofilter at 15 ppt (three samples), and Belsvik= biofilm samples including swab and back-washing sludge from biofilter operated at 22 ppt (five samples).

3.2.2 Microbial community composition

Bacterial communities

The community composition at the class level showed no clear difference in the bacterial communities between the sample groups (Figure 3.6). *Flavobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* dominated the biofilm communities in all samples. A higher abundance of *Nitrospira* was observed in the samples from Havlandet during the production of cod and in the samples from Erko.
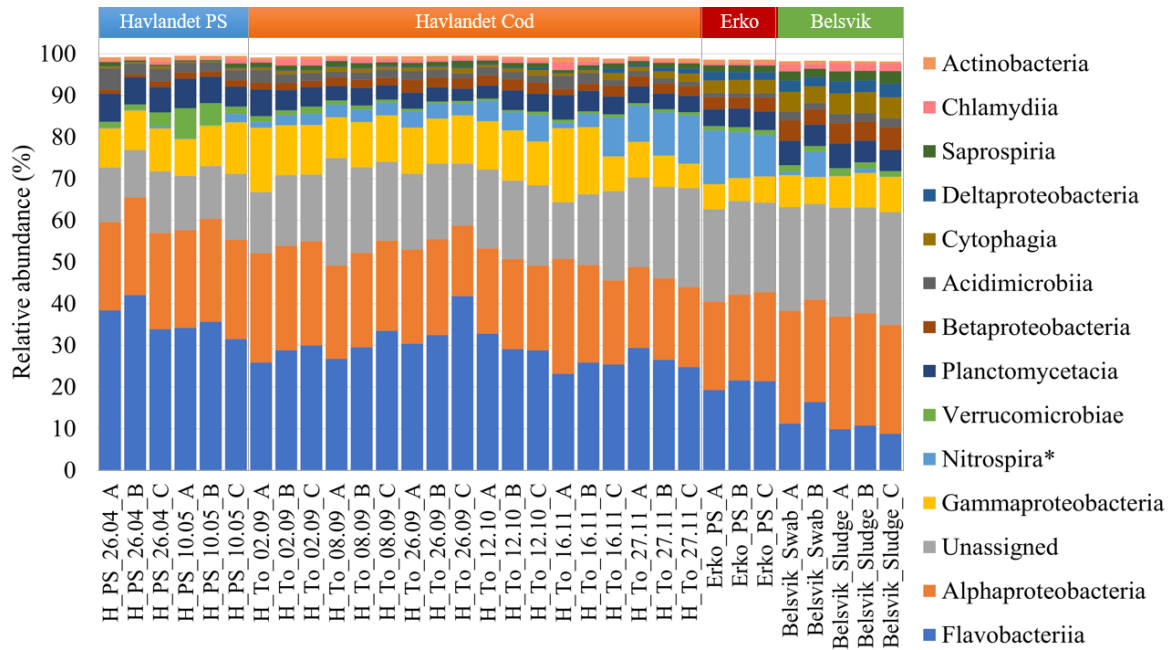
Figure 3.6: Bacterial community composition at the class level. Only classes with a maximum abundance larger than 1% in at least one sample are included. Nitrospira* includes three abundant ASVs classified as the phylum Nitrospirae at a confidence threshold of 0.63. H_PS= biofilm samples from Havlandet biofilter from the period with salmon production at 25 ppt, H_Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 ppt, Erko_PS= biofilm samples from Erko biofilter cultivating post-smolt at 15 ppt, and Belsvik= biofilm samples including swab and back-washing sludge from biofilter operated at 22 ppt. The dates for the sampling are included in the sample names. A, B and C indicate replicate carrier samples.

The bacterial community composition at the ASV level was examined to further investigate the composition of the communities (Figure 3.7). A considerable difference was observed in the community composition between the four different groups A PERMANOVA confirmed a significant difference in the community compositions between all groups, except between Erko and Belsvik (p<0.05). One ASV classified as *Maribacter* was most common in the biofilm samples from Havlandet. The community composition in the biofilm samples from Belsvik stands out from the other groups, with a high abundance of three ASVs classified as Alphaproteobacteria, Flavobacteria, and Saprospira. The biofilm samples from Erko and the last samples

from Havlandet during cod production have a high abundance of an ASV representing Acidobacteria. Two ASVs classified as *Nitrospira*, ASV9 and ASV12, were highly abundant in the samples from Havlandet collected at the last sampling from the cod production and the samples from Erko. These two ASVs were abundant also in the other samples from Havlandet from the period with cod production but at lower relative abundances. *Nitrospira* and *Nitrosomonas* were the most abundant AOBs and NOBs observed in the communities.
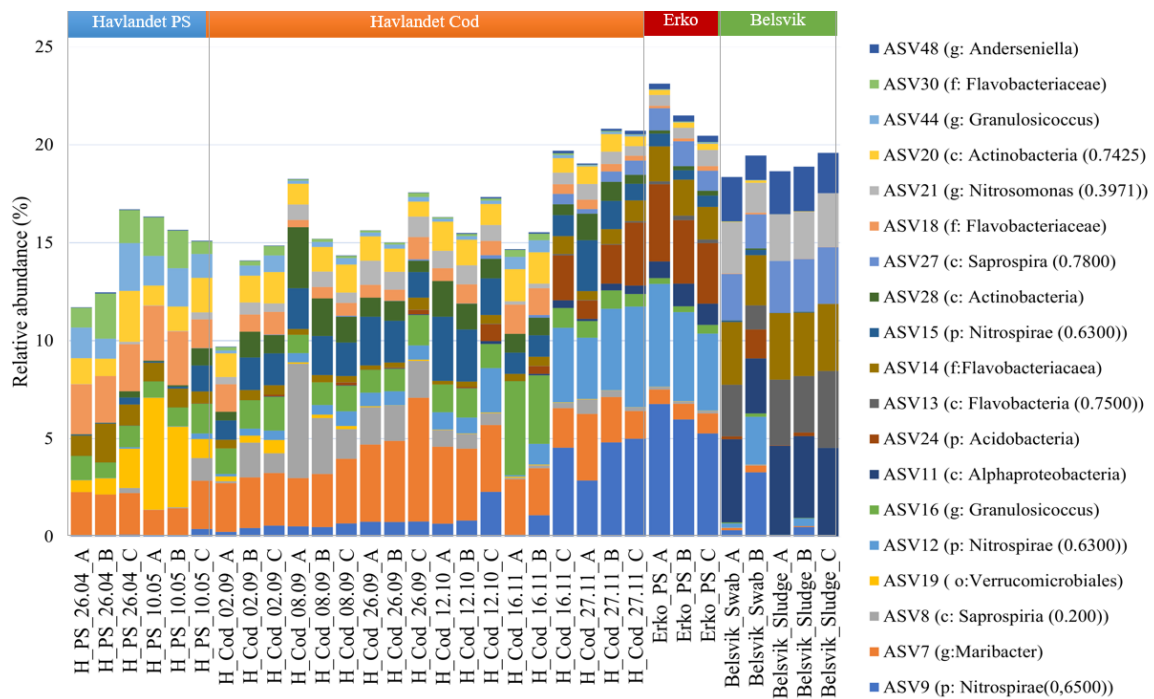
Figure 3.7: Composition at the ASV level for bacterial biofilm communities. Only classes with a maximum abundance larger than 2% in at least one sample are included. For ASVs that could only be classified at high taxonomic levels at the confidence threshold of 0.8, more detailed taxonomic information is presented together with the relevant confidence value. H_PS= biofilm samples from Havlandet biofilter from the period with salmon production at 25 ppt, H_Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 ppt, Erko_PS= biofilm samples from Erko biofilter cultivating post-smolt at 15 ppt, and Belsvik= biofilm samples including swab and backwashing sludge from biofilter operated at 22 ppt. The dates for the sampling are included in the sample names. A, B and C indicate replicate carrier samples.

Archaeal communities

ASV1 classified as *Nitrosopumilus* almost completely dominated the communities for the samples from Havlandet and Erko. ASV1 was also observed in samples from Belsvik, however, ASV3, also classified as *Nitrosopumilus*, dominated the communities. All ASVs observed were classified as either *Nitrosopumilus*, known for ammonia-oxidizing, or *Woesearcheaota*.
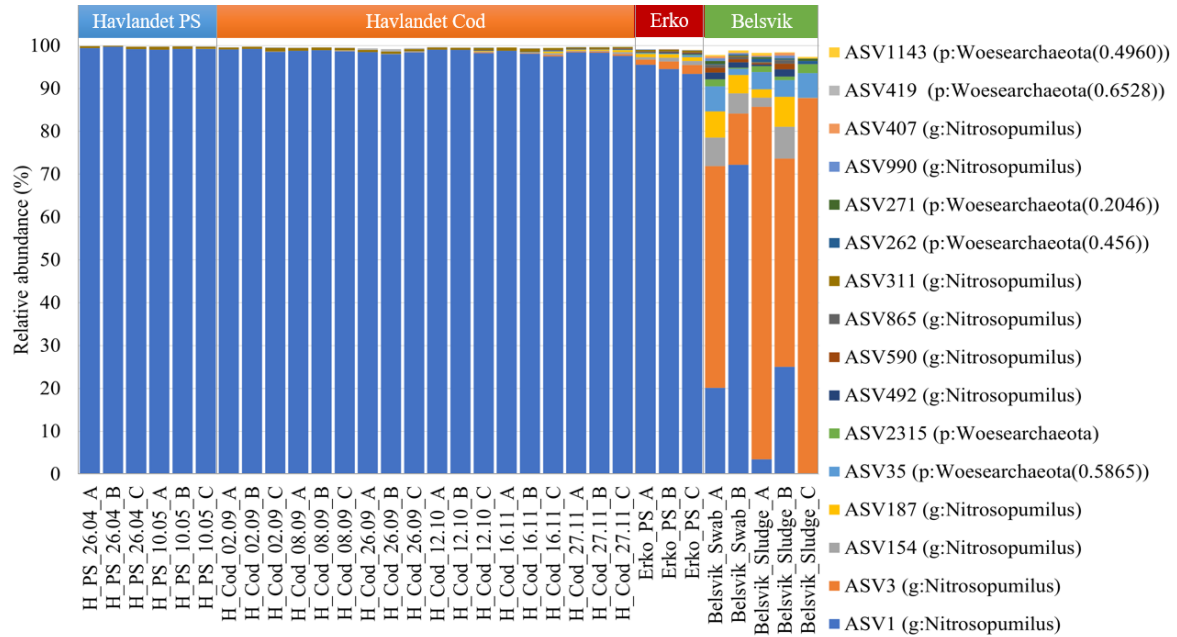
Figure 3.8: Composition at the ASV level for archaeal biofilm communities. Only ASVs with a maximum abundance larger than 0.5% in at least one sample are included. For ASVs that could only be classified at high taxonomic levels at the confidence threshold of 0.8, more detailed taxonomic information is presented together with the relevant confidence value. H_PS= biofilm samples from Havlandet biofilter from the period with salmon production at 25 ppt, H_Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 ppt, Erko_PS= biofilm samples from Erko biofilter cultivating post-smolt at 15 ppt, and Belsvik= biofilm samples including swab and back-washing sludge from biofilter operated at 22 ppt. The dates for the sampling are included in the sample names. A, B and C indicate replicate carrier samples.

3.2.3 Beta-diversity, comparison of communities between sample groups

Bacterial communities

A PCoA ordination based on Bray-Curtis similarities was performed to examine the difference in the bacterial communities between the samples from different facilities (Figure 3.9). The PCoA plot indicated that the community composition was different in the samples from the different facilities. A PERMANOVA confirmed a significant difference between Havlandet and Erko, and Havlandet and Belsvik ($p<0.05$). No significant difference was found between the samples from Erko and Belsvik.
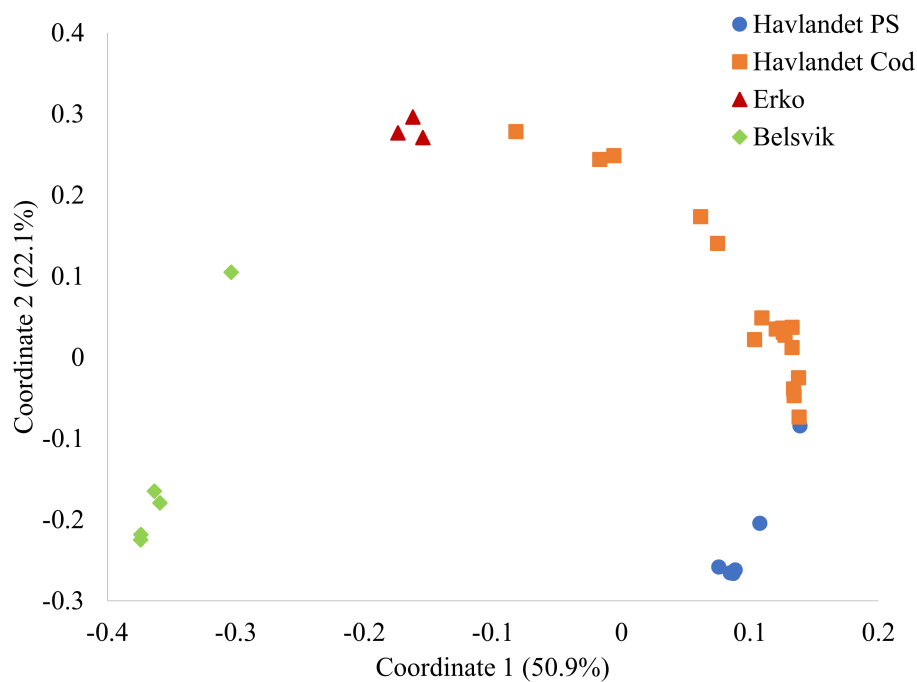
Figure 3.9: PCoA ordination based on Bray-Curtis similarities for comparison of the bacterial communities in the biofilters from Havlandet, Erko and Belsvik. Havlandet PS= biofilm samples from Havlandet biofilter from the period with post-smolt production at 25 ppt, Havlandet Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 pp, Erko= biofilm samples from Erko biofilter from post-smolt production at 15 ppt, and Belsvik= biofilm samples including swab and back-washing sludge from biofilter for post-smolt production at 22 ppt.

Bray-Curtis similarities for comparison of biofilm communities within and between the four facility groups are showed relatively high similarity within groups with average Bray-Curtis similarities between 0.7 and 0.9 (Figure 3.10). The similarities between samples within Havlandet cod range from 0.35 to 0.9, indicating considerable variation in Bray-Curtis similarity between the samples in this group. Samples from Havlandet were the only samples originating from one single facility covering an extended time period, indicating a change in the biofilm communities over time. Comparison of Bray-Curtis similarities between groups showed generally low similarity.
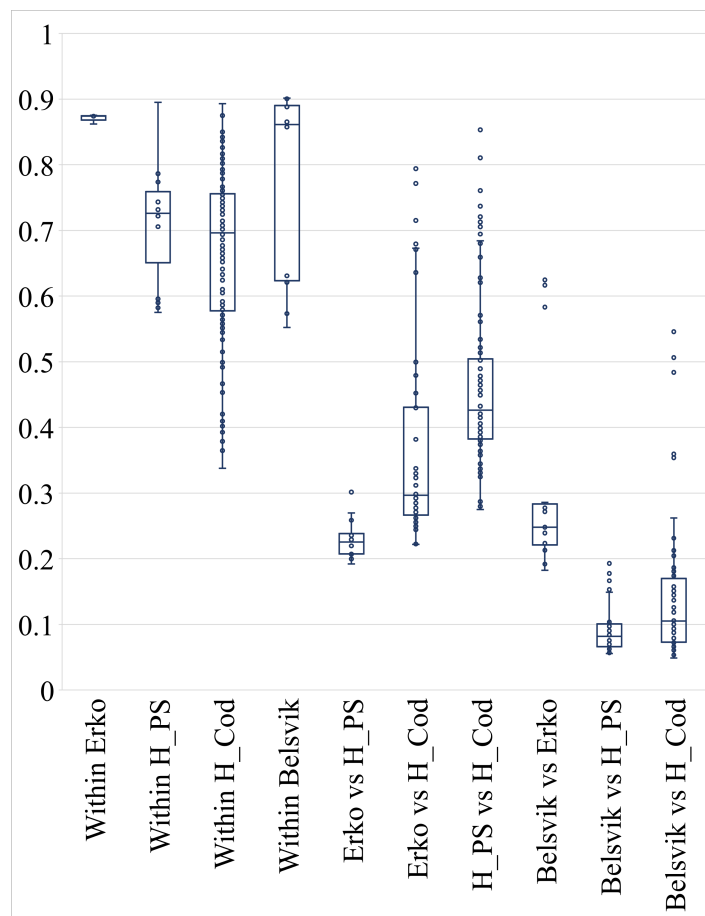
Figure 3.10: Box-plot based on Bray-Curtis similarities for comparison of biofilm communities within and between the four groups; Havlandet post-smolt (H_PS), Havlandet cod (H_Cod), Erko, and Belsvik. The dots represent individual data points, the solid line represents the median, the box for the middle 50% of the data (the interquartile range or IQR), and whiskers that extend to show the range of observations within 1.5 times the IQR.

To study the potential changes in the bacterial communities in samples from Havlandet over time, a PCoA ordination based on Bray-Curtis similarity was plotted to visualize the variation in the communities between sampling times (Figure 3.11). The plot suggested a difference in the communities in samples originating from the period with post-smolt production compared to the period with cod production. A PERMANOVA confirmed a significant difference in bacterial communities between samples from post-smolt and cod production. Temporal dynamics of the biofilm communities in samples from Havlandet were investigated further.

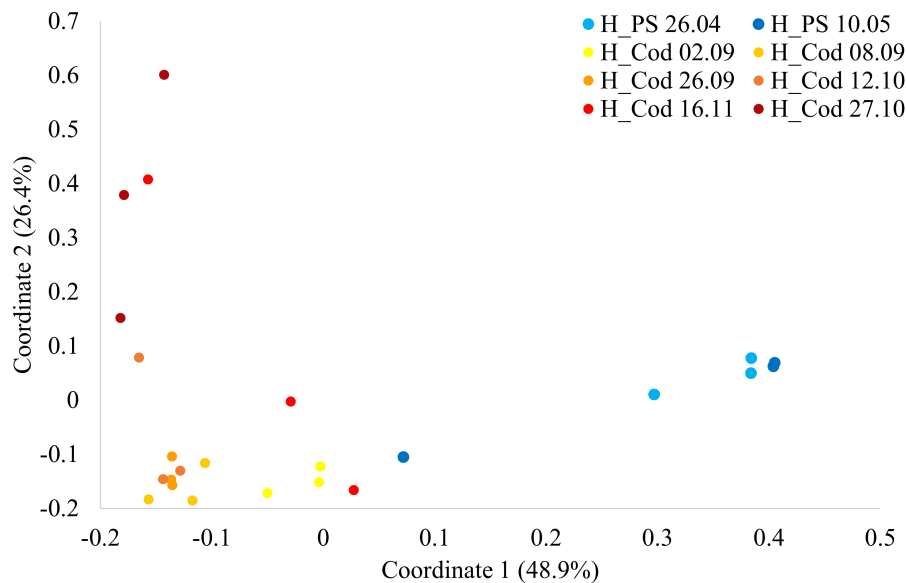**Figure 3.11: PCoA-ordination based on Bray-Curtis similarities for comparison of the bacterial communities in samples from Havlandet following the change in production from post-smolt to cod.** Havlandet PS= biofilm samples from Havlandet biofilter from the period with post-smolt production at 25 ppt, Havlandet Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 pp. The date of sampling is indicated in the group name.

The Bray-Curtis similarity for comparison of the biofilm communities in the samples from Havlandet showed a decrease in similarities over time (Figure 3.12). Comparing Bray-Curtis similarity between the samples from the last sampling from Havlandet during post-smolt production and the samples from the last sampling day at Havlandet during cod production gave an average similarity of 0.35. A Tukey HSD confirmed a significant difference in Bray-Curtis similarities between samples from the first and last sampling day at Havlandet during cod production.
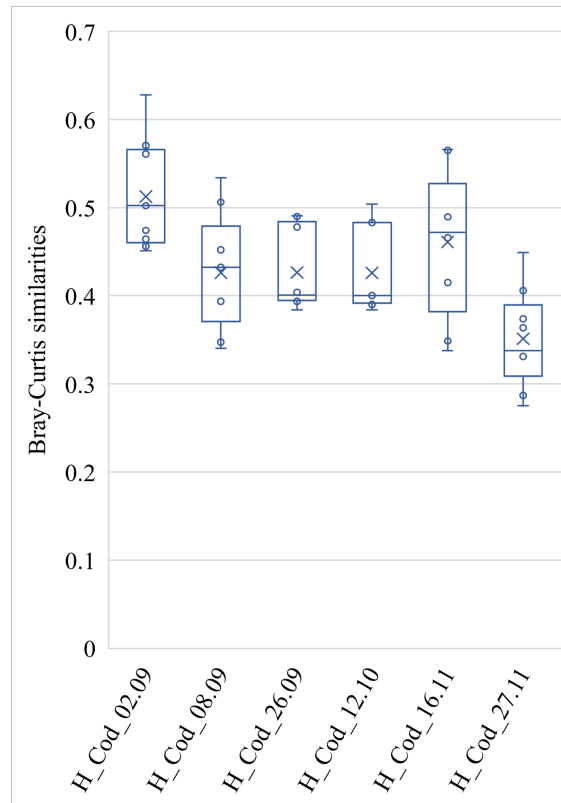
Figure 3.12: Box-plot based on Bray-Curtis similarities comparing biofilm communities between samples from different sampling times at Havlandet. Each comparison is done between the sample from the last sampling time at Havlandet during the production of post-smolt. H_Cod= biofilm samples from Havlandet during cod production. The date of sampling is indicated in the name. Dots represent individual data points, the solid line represents the median, the box for the middle 50% of the data (the interquartile range or IQR), and whiskers that extend to show the range of observations within 1.5 times the IQR.

To identify which ASVs contributed most to the difference in the bacterial communities between the samples from Havlandet during post-smolt and cod production period, a SIMPER analysis based on Bray-Curtis dissimilarities was performed. The most contributing ASV was ASV9, suggested to represent the phylum Nitrospirae, but at a confidence level of 0.65. It accounted for nearly 4% of the difference in the communities between samples from Havlandet during the production of post-smolt and cod. When comparing the first and last samples from Havlandet (H_PS_26.04 and H_Cod_27.11), the average relative abundance of ASV9 increased by over 5%. The second most contributing ASV was ASV12, also suggested to represent the phylum Nitrospirae, but at a confidence level of 0.63. The average relative abundance of this ASV increased by 4% from the first to the last sampling day. The SIMPER analysis also showed that ASV9 and ASV12 were the ASVs that contributed the most to the difference in bacterial communities in samples from Havlandet post-smolt and Erko, as well as between Erko and Belsvik, with a total contribution of 6.8% and 6.7%, respectively. The highest abundance of ASV9 and ASV12 was found in the samples from Erko.

Archaeal communities

A PCoA ordination was generated to compare the archaeal community in the samples from four different groups (Figure 3.13a). The plot indicated a difference between Belsvik and the three other groups. A PERMANOVA confirmed a significant difference between the archaeal community in samples from Belsvik compared to samples from the three other facilities ($p<0.0001$). The samples from Belsvik were excluded, and a second PCoA-plot suggested a difference between samples from Havlandet during post-smolt production, Havlandet during cod production and Erko (Figure 3.13b). A PERMANOVA confirmed this difference to be statistically significant ($p<0.02$).

(a)

(b)
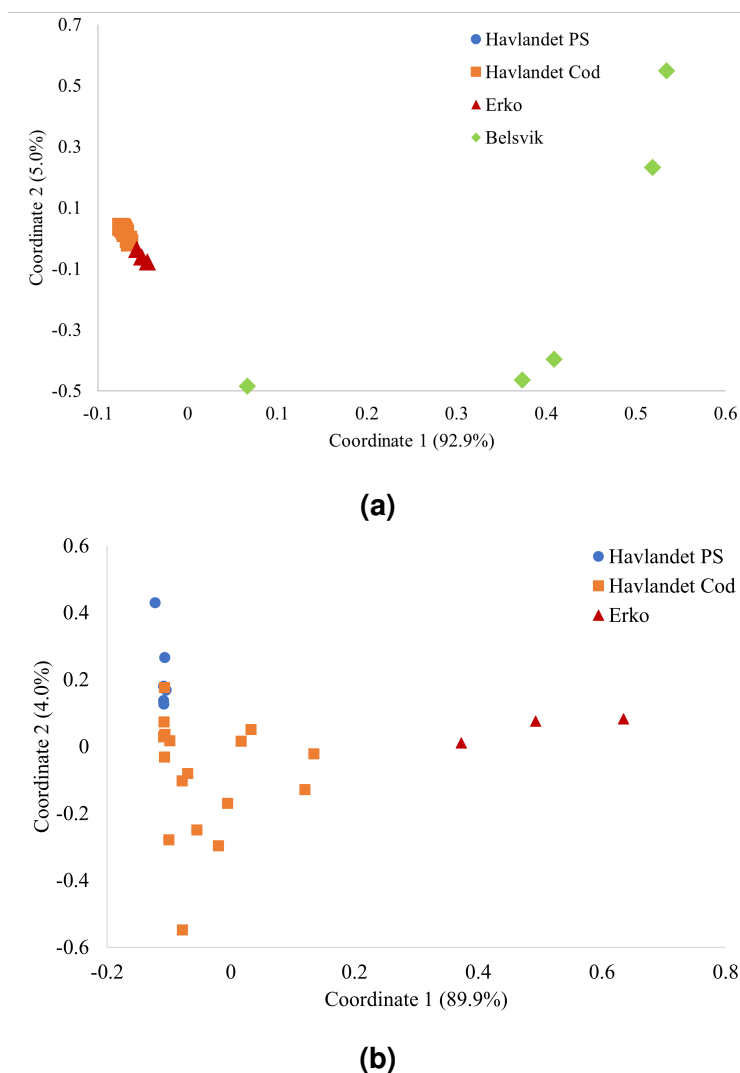
Figure 3.13: PCoA ordination based on Bray-Curtis similarities for comparison of the archaeal community composition in the biofilm samples. (a) Including all four groups; Havlandet PS= biofilm samples from Havlandet biofilter from the period with post-smolt production at 25 ppt, Havlandet Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 pp, Erko= biofilm samples from Erko biofilter from post-smolt production at 15 ppt, and Belsvik= biofilm samples including swab and back-washing sludge from biofilter for post-smolt production at 22 ppt. (b) excluding Belsvik.

A high similarity in the archaeal communities within groups was found for all groups except Belsvik (Figure 3.14). A high similarity was observed in the comparison between groups, also here in exception to comparing groups with Belsvik, indicating variations in the communities between the samples from Belsvik.
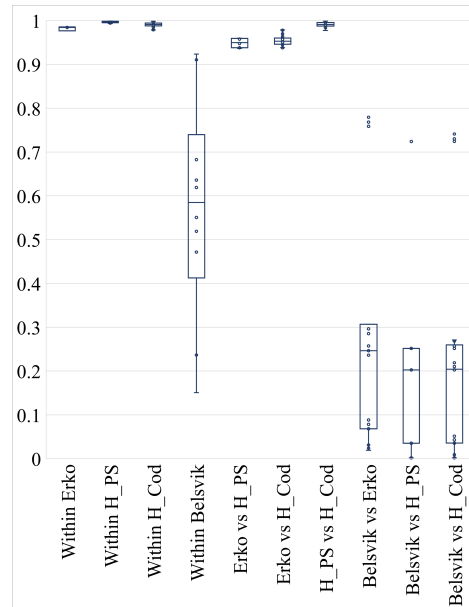
Figure 3.14: Box-plot based on Bray-Curtis similarities for comparison of biofilm communities within and between the four groups; Havlandet post-smolt (H_PS), Havlandet cod (H_Cod), Erko, and Belsvik. Dots represent individual data points, the solid line represents the median, the box for the middle 50% of the data (the interquartile range or IQR), and whiskers that extend to show the range of observations within 1.5 times the IQR.

Potential changes in the archaeal community in the biofilm samples collected from Havlandet before and after the change of cultivated species and the change over time were visualized in a PCoA ordination. A distinct difference in communities was not clear from the PCoA-plot. A PERMANOVA confirmed no significant difference in the archaeal community between any of the samples collected at different time points at Havlandet (p>0.05).
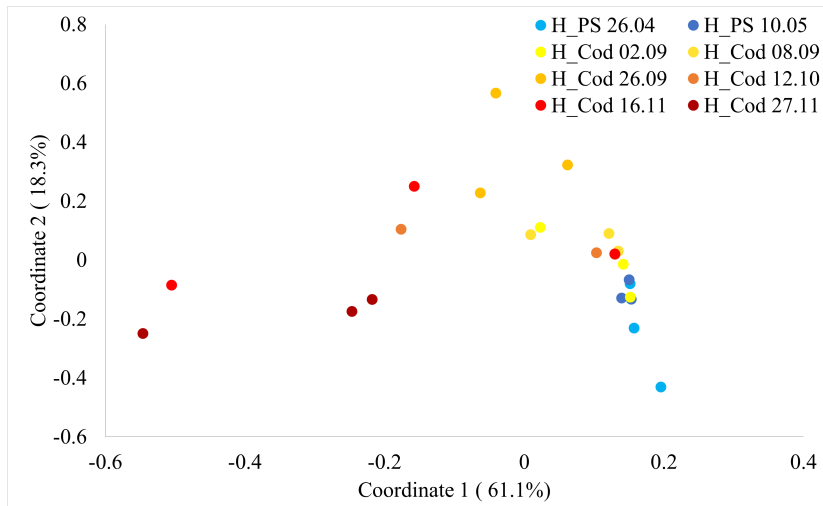
Figure 3.15: PCoA-ordination based on Bray-Curtis similarities for comparison of the archaeal communities in samples from Havlandet following the change in production from post-smolt to cod. Havlandet PS= biofilm samples from Havlandet biofilter from the period with post-smolt production at 25 ppt, Havlandet Cod= biofilm samples from Havlandet biofilter from the period with cod production at 35 ppt. The date of sampling is indicated in the group name.

3.2.4 Nitrifying communities of the marine biofilm samples

By manually expecting the bacterial ASV table, all ASVs representing nitrifiers were identified. A considerably higher abundance of nitrifiers was found in the samples from Erko and the samples from Havlandet collected at the end of the cod production period (Figure 3.16). On average, the bacterial community in the biofilm samples from Erko had a relative abundance of nitrifier of 18%, approximately double the abundance compared to the samples from Havlandet during cod production and the samples from Belsvik, with a relative abundance of 8% and 7%, respectively. The relative abundance of nitrifiers in the biofilm samples from Havlandet during post-smolt production was on average as low as 1%.
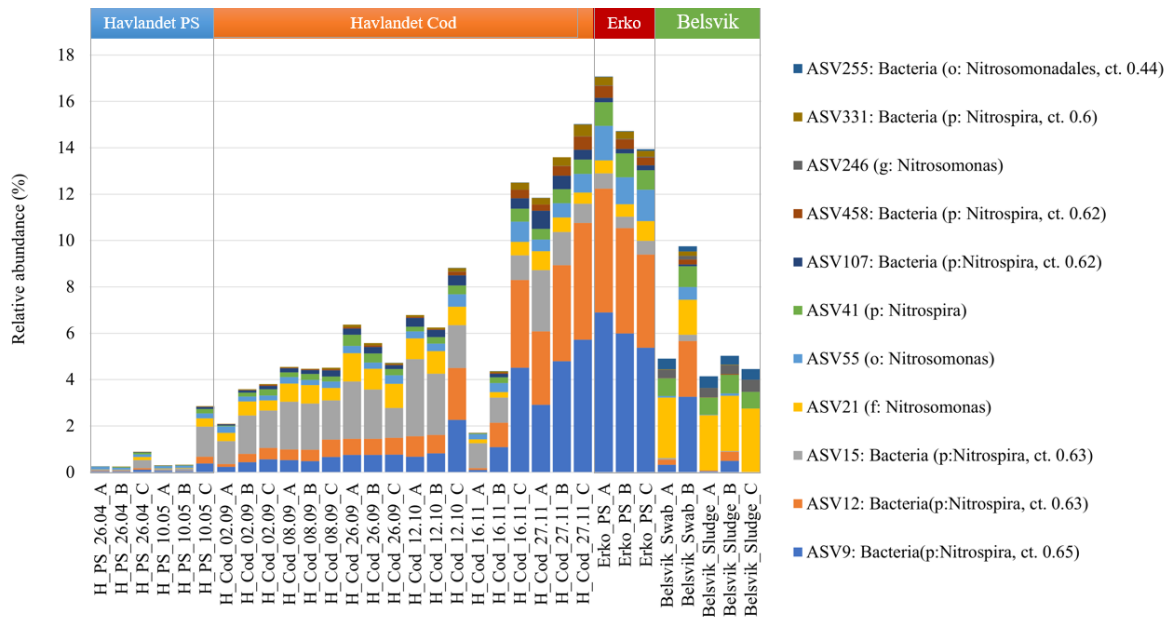
Figure 3.16: Relative abundance of ASVs classified as nitrifying bacteria in the biofilm samples. Only ASVs with a maximum abundance larger than 0.5% in at least one sample are included. The taxonomy for the ASV is given at the lowest level obtained at the 0.8 level of confidence. For ASVs where this did not indicate a nitrifying taxon, the taxonomy obtained at a lower confidence threshold (ct) is also specified.

The nitrite-oxidizing phylum Nitropira was dominant among the nitrifiers in most of the samples. A BLAST search was performed, showing that ASV9, ASV12 and ASV15 were all found to be closely related to *N.salsa*. ASV41, the fourth most abundant ASV representing *Nitrospira* in the biofilm samples was closely related to *N. marina*. A Neigbour-joining phylogenetic tree was generated, confirming previously mentioned ASVs to be closely related to *N.salsa* and *N. marina* (Figure 3.17). No ASVs were closely related to comammox *Nitrospira*. *Nitrosomonas*, a genus known for ammonia-oxidizing, was the second most abundant nitrifier. Generally, all bacterial ASVs identified as nitrifiers were classified as either the phylum Nitrospirae (NOB) or the order Nitrosomonadales (AOB). *Nitrospira* was on average six times more abundant than *Nitrosomonas* in the samples from Havlandet during cod production and in the samples from Erko, giving a high NOB:AOB ratio. In the samples from Havlandet during the production of post-smolt, *Nitrospira* was three times more abundant, and in the samples from Belsvik, the relative abundance of *Nitrospira* and *Nitrosomonas* was approximately equal.
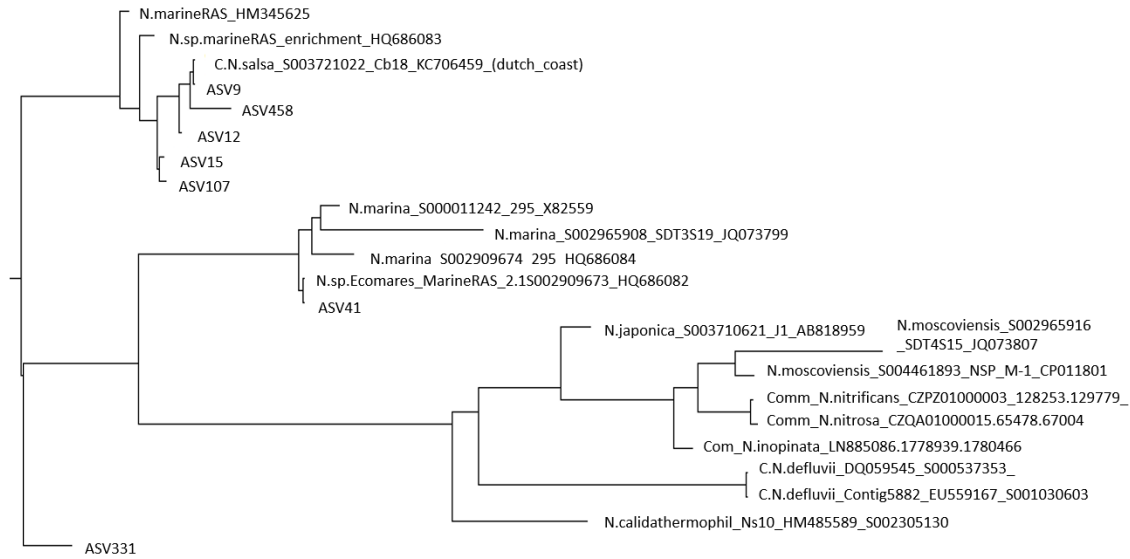
Figure 3.17: A Neighbour-joining tree comparing most abundant ASVs classified as Nitrospira to previously described Nitrospira representing marine and comammox Nitrospira. The sequences were retrieved from the NCBI Genbank or RDP database. The accession numbers are specified with the species names. Comm is indicating comammox. The tree was generated using the Multi-way alignment tool in Clone Manager (v.9).

The archaeal communities consisted of ASVs classified as only *Nitrosopumilus* or *Woesearchaeota*. *Nitrosopumilus* is a known ammonia-oxidizing archaea [48], while Woesearchaeota has no documented nitrifying activity [49]. Nitrifying archaea account for over 90% of the archaeal communities in samples from the three facilities (Figure 3.18).
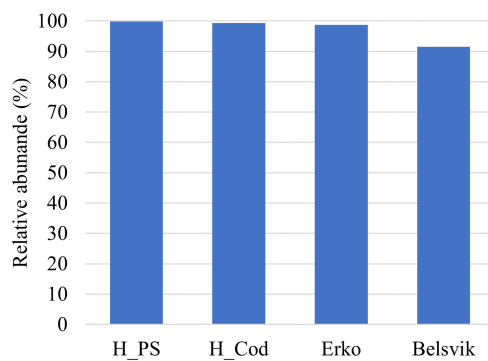
Figure 3.18: Relative abundance of archaeal ASVs classified as nitrifiers. H_PS=biofilm samples from Havlandet during the production of post-smolt at 25 ppt salinity, H_Cod= biofilm samples from Havlandet during the production of cod at 35 ppt salinity, Erko= biofilm samples from Erko during post-smolt production at 15 ppt, Belsvik= biofilm and biofilter back-wash sludge samples from Belsvik during the production of post-smolt at 22 ppt salinity.

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3.3 Archaeal ammonia-oxidizers of the marine nitrifying biofilters

In the biofilm samples from Havlandet and Erko, the nitrifying communities were dominated by Nitrospira, but a low relative abundance of AOB was found. The amplicon sequencing of the archaeal communities indicated the presence of AOA, and the biofilm communities from Havlandet and Erko suggest that only one AOA population dominated. The type and quantity of the *amoA* gene were investigated by end-point PCR followed by Sanger sequencing and qPCR. This was performed on a selection of samples representing Havlandet, Erko and Belsvik.

3.3.1 Quantification of the archaeal *amoA* gene copies in the biofilm samples

The primer sets AmoANSPA and AmoANSPB was designed to amplify the *amoA* gene. In order to perform absolute quantification of the gene, standard curves correlating the C_T-values and the copy numbers of the target gene were made. (Figure 3.19a and 3.19b).
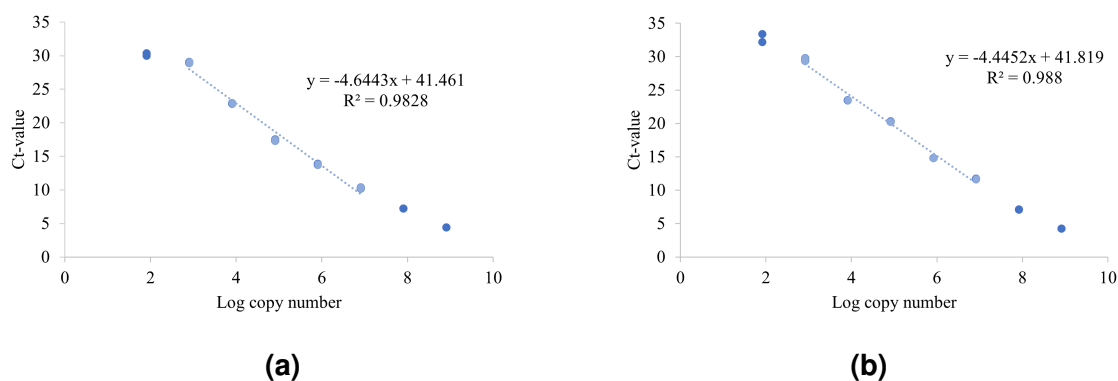
Figure 3.19: qPCR standard curves for primer set targeting the Nitrosopumilus amoA-gene:(a) AmoANSPA, (b) AmoANSPB. Copy number was calculated as described in Section 2.6.1. The standard curve for *amoA* gene was based on a dilution series of synthetic oligonucleotides of (a) 107 bp and (b) 112 bp used as specific targets for the relevant primer pairs. Linear regression was used to determine the relationship between DNA concentration and C_T-value, including the points shown in light blue points.

The amplification efficiencies were calculated (Equation 2.2) based on linear regression. The amplification efficiency for AmoANSPA was found to be 64.2% and for AmoANSPB 67.9%. This was lower than expected. The low amplification efficiencies indicated that the PCR conditions were not optimal, and can lead to inaccurate measurements of the abundance of the *amoA* gene.

The standard curves were used to determine the copy number of the *amoA* gene in the biofilm samples (Figure 3.20). In general, amplification with the AmoANSPB

primer set gave a higher concentration of the *amoA* gene than with the AmoANSPA primer. A considerably higher abundance of the *amoA* gene was found in samples from Havlandet during the production of cod and Erko than in the samples from Havlandet during the production of post-smolt and Belsvik.
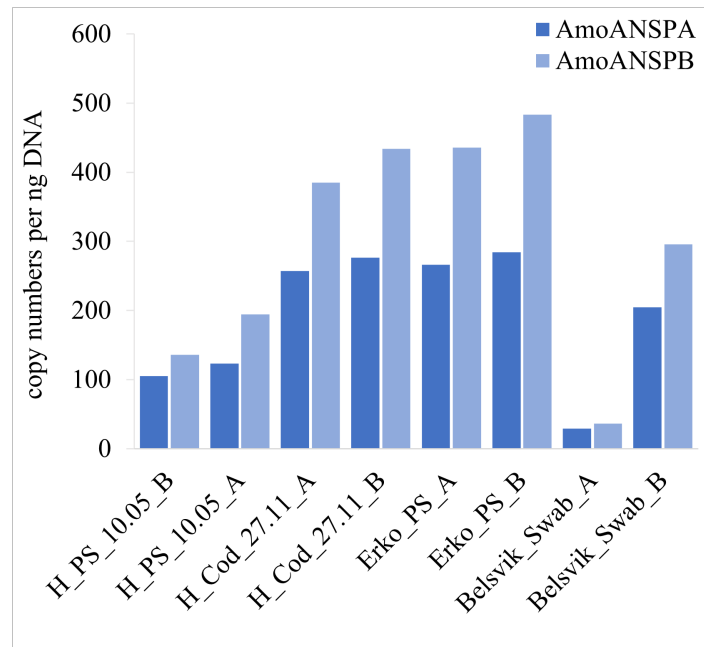
Figure 3.20: Estimated copy number of the amoA gene based on qPCR with the primer sets AmoANSPA and AmoANSPB. The standard curves (Figure 3.19) were used to correlate measured C_T-values for the samples with DNA concentration. The copy number was determined based on C_T-values and then normalized to the concentration av DNA in the original sample. H_PS=sample from Havlandet during post-smolt production, H_Cod=sample from Havlandet during cod production, Erko_PS=sample from Erko during post-smolt production, and Belsvik_Swab= swab sample from biofilter at Belsvik. The sampling date for samples from Havlandet is indicated in the names. A and B indicate replicate carrier/swab samples.

The products after qPCR were analysed by melting point analysis (Figure 3.21). The melting point analysis indicated in general a specific PCR amplification for both primer sets. The melt point curve for all samples amplified with both primer sets has one single peak, but the peaks are slightly broad. The melt curve for Belsvik_Swab_A when amplified with both sets of primers, had a lower and broader peak than the rest of the samples, indicating the presence of non-specific products or poor-quality amplification.
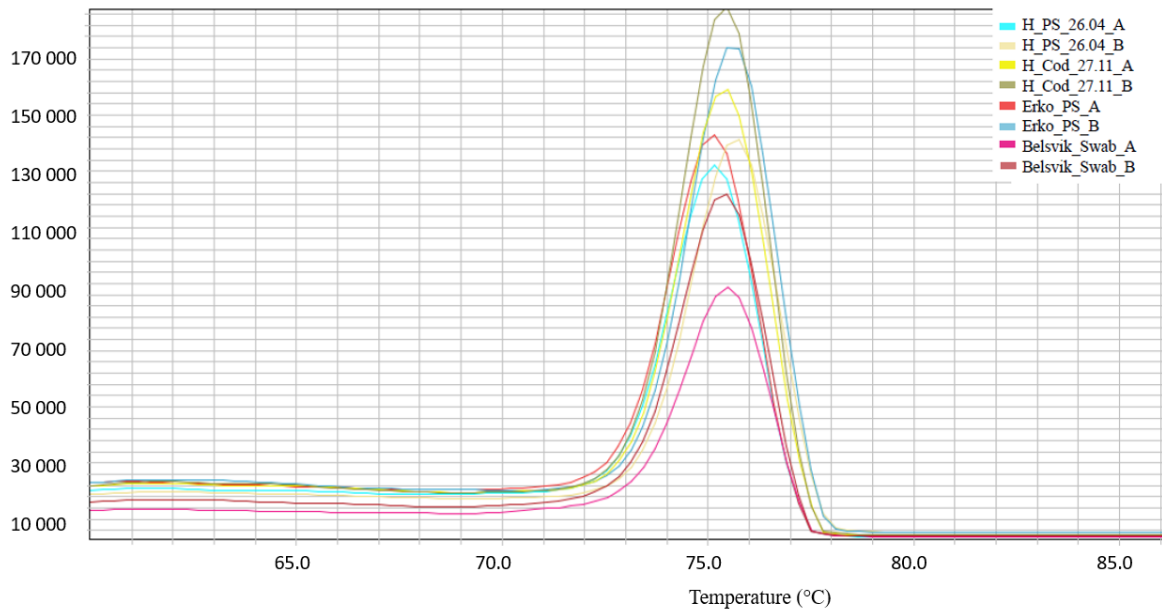
(a)
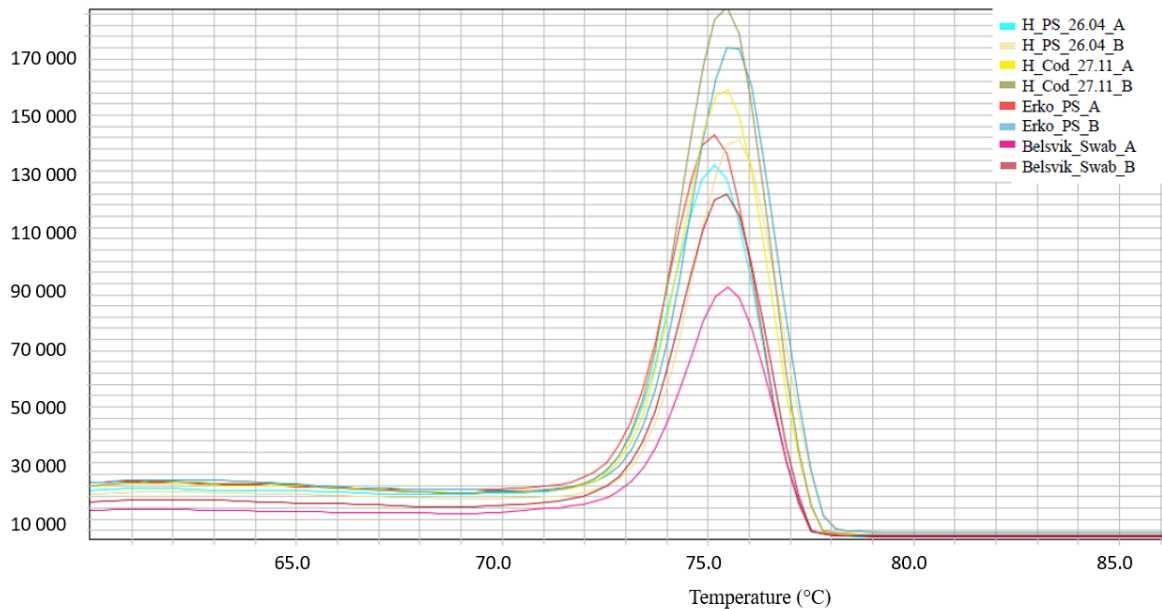
(b)

Figure 3.21: Melt point curves after qPCR of the amoA gene amplified with primer (a)AmoANSPA and (b) AmoANSPB. The average of three parallels for each sample was used. H_PS=samples from Havlandet during post-smolt production, H_Cod=samples from Havlandet during cod production, Erko_PS= samples from Erko during post-smolt production, Belsvik_Swab= swab samples from biofilter at Belsvik during post-smolt production. The sampling dates for samples from Havlandet are indicated in the names. A and B indicate replicate biofilm samples.

3.3.2   Determination of the archaeal *amoA* gene sequences

Parts of the archaea *amoA* gene were amplified with two primer sets targeting the *amoA* genes; one broad coverage targeting the archaeal *amoA* gene (AmoAArch), and one targeting the Nitrosopumilus *amoA* gene (EPPCRNSP). The PCR resulted in specific PCR products of the expected length for both primer sets for all three samples (Figure 3.22).
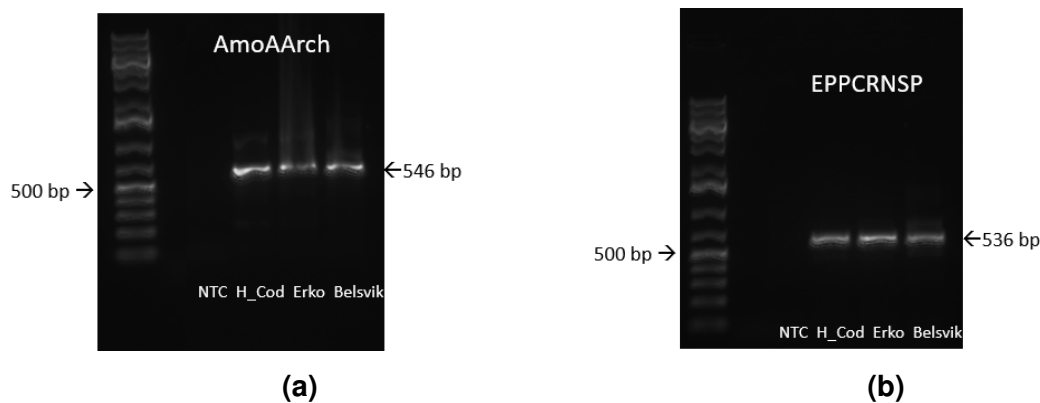
Figure 3.22: Agarosegel (1%) with PCR products amplified with primer set targeting (a) the archeal *amoA* gene and (b) targeting the *amoA* gene in *Nitrosopumilus*. AmoAArch was a broad coverage primer targeting the archaea amoA-genes. EPPCRNSP was targeting the amoA-gene in *Nitrosopumilus*. NTC=non-template control, H_Cod=biofilm sample from Havlandet during cod production at 35 ppt salinity, Erko= biofilm sample from Erko during post-smolt production at 15 ppt salinity, Belsvik= biofilm sample from Belsvik during production og post-smolt at 22 ppt salinity. One sample from each facility was included in the PCR. The expected lengths of PCR products are shown in the figure.

The PCR products were sequenced by Sanger sequencing, with both forward and reverse PCR primers as sequencing primers. For the samples from Havlandet and Erko, sequencing of AmoAArch and EPPCRNSP PCR products resulted in sequences of high quality (Appendix G). For the sample from Belsvik, sequencing of AmoAArch and EPPCRNSP PCR product resulted in sequences of relatively good quality, with some double signals for some positions, around 10-20%, yet due to lower quality, non of the sequencing data was used in further analysis. A summary of the sequencing results is presented in (Table 3.2). The sequences of high quality were investigated further.

Table 3.2: Overview of sequence quality obtained from Sanger sequencing. PCR products were sequenced with primer sets AmoAArch (broad coverage primer targeting archaea amoA gen) and EPPCRNSP (targeting the amoA-gene in Nitrosopumilus). The length of the resulting sequences is indicated in the table.

PCR product	Havlandet	Erko	Belsvik
AmoAArch	high quality (496 bp)	high quality (496 bp)	15% double signal
EPPCRNSP	high quality (496 bp)	high quality (496 bp)	20% double signal

The sequencing resulted in two sequences, one for the AmoAArch and one for EP-PCRNSP PCR products. An alignment in Clone Manager showed that the two sequences were identical. Next, a Nucleotide BLAST search (NCBI) showed that the sequence was identical to the *amoA* gene of *Nitrosopumilus oxyclinae* which is classified as an AOA.

4   Discussion

4.1   The nitrification capacity of marine biofilm carriers determined in batch experiments

The lab-scale batch experiments were conducted with biofilm carriers from marine biofilters at Havlandet and Erko to determine the nitrification capacity and salinity sensitivity of the biofilm carriers. Three parallels with different salinities, 31 ppt, 15 ppt, and 0 ppt, were run in each batch experiment.

The biofilm carriers from Havlandet originated from a biofilter in a RAS for the production of cod at 35 ppt. The highest nitrification capacity was observed in the seawater and brackish water reactors, while in freshwater, the capacity was halved (Figure 3.1). In a study by Hüpeden et al. [50], a salinity tolerance test showed that the nitrification capacity of brackish and marine nitrifiers was highly resistant and maintained its nitrification activity over a wide range of salt concentrations. Like in the test Hüpeden et al. conducted, the biofilm carriers from Havlandet, with nitrifiers adapted to seawater, maintained their nitrification activity in seawater and brackish water.

Regardless of the salinity, the biofilm carriers from Erko, which originated from a brackish RAS biofilter operated at 15 ppt for post-smolt production, showed approximately the same nitrification activity for all three salinities (Figure 3.2). The study by Hüpeden et al. [50] also showed that for the brackish nitrifiers, the highest nitrification activity was observed at 0 ppt. Although the capacity for the Erko biofilm carriers was approximately the same in all salinities, a slightly higher capacity was observed in freshwater. The results from this batch experiment suggest that brackish biofilters were more robust to salinity changes.

The biofilm carriers from Erko demonstrated superior nitrification capacity compared to those from Havlandet, particularly in freshwater conditions (Figure 3.3). On average, a three times higher nitrifying capacity per surface area, and in freshwater five times higher capacity, was observed for the biofilm carriers from Erko compared to those from Havlandet. These biofilm carriers originated from two separate RAS facilities, operated at different salinities, and considering that the batch experiment was performed with identical conditions, it is conceivable that the nitrifying communities differ between the biofilters. Biofilm carriers from Erko were used as inoculums during the biofilter start-up at Havlandet, indicating that the microbial community compositions of the two biofilters have evolved to be different. Further investigation was carried out to characterize the biofilm communities present on the biofilm carriers to identify the nitrifying communities and investigate similarities and differences in the microbial communities.

The increase in nitrate concentration did not correspond with the decrease in TAN concentration in seawater and brackish water reactors (Figure 3.1 and 3.2). To eliminate the interference in nitrate measurements caused by the salt in the water, a chloride elimination kit was used for the water samples from brackish and seawater. In freshwater, the increase in nitrate concentrations corresponded to the decrease in TAN concentrations. This indicated that the chloride-elimination kit may have failed to avoid interference. However, since all the water samples from brackish water and seawater were treated equally, it can be assumed that although the measured concentration may not be precise, the comparison between them was still valid. The concentration of TAN in the freshwater reactors indicated that the issue only applies to the measurements of nitrate concentration.

4.2 Microbial community composition

4.2.1 Bacterial communities

The bacterial community composition at the class level showed no clear difference in the communities between the different samples (Figure 3.6). Nevertheless, the community composition at the ASV level revealed a significant difference (Figure 3.7). *Maribacter* was the most common ASV in the biofilm samples from Havlandet, Nitrospira was the most common ASV in the biofilm samples from Erko, and three ASVs classified as *Alphaproteobacteria*, *Flavobacteria* and *Saprospira* were most common in the biofilm samples from Belsvik. *Maribacter* is a member of the family Flavobacteriaceae and has been isolated from different marine environments [51]. Flavobacteria has been found to be one of the most abundant taxa in marine consortia [32]. Flavobacteria, Alphaproteobacteria, Saprospira and Nitrospira are commonly found in biofilters [32] [27].

The presence and abundance of ASV9 and ASV12, representing *Nitrospira*, were found to be key contributors to the dissimilarities observed in the biofilm samples, as confirmed by a SIMPER analysis. The relative abundance of these two ASVs varied between the three facilities, where the highest abundance was found in biofilm samples from Erko. *Nitrospira* is commonly found in nitrifying biofilters and is believed to be the primary nitrifier in RAS biofilters [34] [28].

There was a significant difference between the bacterial communities of the biofilm samples from Havlandet during post-smolt production and the biofilm samples from Erko. Considering Erko was used as inoculum under the start-up of the biofilter at Havlandet for post-smolt production, the bacterial community composition in the samples from Erko was more similar to the samples from Havlandet during the production of cod. The difference was mainly distinct not considering the ASV present, but the relative abundance of specific ASVs. The microbial communities seem to

have evolved differently, indicating that a difference in operational conditions, like salinity, creates different selection pressure. A study by Navada et al. [52] looking into the effect of priming (prior exposure to seawater) on the nitrifying communities showed that it affected the microbial community composition but not the nitrifying taxa.

4.2.2 Archaeal communities

The archaeal communities in the biofilm samples from Havlandet and Erko were entirely dominated by one single ASV representing *Nitrosopumilus* (Figure 3.8). In contrast, the archaeal community in the biofilm samples from Belsvik had a higher diversity and was mainly dominated by a different ASV representing *Nitrosopumilus*. These findings suggested the presence of two distinct populations present in the biofilm samples from Belsvik. All archaeal ASVs present in the biofilm samples were either classified as *Nitrosopumilus* or *Woesearchaeota*. *Nitrosopumilus* is an AOA previously found in biofilm communities in marine RAS for the cultivation of shrimp [36]. *Nitrosopumilus* was the only AOA identified in the biofilm samples. This is a genus in the phylum Thaumarchaeota, also found in RAS biofilters, where they play an important role in nitrogen removal [53]. *Nitrosopumilus* dominated the archaeal communities in biofilm samples from all three facilities, with a relative abundance above 90% (Figure 3.18).

The highest abundance of ASVs classified as *Woesearchaeota* was found in the biofilm samples from Belsvik. A recent study looking into the ecology, evolution and metabolism of *Woesearchaeota* suggested that this phylum may play a role in organic carbon degradation, as well as being involved in nitrogen cycling [54]. *Woesearchaeota* has been found to be abundant in libraries of wastewater treatment systems, and some genes involved in the nitrogen cycle have been detected. These genes are, amounts others, responsible for denitrification, converting nitrite to dinitrogen. As mentioned, the biofilm samples from Belsvik originate from an FBBR, where anoxic zones can appear in the biofilm. No genes involved in nitrite oxidation have yet been found in *Woesearchaeota* [49]. Therefore, further research is needed to understand the exact role of *Woesearchaeota* in a nitrifying biofilm.

4.2.3 Nitrifying communities

Nitrospira and *Nitrosomonas* were the most abundant AOB and NOB in the biofilm samples. *Nitrospira* dominated the nitrifying communities in the biofilm samples from Havlandet and Erko (Figure 3.7). The bacterial communities in samples collected from Havlandet during the early stage of cod production were mostly dominated by ASV15, while samples collected at a later stage during cod production, in addition to

biofilm samples from Erko, were mostly dominated by ASV9 and ASV12, all representing *Nitrospira*. The bacterial nitrifying communities in the biofilm samples from Belsvik were dominated by ASV21, classified as *Nitrosomonas*, commonly found in RAS biofilters [53]. From the phylogenetic tree, ASV9 and ASV12 were closely related to *N.salsa*, and ASV41 was closely related to *N. marina*. *N. salsa* has been found in marine biofilters [55] and has previously been found to oxidate nitrite in marine environments in the Dutch coastal North Sea [55]. *N. marina* has previously been found in saltwater aquaria [29] and marine biofilters [56], and was the most dominant NOB in a study investigating ammonia- and nitrite oxidizers in marine aquaculture biofilms [57].

A higher NOB: AOB was found for biofilm samples from Havlandet during cod production and Erko, compared to the biofilm samples from Havlandet during post-smolt production and Belsvik. A high NOB:AOB has previously been found in a study comparing two seawater adaptation strategies [15]. A general low abundance of AOBs in the communities indicated that the AOA were important ammonia-oxidizers in these biofilms. Brown et al. [36] found a higher abundance of the archeal *amoA* gene than the bacterial *amoA* gene in a marine biofilter for shrimp production, suggesting that AOBs played a minor role in the nitrification activity in the biofilter.

Due to a high relative abundance of *Nitrospira*, and a low relative abundance of AOBs, it was investigated if any of the ASVs representing *Nitropsira* was related to complete ammonia oxidizers, comammox. Comammox *Nitrospira* has been found in freshwater biofilters, and could possibly be found in marine biofilters [34]. A high NOB:AOB could indicate the presence of comammox, yet, no highly abundant ASVs representing *Nitrospira* in the biofilm samples were related to comammox *Nitrospira* based on the phylogenetic tree (Figure 3.17).

A difference in the bacterial nitrifying communities was observed when comparing the relative abundance of nitrifiers in the biofilm samples from the different facilities. The relative abundance of bacterial nitrifiers ranged from 1% to 18% between the biofilm samples. The abundance of nitrifiers in marine biofilters has been reported to range between 0-20% [15], and Navada et al. reported a relative abundance of nitrifiers under 30% during the start-up of a marine biofilter [18]. A study by Tesdal [27] reported a surprisingly high abundance of potential ASVs representing nitrifiers of 77% during the start-up of a marine biofilter. Successful nitrification was reported for the biofilters from all three facilities, also during the production of post-smolt at Havlandet, suggesting that a high abundance of nitrifiers might not be necessary to achieve efficient nitrification.

4.2.4 Change in microbial communities in biofilm samples from Havlandet after a switch in salinity and cultivated species

By analysing samples taken from the biofilter at Havlandet both before and after the switch from salmon to cod, as well as samples collected over a three-month period after the introduction to cod, the impact of changes in both salinity and cultivated fish species on the dynamics of the biofilm communities could be investigated.

Between the samples taken from the Havlandet biofilter during the production of post-smolt (at 22 ppt salinity) and during the production of cod (35 ppt salinity), a significant difference between the bacterial communities was found(Figure 3.11). Comparing biofilm communities for samples collected at various time points during the period of cod production with the biofilm samples collected on the last sampling day during the production of post-smolt, revealed a decline in community similarity over time (Figure 3.12). Notably, no discernible shift in similarity was observed between September 8th and November 16th, indicating a relatively stable bacterial community during this period. However, a significant decrease in Bray-Curtis similarity was observed between the first and last samples collected during cod production, indicating a shift in the bacterial community dynamics. A significant difference was found for the archaeal communities from the biofilm samples from Havlandet during the production of post-smolt and cod, yet no significant difference between any of the samples over time was found (Figure 3.15).

From the ASV table (Figure 3.7), no ASV representing nitrifying bacteria with a maximum abundance larger than 2% in at least one sample was present in the biofilm samples from Havlandet during post-smolt production. A slight increase in abundance was observed when comparing these samples to some of the first samples taken from Havlandet during cod production. An increase in the abundance of especially two ASVs representing *Nitrospira*, ASV9 and ASV 12, was evident between the samples collected from Havlandet during the production of cod (Figure 3.7). In general, a noticeable increase in the relative abundance of ASVs classified as nitrifying bacteria was observed (Figure 3.16). An increase from 4% to 14% in the relative abundance of bacterial nitrifiers was observed between the samples from the first and last sampling day at Havlandet during cod production. A SIMPER analysis confirmed that these two ASVs contribute most to the difference in bacterial communities between the samples from Havlandet during cod production.

4.2.5 Differences in microbial communities affecting the nitrifying capacity

As previously discussed, the biofilm carriers from Erko exhibited a significantly higher nitrification capacity than the biofilm carriers from Havlandet, on average three times higher capacity (Figure 3.3) and relative abundance of bacterial nitrifiers (Figure

3.16). It is plausible that the abundance of nitrifiers in the biofilters correlates with the nitrification capacity. Salinity has been found to extensively influence and regulate the community compositions and the abundance of ammonia oxidizers and nitrifying activity in RAS [58]. It should be noted that the characterization of the archaeal communities indicated that AOAs were present in the biofilm and that these archaeal nitrifiers also contribute to the nitrification activity in the biofilter. As there was no significant difference in the abundance of AOAs between Havlandet and Erko, the difference in nitrification capacity, in this case, may be linked to the abundance of bacterial nitrifiers in the biofilm communities.

Three times higher NOB: AOB ratio was found in the biofilm carriers from Erko used in the batch experiments, compared to the biofilm carrier from Havlandet. Gonzales et al. [33] reported a fourfold higher NOB population than AOB in the freshwater reactor with almost complete ammonium conversion. These findings are in agreement with Figuerola and Erijman [59] who reported that a high NOB:AOB ratio appeared to correlate with good nitrification. The high NOB: AOB ratio in the biofilm samples from Erko might be a cause of good nitrification activity in freshwater.

ASV246 and ASV255, classified as *Nitrosomonas*, were the only two ASVs, with a maximum abundance higher than 0.5% in at least one sample, present in just the biofilm samples from Erko and, with an average relative abundance of 0.7%. One ASV with a low relative abundance of 0.1% representing Nitrospira was also only present in the biofilm samples from Erko. As the biofilm carriers were more robust to salinity changes and exhibited high nitrifying capacity in freshwater, these ASV might represent bacteria with a higher tolerance to freshwater. As the biofilter at Erko was operated at a lower salinity than the biofilter at Havlandet, the nitrifying communities might be more robust to adapt to freshwater.

The number of biofilm carriers used in the batch experiments was taken into account when calculating the nitrifying capacity per unit surface area. However, the number of microbes per surface area could be different for the two biofilm carriers. The higher nitrifying capacity for the biofilm carriers from Erko could therefore not only be due to a higher relative abundance of nitrifying bacteria present but also because of a larger number of bacterial- and archeal cells present per biofilm carrier used in the experiment.

As both the bacterial and archeal nitrifying community in the biofilm samples from Belsvik differed clearly from biofilm samples from Havlandet and Erko, it would be interesting to look into the nitrification capacity of this biofilter. As the biofilter at Belsivk was an FBBR, it was not possible to conduct a similar batch experiment for investigating the nitrification capacity of this biofilter.

4.3 Identifying and quantifying the ammonia-oxidizers archaea in the biofilm samples

The archaeal communities were examined using amplicon sequencing, which revealed the presence of *Nitrosopumilus*, known to oxidize ammonia. The biofilm communities from Havlandet and Erko showed the dominance of a single archaeal ASV (Figure 3.8). To further investigate, a subset of samples representing Havlandet during cod production, Erko, and Belsvik were subjected to a qPCR followed by an end-point PCR and Sanger sequencing to determine the presence and quantity of the *amoA* gene in the biofilm samples.

The results from the end-point PCR and Sanger sequencing indicated that there in the biofilm samples from Havlandet and Erko was one archaea-*amoA*-sequence dominating. This sequence was identical in these biofilm samples. This sequence was found to be identical to parts of the *amoA* gene in *N. oxyclinae*, previously isolated from marine environments [60] [61]. For the biofilm samples from Belsvik, the Sanger sequencing resulted in messy results, indicating multiple targets for the primer and possibly multiple *amoA* genes present in the biofilm sample, or unspecific binding and amplification. However, from the gel-electrophoresis, the PCR products were of the expected length (Figure 3.22). As the archaeal communities in the biofilm samples from Belsvik were dominated mainly by a different ASV representing Nitrosopumilus than Havlandet and Erko (Figure 3.8), a different variant of the *amoA* gene could be present in the biofilm samples from Belsvik.

The archaeal communities might contribute to large parts of the nitrification activity in regards to ammonia oxidation, as *Nitrosopumilus*, a known AOA, dominated the archaeal communities in all the biofilm samples. However, without knowledge about the relationship between the abundance of bacteria and archaea in the communities, it is uncertain whether the archeal nitrifying communities have a high relative abundance in the microbial communities, or if it is the bacterial nitrifying community, dominated by *Nitrospira* and *Nitrosomonas*, that is primarily responsible for the nitrifying activity of the biofilter.

A higher concentration of the *amoA*-gene was found in qPCR products amplified with AmoANSPB (Figure 3.20). qPCR with primers AmoANSPA and AmoANSPB, targeting the *amoA* gene in *Nitrosopumilus* was performed with low amplification efficiency (64.2% and 67.9%, respectively), indicating that the PCR conditions were non-optimal. As AmoANSPB had a higher amplification efficiency than AmoANSPA, that might explain the higher concentration of the *amoA* gene identified in the qPCR with the AmoANSPB primers. One implication of low amplification efficiency is that it may lead to inaccurate quantification of the target DNA.

4.4 Further work

In this master thesis, *Nitrospira* and *Nitrosopumilus* were found to be important contributors to the nitrifying community composition of the biofilm samples from RAS operated at high salinities. It could be interesting to investigate further why the biofilm carriers from Erko had such a high nitrifying capacity and tolerance in both high and low salinity. Continuous lab-scale MBBR would be run over a longer period of time at high and low salinity, investigating how it affects the nitrifying communities. That could give an insight into which populations in a biofilm community are the most important at different salinities. Similar lab-scale batch experiments could be performed on biofilm carriers from multiple RAS operated at varying salinities to further investigate the difference in nitrifying capacity and salinity tolerance and to examine if there was a correlation between nitrifying capacity and the abundance of nitrifiers in the biofilm.

Considering the superior nitrifying capacity and salinity tolerance of the biofilm carriers from Erko, it could be interesting to investigate the potential of these biofilm carriers in a start-up of a new biofilter for a RAS operated at high salinities and investigate how the biofilm community composition evolves and changes on new biofilm carriers.

qPCR could be performed with primers targeting the 16S rDNA or the *amoA* gene in both bacteria and archeal. From this, the ratio between bacteria and archaea in the total community could be determined, giving insight into the relative abundance and importance of archeal nitrifiers in the biofilm. In this way, the ratio between ammonia oxidizers and nitrite oxidisers could be further investigated, and specific primers targeting the *amoA* gene and *nxr* gene could be used in Illumina sequencing to investigate the origin of the nitrifying genes. A challenge would be to design primers targeting these genes in all microbes. The presence of comammox *Nitrospira* could be investigated by using primers targeting the comammox *amoA* gene.

5 Conclusion

The biofilm carriers from Havlandet demonstrated higher nitrification capacity in seawater and brackish water than in freshwater, where the capacity was reduced by around 50%. In contrast, the biofilm carriers from Erko showed similar nitrification capacity across all salinities. These results suggested that brackish biofilters may be more robust to salinity changes. At 31 ppt and 15 ppt salinity, the biofilm carriers from Erko had twice as high nitrification capacity as those from Havlandet, and in freshwater, the capacity was as much as five times higher. The difference in the nitrification capacity and salinity tolerance could be linked to the relative abundance of bacterial nitrifiers in the biofilm communities.

The biofilm community composition in the samples from the different facilities showed significant differences. *Maribacter* was common in the biofilm samples from Havlandet during cod production, while three ASVs representing *Alphaproteobacteria*, *Flavobacteria* and *Saprospira* were more common in the samples from Belsvik. The relative abundance of nitrifiers in the biofilm samples ranged from an average of 1% in the biofilm samples from Havlandet during post-smolt production to 18% in the biofilm samples from Erko.

Nitrospira and *Nitrosomonas* were the most important NOBs and AOBs in the biofilm samples. *Nitrospira* dominated the bacterial nitrifying communities in the biofilm samples from Havlandet and Erko, and ASVs representing *Nitrospira* were the most significant contributors to the difference in biofilm community compositions between the facilities. An ASV representing *Nitrosomonas* was the most common bacterial nitrifier in the biofilm samples from Belsvik, and other ASVs representing *Nitrospira* were more common in the samples from Belsvik than the two ASVs representing *Nitrospira* in the samples from Erko and Havlandet.

The archaeal communities in the biofilm samples from Havlandet and Erko were dominated by one single ASV representing *Nitrosopumilus*, known to be ammonia oxidizing. The biofilm communities in the samples from Belsvik were dominated by another ASV, also representing *Nitrosopumilus*. The ASVs representing archaeal nitrifiers had a relative abundance above 90% of the archeal biofilm communities in the samples.A low abundance of AOBs indicated that the archeal nitrifiers were important in the nitrifying activity in the biofilm. qPCR and Sanger sequencing confirmed the presence of the *Nitrsopulimus amoA* gene in the biofilm samples from Erko and Havlandet, shown to be identical to the *amoA* gene of *N. oxyclinae* . The results from the Sanger sequencing further suggested the presence of multiple *amoA* gene sequences in the samples from Belsvik.

References

[1] Timmons, M., Guerdat, T. and Vinci, B. *Recirculating Aquaculture*. 4. ed. Ithaca Publishing Company LLC; 2018.

[2] Sommerset, I., Walde, C. S., Bang Jensen, B., Wiik-Nielsen, J., Bornø, G., Oliveira, V. H. S. d., Haukaas, A. and Brun, E. *Fiskehelserapporten 2021*. Tech. rep. 2a. Veterinærinstituttet, 2022. URL: https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2022/fiskehelserapporten-2021.

[3] *Annual report*. Tech. rep. Federation of European Aquaculture Producers, 2022. URL: https://feap.info/wp-content/uploads/2023/03/feap-annual-report-feap-2022.pdf.

[4] *Atlantic Salmon (Protected) — NOAA Fisheries*. 2023. URL: https://www.fisheries.noaa.gov/species/atlantic-salmon-protected.

[5] Puvanendran, V., Mortensen, A., Johansen, L.-H., Kettunen, A., Hansen, Ø. J., Henriksen, E. and Heide, M. 'Development of cod farming in Norway: Past and current biological and market status and future prospects and directions'. In: *Rev Aquac*. 14.1 (2022), pp. 308–342. DOI: https://doi.org/10.1111/raq.12599.

[6] fiskeridepartementet, N.-o. *Norsk havbruksnæring*. 2021. URL: https://www.regjeringen.no/no/tema/mat-fiske-og-landbruk/fiskeri-og-havbruk/1/oppdrettslaksen/Norsk-havbruksnaring/id754210/ (visited on 21st Mar. 2023).

[7] Thodesen, J. and Gjedrem, T. 'Breeding programs on Atlantic salmon in Norway: lessons learned'. In: (2006). URL: https://hdl.handle.net/20.500.12348/1861.

[8] Ebeling, J. M. and Timmons, M. B. *Recirculating Aquaculture Systems*. John Wiley & Sons, Ltd, 2012. ISBN: 978-1-118-25010-5.

[9] Hussan, A., Adhikari, S., Das, A., Hoque, F. and Pillai, B. 'Fish Culture Without Water. Does Aquaculture contribute to Water Scarcity? Is Aquaculture without Water Possible?' In: 7 (2019), pp. 39–48.

[10] Lekang, O.-I. *Aquaculture Engineering*. 3. ed. Wiley-Blackwell, 2020. DOI: 10.1002/9781119489047.

[11] Summerfelt, S. T., Vinci, B. J. and Piedrahita, R. H. 'Oxygenation and carbon dioxide control in water reuse systems'. In: *Aquac Eng* 22.1 (2000), pp. 87–108. DOI: https://doi.org/10.1016/S0144-8609(00)00034-0.

[12] Randall, D. J. and Wright, P. A. 'Ammonia distribution and excretion in fish'. In: *Fish Physiol Biochem* 3.3 (1987), pp. 107–120. DOI: 10.1007/BF02180412.

[13] Timmons, M. B. and Ebeling, J. M. 'Chapter 7 Biofiltration'. In: *Recirculating Aquaculture*. 3rd ed. Ithaca Publishing Company LLC, 2013.

[14] Vaccari, D. A., Strom, P. F. and Alleman, J. E. 'Chapter 13.2 Microbial Transformations Nitrogen'. In: *Environmental Biology for Engineers and Scientists*. Hoboken, New Jersey: John Wiley and Sons, 2006. ISBN: 978-0-471-72239-7.

[15] Fossmark, R. O., Attramadal, K. J. K., Nordøy, K., Østerhus, S. W. and Vadstein, O. 'A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (Salmo salar) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish'. In: *Aquaculture* 532 (2021), p. 735973. DOI: https://doi.org/10.1016/j.aquaculture.2020.735973.

[16] Berry, E. D. and Foegeding, P. M. 'Cold Temperature Adaptation and Growth of Microorganisms†'. In: *J. Food Prot.* 60.12 (1997), pp. 1583–1594. DOI: https://doi.org/10.4315/0362-028X-60.12.1583.

[17] Csonka, L. N. 'Physiological and genetic responses of bacteria to osmotic stress'. In: *Microbiol. Rev.* 53.1 (1989), pp. 121–147. DOI: 10.1128/mr.53.1.121-147.1989.

[18] Navada, S., Vadstein, O., Tveten, A.-K., Verstege, G. C., Terjesen, B. F., Mota, V. C., Venkataraman, V., Gaumet, F., Mikkelsen, Ø. and Kamstra, A. 'Influence of rate of salinity increase on nitrifying biofilms'. In: *J. Clean. Prod.* 238 (2019), p. 117835. DOI: https://doi.org/10.1016/j.jclepro.2019.117835.

[19] Grindheim, J. *Erko Settefisk med mange byggeplanar*. 2017. URL: https://www.intrafish.no/nyheter/erko-settefisk-med-mange-byggeplanar/2-1-120422 (visited on 21st Jan. 2023).

[20] *FREDRIKSTAD SEAFOOD*. 2023. URL: https://fredrikstadseafoods.no/about-us/ (visited on 6th Feb. 2023).

[21] *Lerøys historie*. URL: https://www.leroyseafood.com/no/om-leroy/historikk/ (visited on 6th Feb. 2023).

[22] *Fikk tillatelse for Norges første settefiskanlegg for torsk med RAS teknologi*. 2021. URL: https://www.landbasedaq.no/landbasert-ras-torsk/fikk-tillatelse-for-norges-forste-settefiskanlegg-for-torsk-med-ras-teknologi/540464 (visited on 6th Feb. 2023).

[23] *Derfor døde fisken hos Havlandet*. 2023. URL: https://www.landbasedaq.no/h2s-havlandet-torsk/derfor-dode-fisken-hos-havlandet/1473369 (visited on 21st Jan. 2023).

[24] Jensen, B.-A. 'Regjeringen heier på torskeoppdrett'. In: (2022). URL: https://www.intrafish.no/politikk/regjeringen-heier-pa-torskeoppdrett/2-1-1196250.

[25] Ward, B. B. 'Chapter 5 - Nitrification in Marine Systems'. In: *Nitrogen in the Marine Environment (Second Edition)*. Ed. by Capone, D. G., Bronk, D. A., Mulholland, M. R. and Carpenter, E. J. San Diego: Academic Press, 2008, pp. 199–261. ISBN: 978-0-12-372522-6. DOI: 10.1016/B978-0-12-372522-6.00005-0.

[26] Navada, S., Sebastianpillai, M., Kolarevic, J., Fossmark, R. O., Tveten, A.-K., Gaumet, F., Mikkelsen, Ø. and Vadstein, O. 'A salty start: Brackish water start-up as a microbial management strategy for nitrifying bioreactors with variable salinity'. In: *Sci. Total Environ.* 739 (2020), p. 139934. DOI: 10.1016/j.scitotenv.2020.139934.

[27] Tesdal, V. 'Start-up of a nitrifying MBBR biofilter for a pilot-RAS operated at high salinity for grow-out salmon at Havlandet'. Accepted: 2021-09-25T16:16:14Z. MA thesis. NTNU, 2021. URL: https://ntnuopen.ntnu.no/ntnu-xmlui/handle/11250/2782673.

[28] Lee, D.-E., Lee, J., Kim, Y.-M., Myeong, J.-I. and Kim, K.-H. 'Uncultured bacterial diversity in a seawater recirculating aquaculture system revealed by 16S rRNA gene amplicon sequencing'. In: *Journal of Microbiology* 54.4 (2016), pp. 296–304. DOI: 10.1007/s12275-016-5571-4.

[29] Hovanec, T. A., Taylor, L. T., Blakis, A. and Delong, E. F. 'Nitrospira-like bacteria associated with nitrite oxidation in freshwater aquaria'. eng. In: *Appl Environ Microbiol* 64.1 (1998), pp. 258–264. DOI: 10.1128/AEM.64.1.258-264.1998.

[30] Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K. H., Pommerening-Röser, A., Koops, H. P. and Wagner, M. 'Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations'. In: *Appl. Environ. Microbiol.* 64.8 (Aug. 1998), pp. 3042–3051. DOI: 10.1128/AEM.64.8.3042-3051.1998.

[31] Kruse, M., Keuter, S., Bakker, E., Spieck, E., Eggers, T. and Lipski, A. 'Relevance and Diversity of Nitrospira Populations in Biofilters of Brackish RAS'. In: *PLoS ONE* 8.5 (2013), e64737. DOI: 10.1371/journal.pone.0064737.

[32] Kumar, V. J. R., Sukumaran, V., Achuthan, C., Joseph, V., Philip, R. and Singh, I. S. B. 'Molecular characterization of the nitrifying bacterial consortia employed for the activation of bioreactors used in brackish and marine aquaculture systems'. In: *Int. Biodeterior. Biodegradation.* 78 (2013), pp. 74–81. DOI: https://doi.org/10.1016/j.ibiod.2013.01.002.

[33] Gonzalez-Silva, B. M., Jonassen, K. R., Bakke, I., Østgaard, K. and Vadstein, O. 'Nitrification at different salinities: Biofilm community composition and physiological plasticity'. In: *Water Res* 95 (2016), pp. 48–58. DOI: https://doi.org/10.1016/j.watres.2016.02.050.

[34] Bartelme, R. P., McLellan, S. L. and Newton, R. J. 'Freshwater Recirculating Aquaculture System Operations Drive Biofilter Bacterial Community Shifts around a Stable Nitrifying Consortium of Ammonia-Oxidizing Archaea and Comammox Nitrospira'. In: *Front. Microbiol.* 8 (2017). DOI: https://doi.org/10.3389/fmicb.2017.00101.

[35] Wang, D.-Q., Zhou, C.-H., Nie, M., Gu, J.-D. and Quan, Z.-X. 'Abundance and niche specificity of different types of complete ammonia oxidizers (comammox) in salt marshes covered by different plants'. In: *Sci. Total Environ.* 768 (2021), p. 144993. DOI: 10.1016/j.scitotenv.2021.144993.

[36] Brown, M. N., Briones, A., Diana, J. and Raskin, L. 'Ammonia-oxidizing archaea and nitrite-oxidizing nitrospiras in the biofilter of a shrimp recirculating aquaculture system'. In: *FEMS Microbiol. Ecol.* 83.1 (2013), pp. 17–25. DOI: 10.1111/j.1574-6941.2012.01448.x.

[37] Madigan, M. T., Bender, K. S., Buckley, D. H., Sattley, W. M. and Stahl, D. A. *Brock Biology of Microorganisms*. 15th ed. Pearson Education, 2019.

[38] Ogunseitan, O. *Microbial Diversity*. Blackwell Science Ltd, 2005.

[39] Head, I., Saunders, J. and Pickup, R. 'Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms'. In: *Microbial Ecology* 35.1 (1998), pp. 1–21. DOI: 10.1007/s002489900056.

[40] Tringe, S. G. and Hugenholtz, P. 'A renaissance for the pioneering 16S rRNA gene'. In: *Curr. Opin. Microbiol.* 11.5 (2008), pp. 442–446. DOI: https://doi.org/10.1016/j.mib.2008.09.011.

[41] Mardis, E. R. 'The impact of next-generation sequencing technology on genetics'. In: *Trends Genet.* 24.3 (2008), pp. 133–141. DOI: https://doi.org/10.1016/j.tig.2007.12.007.

[42] Pester, M., Maixner, F., Berry, D., Rattei, T., Koch, H., Lücker, S., Nowka, B., Richter, A., Spieck, E., Lebedeva, E., Loy, A., Wagner, M. and Daims, H. 'NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing Nitrospira'. In: *Environ. Microbiol.* 16.10 (2014). _eprint: https://ami-journals.onlinelibrary.wiley.com/doi/pdf/10.1111/1462-2920.12300, pp. 3055–3071. DOI: https://doi.org/10.1111/1462-2920.12300.

[43] Edgar, R. C. 'Search and clustering orders of magnitude faster than BLAST'. In: *Bioinformatics* 26.19 (2010), pp. 2460–2461. DOI: 10.1093/bioinformatics/btq461.

[44] Edgar, R. C. 'UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing'. In: *bioRxiv* (2016). DOI: 10.1101/081257.

[45] Edgar, R. C. 'SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences'. In: *bioRxiv* (2016). DOI: 10.1101/074161.

[46] Hammer, Ø., Harper, D. and Ryan, P. *PAST: Paleontological statistics software pachage for education and data analysis.* Palaeontologia Electrica, 2001. URL: http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

[47] Hollister, E. B., Brooks, J. P. and Gentry, T. J. 'Chapter 21 - Bioinformation and 'Omic Approaches for Characterization of Environmental Microorganisms'. In: *Environmental Microbiology (Third Edition)*. Ed. by Pepper, I. L., Gerba, C. P. and Gentry, T. J. Third Edition. San Diego: Academic Press, 2015, pp. 483–505. ISBN: 978-0-12-394626-3. DOI: https://doi.org/10.1016/B978-0-12-394626-3.00021-1.

[48] You, J., Das, A., Dolan, E. M. and Hu, Z. 'Ammonia-oxidizing archaea involved in nitrogen removal'. In: *Water Res* 43.7 (2009), pp. 1801–1809. DOI: 10.1016/j.watres.2009.01.016.

[49] Liu, X., Wang, Y. and Gu, J.-D. 'Ecological distribution and potential roles of Woesearchaeota in anaerobic biogeochemical cycling unveiled by genomic analysis'. In: *Comput. Struct. Biotechnol. J.* 19 (2021), pp. 794–800. DOI: 10.1016/j.csbj.2021.01.013.

[50] Hüpeden, J., Wemheuer, B., Indenbirken, D., Schulz, C. and Spieck, E. 'Taxonomic and functional profiling of nitrifying biofilms in freshwater, brackish and marine RAS biofilters'. In: *Aquac Eng.* 90 (2020), p. 102094. DOI: 10.1016/j.aquaeng.2020.102094.

[51] Nedashkovskaya, O. I., Kim, S. B., Han, S. K., Lysenko, A. M., Rohde, M., Rhee, M.-S., Frolova, G. M., Falsen, E., Mikhailov, V. V. and Bae, K. S. 'Maribacter gen. nov., a new member of the family Flavobacteriaceae, isolated from marine habitats, containing the species Maribacter sedimenticola sp. nov., Maribacter aquivivus sp. nov., Maribacter orientalis sp. nov. and Maribacter ulvicola sp. nov.' In: *Int. J. Syst. Evol. Microbiol.* 54.4 (2004), pp. 1017–1023. DOI: https://doi.org/10.1099/ijs.0.02849-0.

[52] Navada, S., Vadstein, O., Gaumet, F., Tveten, A.-K., Spanu, C., Mikkelsen, Ø. and Kolarevic, J. 'Biofilms remember: Osmotic stress priming as a microbial management strategy for improving salinity acclimation in nitrifying biofilms'. In: *Water Res.* 176 (2020), p. 115732. DOI: https://doi.org/10.1016/j.watres.2020.115732.

[53] Ruiz, P., Vidal, J., Sepúlveda, D., Torres, C., Villouta, G., Carrasco, C., Aguilera, F., Ruiztagle, N. and Urrutia, H. 'Overview and future perspectives of nitrifying bacteria on biofilters for recirculating aquaculture systems'. In: *Rev Aquacult* 12 (2019). DOI: 10.1111/raq.12392.

[54] Liu, X., Li, M., Castelle, C. J., Probst, A. J., Zhou, Z., Pan, J., Liu, Y., Banfield, J. F. and Gu, J.-D. 'Insights into the ecology, evolution, and metabolism of the widespread Woesearchaeotal lineages'. In: *Microbiome* 6.1 (2018), p. 102. DOI: 10.1186/s40168-018-0488-2.

[55] Haaijer, S. C. M., Ji, K., Niftrik, L. van, Hoischen, A., Speth, D., Jetten, M. S. M., Damsté, J. S. S. and Op den Camp, H. J. M. 'A novel marine nitrite-oxidizing Nitrospira species from Dutch coastal North Sea water'. eng. In: *Front Microbiol* 4 (2013), p. 60. DOI: 10.3389/fmicb.2013.00060.

[56] Tal, Y., Watts, J. E. M., Schreier, S. B., Sowers, K. R. and Schreier, H. J. 'Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system'. In: *Aquaculture* 215.1 (2003), pp. 187–202. DOI: https://doi.org/10.1016/S0044-8486(02)00372-1.

[57] Foesel, B. U., Gieseke, A., Schwermer, C., Stief, P., Koch, L., Cytryn, E., Torré, J. R. de la, Rijn, J. van, Minz, D., Drake, H. L. and Schramm, A. 'Nitrosomonas Nm143-like ammonia oxidizers and Nitrospira marina-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm'. In: *FEMS Microbiol Ecol* 63.2 (2008), pp. 192–204. DOI: 10.1111/j.1574-6941.2007.00418.x.

[58] Bernhard, A. E., Donn, T., Giblin, A. E. and Stahl, D. A. 'Loss of diversity of ammonia-oxidizing bacteria correlates with increasing salinity in an estuary system'. In: *Environ Microbiol* 7.9 (2005), pp. 1289–1297. DOI: 10.1111/j.1462-2920.2005.00808.x.

[59] Figuerola, E. L. M. and Erijman, L. 'Diversity of nitrifying bacteria in a full-scale petroleum refinery wastewater treatment plant experiencing unstable nitrification'. In: *J. Hazard. Mater.* 181.1 (2010), pp. 281–288. DOI: https://doi.org/10.1016/j.jhazmat.2010.05.009.

[60] Qin, W., Heal, K. R., Ramdasi, R., Kobelt, J. N., Martens-Habbena, W., Bertagnolli, A. D., Amin, S. A., Walker, C. B., Urakawa, H., Könneke, M., Devol, A. H., Moffett, J. W., Armbrust, E. V., Jensen, G. J., Ingalls, A. E. and Stahl, D. A. 'Nitrosopumilus maritimus gen. nov., sp. nov., Nitrosopumilus cobalaminigenes sp. nov., Nitrosopumilus oxyclinae sp. nov., and Nitrosopumilus ureiphilus sp. nov., four marine ammoniaoxidizing archaea of the phylum thaumarchaeo'. In: *Int. J. Syst. Evol. Microbiol.* 67.12 (2017). Type: Article, pp. 5067–5079. DOI: 10.1099/ijsem.0.002416.

[61] Könneke, M., Bernhard, A. E., De La Torre, J. R., Walker, C. B., Waterbury, J. B. and Stahl, D. A. 'Isolation of an autotrophic ammonia-oxidizing marine archaeon'. In: *Nature* 437.7058 (2005), pp. 543–546. DOI: 10.1038/nature03911.

Appendix

A Sample overview

Table A.1: Overview of samples for the microbial community analysis.

Sample name	Collection date	Type of sample	Facility	Salinity	Cultivated species	Biofilter
H_PS_26.04_A	26.04.2022	Biofilm carrier	Havlandet	25 ppt	Atlantic salmon	MBBR
H_PS_26.04_B						
H_PS_26.04_C						
H_PS_10.05_A	10.05.2022					
H_PS_10.05_B						
H_PS_10.05_C						
H_Cod_02.09_A	02.09.2022	Biofilm carrier	Havlandet	35 ppt	Atlantic cod	MBBR
H_Cod_02.09_B						
H_Cod_02.09_C						
H_Cod_08.09_A	08.09.2022					
H_Cod_08.09_B						
H_Cod_08.09_C						
H_Cod_26.09_A*	26.09.2022					
H_Cod_26.09_B						
H_Cod_26.09_C						
H_Cod_12.10_A	12.10.2022					
H_Cod_12.10_B						
H_Cod_12.10_C						
H_Cod_16.11_A	16.11.2022					
H_Cod_16.11_B						
H_Cod_16.11_C						
H_Cod_27.11_A	27.11.2022					
H_Cod_27.11_B						
H_Cod_27.11_C						
Erko_PS_A	14.10.2022	Biofilm carrier	Erko	15 ppt	Atlantic salmon	MBBR
Erko_PS_B						
Erko_PS_C						
Belsvik_Swab_A	28.11.2022	Biofilm swab	Lerøy Belsvik	22 ppt	Atlantic salmon	FBBR
Belsvik_Swab_B						
Belsvik_Sludge_A		Biofilm back-washing sludge				
Belsvik_Sludge_B						
Belsvik_Sludge_C						

* indicates the samples used in the batch experiment.

B Batch experiment medium composition

Table B.1: Addition in 1 L medium to get a concentration of 10 mg L^{-1} TAN-N.

Components	Quantity
$(NH_4)_2SO_4$	0.047 g
$NaH_2PO_4 * 2H_2O$	0.272 g
Na_2CO_3	1 g
Trace metal (stock-solution)	10 mL
pH-adjustment	3-6 drops HCl till pH 7.5

Table B.2: Addition in 1 L stock solution adjusted to pH 7.5.

Components	Quantity
$MgSO_4*7H_2O$	2.5 g
$CaCl_2*2H_2O$	1.5 g
$FeCl_2*4H_2O$	0.2 g
$MnCl_2*4H_2O$	0.55 g
$ZnCl_2$	0.068 g
$CoCl_2*6H_2O$	0.12 g
$NiCl_2*6H_2O$	0.12 g
EDTA Titriplex	2.8 g

C Protocol Hach-Lange

C.1 Hach-Lange kits

Table C.1: Hach-Lange kits for measuring ammonium, nitrite and nitrate with reference range

Measurement of	Product code	Reference range
TAN	LCK303	2.0 - 47.0 mg TAN L^{-1}
Nitrite-N	LCK342	0.6 - 6 mg NO_2^- -N L^{-1}
Nitrate-N	LCK339	0.23 - 13.5 mg NO_3^- -N L^{-1}
Chloride-elimination	LCW925	$<$20 g L^{-1} NaCl

C.2 Ammonium-kit

1. Carefully remove the foil from the screwed-on DosiCap Zip.

2. Unscrew the DosiCap Zip.

3. Carefully pipet **0.2 mL** of **sample**.

4. **Immediately** screw the DosiCap Zip back on; **fluting at the top**.

5. Shake **vigorously**.

6. After **15 minutes**, thoroughly clean the outside of the cuvette and evaluate.

7. Insert the cuvette into the cell holder.
DR 1900: Go to LCK/TNTplus methods.
Select the test, push **READ**.

Figure C.1: Protocol for Hach-Lange LCK 303 for measuring TAN

C.3 Nitrite-kit

1. Carefully remove the foil from the screwed-on DosiCap Zip.

2. Unscrew the DosiCap Zip.

3. Carefully pipet **0.2 mL** of **sample**.

4. **Immediately** screw the DosiCap Zip back on; **fluting at the top**.

5. Shake **vigorously** until the freeze-dried contents are **completely dissolved**.

6. After **10 minutes**, invert a few more times, thoroughly clean the outside of the cuvette and evaluate.

7. Insert the cuvette into the cell holder.
DR 1900: Go to LCK/TNTplus methods.
Select the test, push **READ**.

Figure C.2: Protocol for Hach-Lange LCK 342 for measuring nitrite

C.4 Nitrate-kit

1. Carefully pipet **1.0 mL** of **sample**.

2. Carefully pipet **0.2 mL** of **solution A**.

3. Close the cuvette and invert a few times until **no more streaks** can be seen.

4. After **15 minutes**, thoroughly clean the outside of the cuvette and evaluate.

5. Insert the cuvette into the cell holder.
DR 1900: Go to LCK/TNTplus methods.
Select the test, push **READ**.
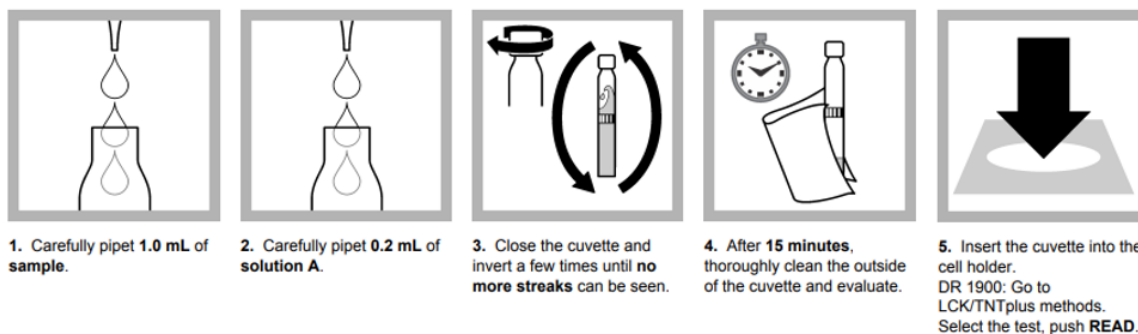
Figure C.3: Protocol for Hach-Lange LCK 339 for measuring nitrate

C.5 Elimination of chloride

Procedure

1. Draw **4.0 mL sample** into the syringe with the silver oxide.

2. Hold the syringe pointing upward and withdraw the piston another **0.5 mL** (see scale) so that air is drawn in.

3. Invert for **1 minute**.

4. Discharge the **sample** into a small beaker. Analyze the **prepared sample** by following the procedure of the cuvette test.

Figure C.4: Protocol for Hach-Lange LCW 925 for elimination of chloride.

D Results Batch experiment

D.1 Biofilters from Havlandet

Table D.1: Concentrations of ammonium, nitrite and nitrate from batch experiment of Havlandet biofilter in seawater, 31 ppt, given in mg L^{-1}

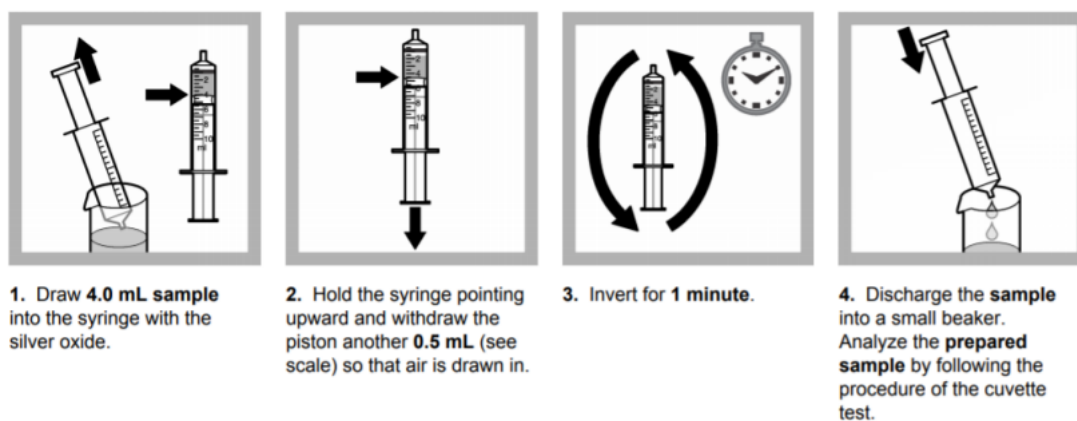| Time [minutes]	TAN [mg L^{-1}]	NO$_2$-N [mg L^{-1}]	NO$_3$-N [mg L^{-1}]
0	6.35	0*	0.499
15	5.9	0.04*	0.739
30	5.88	0.134*	0.810
60	4.63	0.265*	1.29
90	3.10	0.277*	1.73
120	2.25	0.271*	1.95
150	0.941*	0.26*	2.15
180	0.215*	0.131*	2.52

*outside reference range

Table D.2: Concentrations of ammonium, nitrite and nitrate from batch experiment of Havlandet biofilter in brackish water, 15 ppt, given in mg L^{-1}

Time [minutes]	TAN [mg L^{-1}]	NO$_2$-N [mg L^{-1}]	NO$_3$-N [mg L^{-1}]
0	5.22	0*	0.728
15	5.64	0.062*	0.811
30	4.91	0.164*	1.23
60	4.56	0.261*	1.82
90	3.13	0.316*	2.81
120	2.07	0.306*	2.87
150	0.599*	0.297*	4.75
180	0.061*	0.099*	4.65

* outside reference range

Table D.3: Concentration of ammonium, nitrite and nitrate from batch experiment of Havlandet biofilter in freshwater, 0 ppt, given in mg L^{-1}

Time [minutes]	TAN [mg L^{-1}]	NO$_2$-N [mg L^{-1}]	NO$_3$-N [mg L^{-1}]
0	7.3	0*	0.63
15	6.9	0*	0.967
30	6.77	0.043*	1.08
60	6.14	0.107*	1.59
90	5.75	0.106*	2.08
120	5.03	0.150*	2.81
150	4.47	0.203*	3.31
180	3.67	0.198*	3.95

* outside reference range

D.2 Biofilters from Erko

Table D.4: Concentrations of ammonium, nitrite and nitrate from batch experiment of Erko biofilter in seawater, 31 ppt, given in mg L^{-1}

Time [minutes]	TAN [mg L^{-1}]	NO$_2$-N [mg L^{-1}]	NO$_3$-N [mg L^{-1}]
0	6.54	0*	0.421
15	6.13	0.028*	0.515
30	5.24	0.063*	0.580
60	2.15	0.211*	1.35
90	0.207*	0.077*	1.85
120	0*	0*	2.13
150	0.068*	0*	2.47
180	0*	0*	3.64

*outside reference range

Table D.5: Concentrations of ammonium, nitrite and nitrate from batch experiment of Erko biofilter in brackish water, 15 ppt, given in mg L^{-1}

Time [minutes]	TAN [mg L^{-1}]	NO$_2$-N [mg L^{-1}]	NO$_3$-N [mg L^{-1}]
0	6.07	0*	0.826
15	5.38	0.066*	1.34
30	3.79	0.184*	1.88
60	0.466*	0.185*	4.45
90	0*	0*	6.43
120	0*	0*	7.64
150	0*	0*	8.16
180	0*	0*	7.9

*outside reference range

Table D.6: Concentrations of ammonium, nitrite and nitrate from batch experiment of Erko biofilter in freshwater, 0 ppt, given in mg L^{-1}

Time [minutes]	TAN [mg L^{-1}]	NO$_2$-N [mg L^{-1}]	NO$_3$-N [mg L^{-1}]
0	6.66	0*	1.6
15	6.12	0.061*	1.98
30	4.88	0.250*	3.01
60	1.36*	0.773	5.67
90	0.028*	0.180*	7.57
120	0*	0*	7.93
150	0*	0*	8.02
180	0*	0*	8.04

*outside reference range

E   Protocols for preparation of amplicon library

E.1   DNA-extraction kit

October 2020

Quick-Start Protocol

MagAttract® PowerSoil® Pro DNA Kit with KingFisher®

This protocol describes the use of the MagAttract PowerSoil Pro DNA Kit (cat. no. 47109) with the KingFisher Flex instrument. For use with the epMotion® instrument, please refer to the *MagAttract PowerSoil Pro DNA Handbook*.

Solution CD2 should be stored at 2–8°C upon arrival. All other reagents and kit components should be stored at room temperature (15–25°C).

Further information

- *MagAttract PowerSoil Pro DNA Handbook*: **www.qiagen.com/HB-2816**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Use extra-long pipette tips (1000–1250 µl) for collection microtube racks (CMTRs).
- Add 400 µl RNase A Solution to 80 ml Solution CD1 for each 96-well plate to be processed.
- Prepare Buffer QSB1 and Buffer MW1 according to the instructions on the bottles.
- 80% ethanol is required in this protocol and needs to be supplied by the user.

Sample to Insight

QIAGEN

Procedure

1. Spin the PowerBead Pro Plate (cat. no. 19311) or the PowerBead Pro Tube (cat. no. 19301) briefly to ensure that the beads have settled at the bottom of the wells or tube.

2. Add up to 250 mg of soil or 100 mg of stool into the plate/tube, and 800 µl Solution CD1/RNase A Solution. Seal the plate with sealing film or recap the tube.

3. Homogenize samples thoroughly using the TissueLyser II (cat. no. 85300). (For other homogenization methods, please refer to the *MagAttract PowerSoil Pro DNA Handbook*.)

   3a. If using a PowerBead Pro Plate, place a silicone compression mat on top of the sealing film, and then place the sealed plate and mat between 2 adapter plates (cat. no. 11990). Shake for 5 min at 25 Hz.
   Reorient the plates so that the sides that were closest to the machine body are now furthest from it. Shake again for 5 min at 25 Hz.

   3b. If using PowerBead Pro Tubes, place the tubes into a TissueLyser Adapter Set 2 x 24 (cat. no. 69982), or into a 2 ml Tube Holder (cat. no. 11993) and Plate Adapter Set (cat. no. 11990). Fasten the adapter into the TissueLyser II. Shake for 5 min at 25 Hz. Reorient the adapter so that the side that was closest to the machine body becomes furthest from it. Shake again for 5 min at 25 Hz.

4. Centrifuge the PowerBead Pro Plate at 4500 x g for 6 min, or the PowerBead Pro Tubes at 15,000 x g for 1 min.

5. Transfer the supernatant to the CMTRs.

   Note: Expect 500–600 µl. The supernatant may still contain some soil/stool particles.

MagAttract PowerSoil Pro DNA Kit with KingFisher   10/2020

6. Add 300 µl Solution CD2. Seal the CMTRs with the caps provided, and then vortex.

7. Centrifuge the CMTRs at 4500 x g for 6 min at room temperature.

8. Taking care to avoid any residual pellet, transfer no more than 450 µl supernatant from each well to a clean KingFisher deep-well 96 plate.

9. Resuspend MagAttract Suspension G Beads by vortexing. For each 96-well plate to be processed, add 3 ml of the resuspended MagAttract Suspension G Beads to 44 ml Buffer QSB1 and mix well. Immediately transfer to a multichannel pipette reservoir.

   Note: Maintain the MagAttract Suspension G Beads in suspension to ensure uniform distribution.

10. Add 470 µl of the MagAttract Suspension G beads/Buffer QSB1 mix to each well containing lysate in a KingFisher 96 deep-well plate.

11. Place the plate on the robotic deck at the specified location indicated in the program.

12. Add 500 µl Buffer MW1 to each well of one clean KingFisher 96 deep-well plate. Add 80% ethanol (provided by the user) to each well of 2 clean KingFisher 96 deep-well plates. Place the plates on the robotic deck at the specified locations indicated in the program.

13. Add 100 µl Solution C6 to each well of a clean KingFisher 96 microplate and place on the robotic deck at the specified location. Initiate the robotic program.

14. Upon completion of the robotic program, cover the wells of the KingFisher 96 microplate with an appropriate storage seal. DNA is now ready for downstream applications.

X

Document Revision History

Date	Changes
10/2020	Initial release

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

E.2 SequalPrep Normalization plate (96) Kit

invitrogen™

SequalPrep™ Normalization Plate (96) Kit

Catalog no: A10510-01   **Store at room temperature (15–30ºC)**

Contents and Storage

The components included with the SequalPrep™ Normalization Plate (96) Kit are listed in the table below. Sufficient reagents are included to perform 10 × 96 purification/normalization reactions. Upon receipt, **store all components at room temperature (15–30ºC)**. Store plates for up to 6 months.

Components	Quantity
SequalPrep™ Normalization Plate (96)	2 bags of 5 plates each
SequalPrep™ Normalization Binding Buffer	40 ml
SequalPrep™ Normalization Wash Buffer	50 ml
SequalPrep™ Normalization Elution Buffer (10 mM Tris-HCl, pH 8.5)	40 ml

Description

The SequalPrep™ Normalization Plate Kit allows simple, one-step, high-throughput amplicon purification and normalization of PCR product concentration (2–3 fold range) via a limited binding capacity solid phase. Each well of the SequalPrep™ Normalization Plate can bind and elute ~25 ng of PCR amplicon. Eluted PCR amplicon can be subsequently pooled and subjected to a variety of massively parallel sequencing analyses. The SequalPrep™ Normalization Plate is compatible with any automated liquid handling workstations without the need for shakers, magnets, or vacuum. The SequalPrep™ Normalization Plate Kit when used with SequalPrep™ Long PCR Kit provides a complete PCR enrichment and amplicon normalization system that is designed to complement amplicon sequencing workflows such as next-generation sequencing.

The conventional next generation sequencing workflows require laborious sample prep methods consisting of amplicon purification, quantitation, and manual normalization to adjust amplicon concentration. The SequalPrep™ Normalization Plate Kit eliminates the tedious amplicon quantitation and manual normalization steps.

SequalPrep™ Normalization Plate Kits utilize ChargeSwitch® Technology that provides a switchable surface charge depending on the pH of the surrounding buffer to facilitate nucleic acid purification. Under low pH conditions, the positive surface charge of the ChargeSwitch® coating binds the negatively charged nucleic acid backbone. Proteins and other contaminants (such as short oligonucleotide primers) are not bound and are simply washed away.

System Overview

The SequalPrep™ Normalization Plate Kit is a solid phase, high-throughput amplicon purification and normalization system in a 96-well plate format. PCR products (5–25 µl) are added to a SequalPrep™ Normalization Plate well and mixed with the Binding Buffer. DNA binding to the plate is performed at room temperature for 1 hour. The wells are washed with Wash Buffer to efficiently remove contaminants. Purified PCR products are eluted using 20 µl Elution Buffer at normalized concentrations.

System Specifications

Starting Material:	At least 250 ng PCR product (amplicon) per well
DNA Fragment Size:	100 bp to 20 kb
Elution Volume:	20 µl
DNA Yield:	Up to 25 ng per well
Normalization Range:	2–3-fold
Plate Dimensions:	Standard SBS (Society for Biomolecular Screening) footprint, semi-skirted 96-well plate
Plate Capacity:	0.2 ml

Accessory Products

The following products may be used with the SequalPrep™ Normalization Plate Kit. For details, visit www.invitrogen.com.

Product	Quantity	Catalog no.
SequalPrep™ Normalization Wash Buffer	4 × 50 ml	A10510-03
SequalPrep™ Long PCR Kit with dNTPs	1,000 units	A10498
Platinum® PCR Supermix	100 reactions	11306-016
Platinum® PCR Supermix High Fidelity	100 reactions	12532-016
Quant-iT™ PicoGreen® dsDNA Assay Kit	1 kit	P7589
PureLink™ Foil Tape	50 tapes	12261-012
E-Gel® 96 gels 1% (or 2%)	8 gels	G7008-01 (G7008-02)

Part no: 100003531   Rev. date: 5 May 2008

For technical support, email tech_support@invitrogen.com. For country-specific contact information, visit **www.invitrogen.com**.

General Guidelines

- Wear a laboratory coat, disposable gloves, and eye protection when handling reagents and plate.
- Always use proper aseptic techniques when working with DNA and use only sterile, DNase-free tips to prevent DNase contamination.
- If you are using only part of the plate for DNA purification, cover unused wells with the Plate Seal and leave them attached while purifying DNA in the other wells. The plates can be stored at room temperature for up to 6 months.
- The SequalPrep™ Normalization Plates are compatible for use with automated liquid handling workstation; the workstation must be capable of handling and manipulating 96-well plates.
- If you are using automated liquid handling workstations for purification, you may need additional Wash Buffer depending on your type of workstation. See previous page for Wash Buffer ordering information.

Generating PCR Amplicon

You can generate the PCR amplicon using a method of choice. General recommendations for generating PCR amplicons are listed below:

- To obtain the best results, we recommend using the SequalPrep™ Long PCR Kit with dNTPs (page 1) which provides a robust system for long-range, high-fidelity PCR for use in next-generation sequencing applications.
- Other commercially available PCR supermixes and enzymes such as Platinum® PCR Supermix (page 1), Platinum® PCR Supermix High Fidelity (page 1), or equivalent are suitable for use.
- Perform PCR in a separate plate. **Do not** use the SequalPrep™ Normalization Plate to perform PCR.
- You need at least 250 ng amplicon per well to use with the SequalPrep™ Normalization Plate (see below).

Sample Amount

To achieve robust normalization, we recommend adding at least 250 ng/well of amplicon. This input amount is easily achieved using only a fraction of most PCR amplification reactions. An average efficiency PCR (20 µl reaction volume) produces product in the range of 25–100 ng/µl, allowing you to purify 5–10 µl using the SequalPrep™ system.

Elution Options

Depending on the nature of the downstream application and target nucleic acid concentrations desired, the SequalPrep™ kit offers the flexibility to elute purified DNA in a variety of options.

The **standard elution** method described in the protocol below is designed to elute purified DNA from each well using 20 µl elution volume to obtain each amplicon at a concentration of 1–2 ng/µl.

The **optional sequential elution** method is designed to sequentially elute multiple rows or columns using the same 20 µl of elution buffer to obtain higher amplicon concentrations. The amplicon concentrations will be additive as sequential wells are eluted. For example, dispense 20 µl of elution buffer into the first column (A1–H1), mix well, and incubate for 5 minutes at room temperature. Then, simply move this column of elution buffer to the next column (A2–H2), and again incubate for 5 minutes. Continue this step to obtain your specific elution needs for the downstream application of choice.

Materials Needed

- PCR reactions containing amplicons of the desired length (see **Generating PCR Amplicon**, above)
- DNase-free, aerosol barrier pipette tips
- *Optional:* automated liquid handling workstation capable of handling and manipulating 96-well plates
- *Optional:* PureLink™ Foil Tape (see previous page)

Binding Step

1. Transfer the desired volume of PCR product (5–25 µl PCR reaction mix, at least 250 ng amplicon/well) from the PCR plate into the wells of the SequalPrep™ Normalization plate.
2. Add an equivalent volume of SequalPrep™ Normalization Binding Buffer.
   For example: To purify 10 µl of PCR product, add 10 µl SequalPrep™ Normalization Binding Buffer.
3. Mix completely by pipetting up and down, or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate.
4. Incubate the plate for 1 hour at room temperature to allow binding of DNA to the plate surface. Mixing is not necessary at this stage.
   Note: Incubations longer than 60 minutes do not improve results. However, depending on your workflow you may perform overnight incubation at room temperature for the binding step.
5. **Optional:** If >25 ng DNA/well yield is desired, transfer the amplicon/Binding Buffer mixture from Step 4 to another, fresh well/plate to sequentially bind more DNA. Perform DNA binding at room temperature for 1 hour.
   Note: After binding is complete, you can remove the amplicon/Binding Buffer mixture from the well and store at –20ºC for up to 30 days to perform additional purifications at a later time.
6. Proceed to **Washing Step**, next page.

Washing Step

1. Aspirate the liquid from wells. Be sure not to scrape the well sides during aspiration.

   Note: If you wish to store the amplicon/Binding Buffer mixture for additional purifications at a later time, aspirate the liquid from wells into another plate and store at –20ºC for up to 30 days.

2. Add 50 μl SequalPrep™ Normalization Wash Buffer to the wells. Mix by pipetting up and down twice to improve removal of contaminants.

3. Completely aspirate the buffer from wells and discard.

   To ensure complete removal of wash buffer and maximize elution efficiency, you may need to invert and tap the plate on paper towels depending on the pipetting technique or instrument used. A small amount of residual Wash Buffer (1–3 μl) is typical and does not affect the subsequent elution or downstream applications.

4. Proceed to **Elution Step**, below.

Elution Step

Review **Elution Options** (previous page).

1. Add 20 μl SequalPrep™ Normalization Elution Buffer to each well of the plate.

   Note: Do not use water for elution. If you need to elute in any other buffer, be sure to use a buffer of pH 8.5–9.0. If the pH of the buffer is <8.5, the DNA will not elute efficiently.

2. Mix by pipetting up and down 5 times or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate. Ensure that the buffer contacts the entire plate coating (up to 20 μl level).

3. Incubate at room temperature for 5 minutes.

4. Transfer and pool the purified DNA as desired or store the eluted DNA at 4°C (short-term storage) or –20°C (long-term storage) until further use.

Expected Yield and Concentration

The expected DNA concentration is 1–2 ng/μl when using 20 μl elution volume. The expected DNA yield is ~25 ng/well normalized.

Optional: DNA Quantitation

The SequalPrep™ Normalization Plate Kit is designed to eliminate the quantitation and manual dilution steps typically performed for normalization in next-generation sequencing workflows. You can pool the eluted amplicon and use the pooled amplicons directly for your downstream applications without DNA quantitation.

However, if your downstream application requires DNA quantitation, you may determine the yield of the eluted amplicon using Quant-iT™ PicoGreen® dsDNA Assay Kit (page 1). We **do not** recommend using UV spectrophotometric measurements (A_{260}/A_{280} nm), as this method is inaccurate for low DNA concentrations.

Downstream Applications

The SequalPrep™ Normalization Plate Kit is designed to produce purified PCR products with normalized concentrations and substantially free of salts and contaminating primers. PCR amplicons purified from this system can be used individually or pooled in any downstream application for which normalization is an important sample preparation criterion such as next generation sequencing applications.

Pooled amplicons purified using the SequalPrep™ Normalization Plate Kit have produced successful data from massively parallel sequencing-by-synthesis on the Illumina/Solexa Genome Analyzer indicating that the amplicon purity is suitable for other next-generation sequencing platforms (Roche/454 FLX, Applied Biosystems SOLiD™ system). For detailed sample preparation guidelines, refer to the instrument manufacturer's recommendations.

Troubleshooting

Problem	Cause	Solution
Low DNA yield	Insufficient starting material	Be sure to input at least 250 ng amplicon per well for best results.
	PCR conditions not optimal	Check amplicon on gel to verify the PCR product prior to purification. Use SequalPrep™ Long Polymerase (page 2) for best results.
	Incorrect binding conditions	Be sure to add an equivalent volume of SequalPrep™ Normalization Binding Buffer, mix completely, and incubate for 1 hour during the Binding Step.
	Incorrect elution conditions	Use 20 µl SequalPrep™ Normalization Elution Buffer for elution and ensure that the buffer contacts the entire plate coating (up to 20 µl level). **Do not** use any water for elution.
DNA degraded	DNA contaminated with DNase	Follow the guidelines on page 2 to prevent DNase contamination.
Poor normalization	Insufficient starting material	Be sure to input at least 250 ng amplicon per well for best results.
	Inconsistent pipetting or handling	Avoid introducing bubbles while pipetting and do not scratch the plate surface while pipetting. To avoid pipetting inconsistencies, we recommend using automated liquid handling workstations.
	Incorrect binding conditions	Be sure to add an equivalent volume of SequalPrep™ Normalization Binding Buffer, mix completely, and incubate for 1 hour during the Binding Step.
	Too much (>3 µl) wash buffer remaining	Completely remove wash buffer and if needed, invert and tap the plate on paper towels to remove any remaining wash buffer.

Quality Control

The Certificate of Analysis provides quality control information for this product, and is available by product lot number at www.invitrogen.com/cofa. Note that the lot number is printed on the kit box.

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E.3 Amicon Centrifugal Filter Device

How to Use Amicon Ultra-0.5 Centrifugal Filter Devices

1. Insert the Amicon Ultra-0.5 device into one of the provided microcentrifuge tubes.
2. Add up to 500 µL of sample to the Amicon Ultra filter device and cap it.
3. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.
4. Spin the device at 14,000 × g for approximately 10–30 minutes depending on the NMWL of the device used. Refer to Figure 1 and table 3 for typical spin times.

Add sample   Cap   Spin at 14,000 × g
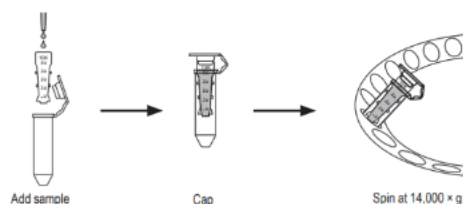
How to Use Amicon Ultra-0.5 Filter Devices, continued

5. Remove the assembled device from the centrifuge and separate the Amicon Ultra filter device from the microcentrifuge tube.
6. To recover the concentrated solute, place the Amicon Ultra filter device upside down in a clean micro centrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 × g to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.
   NOTE: For optimal recovery, perform the reverse spin immediately.

Separate device   Turn device upside   Filtrate Concentrate
from tube   down in clean tube   Spin at 1,000 × g
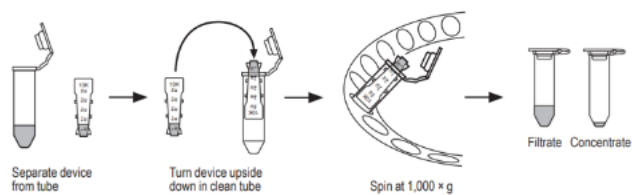
E.4 QIAquick PCR purification Kit Protocol

QIAquick PCR Purification Kit Protocol
using a microcentrifuge

Important points before starting

■ Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

■ All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.

■ Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 μl pH indicator I to 30 ml Buffer PB or add 600 μl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5.

■ Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.

■ If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

   For example, add 500 μl of Buffer PB to 100 μl PCR sample (not including oil).

2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 μl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**

4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**

5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**

7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

F Primer design

Primer design

The ammonia monooxygenase sub-unit (amoA) gene encodes for the ammonia monooxygenase, a key enzyme catalyzing the oxidation of ammonia (Arp et al., 2002). The abundance of amoA gene has been therefore used to infer the nitrification activity in the prokaryote communities (Bartelme et al., 2017; Hu et al., 2022). In this thesis, primer pairs were designed to target the amoA gene. Using the NCBI gene database, the amoA gene sequences in the Archaea domain were searched with the keywords: "ammonia monooxygenase" [All Fields] AND (Archaea [Organism] OR archaea [All Fields])" and then retrieved. The search and retrieval of sequences were implemented in R using the REntrez package (Winter, 2017). The retrieved sequences were manually inspected before further analysis.

A maximum likelihood (ML) phylogenetic tree was constructed to investigate the evolutionary relationship and diversity of this gene.
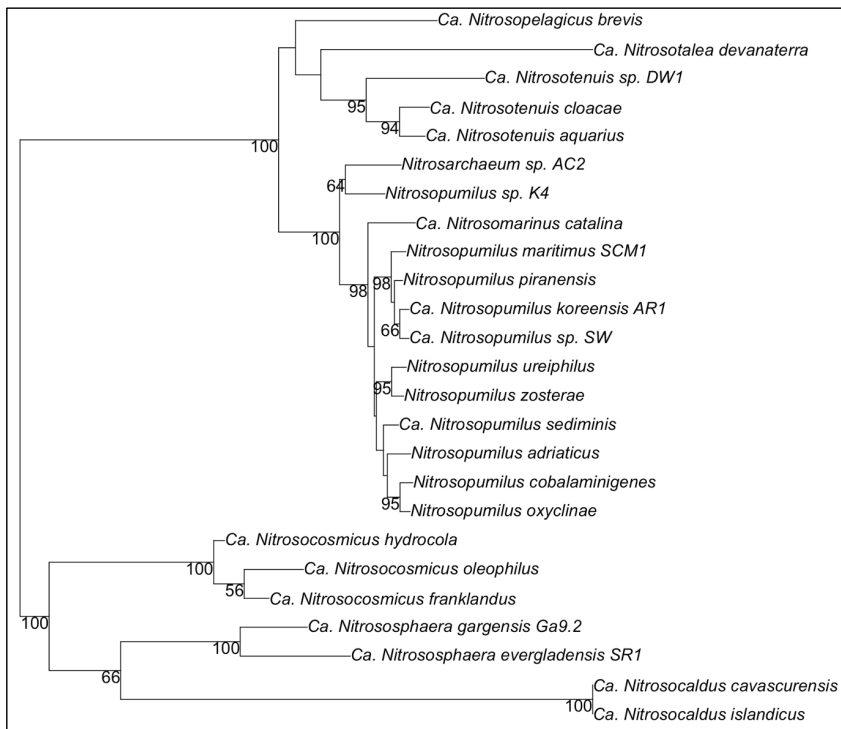

Figure 1: Phylogenetic tree of ammonia monooxygenase sub-unit (amoA) gene in the Archeal domain constructed by maximum likelihood method. The numbers below the branch points denote the confidence levels of the relationship of the paired sequences determined by boot strap statistical analysis.
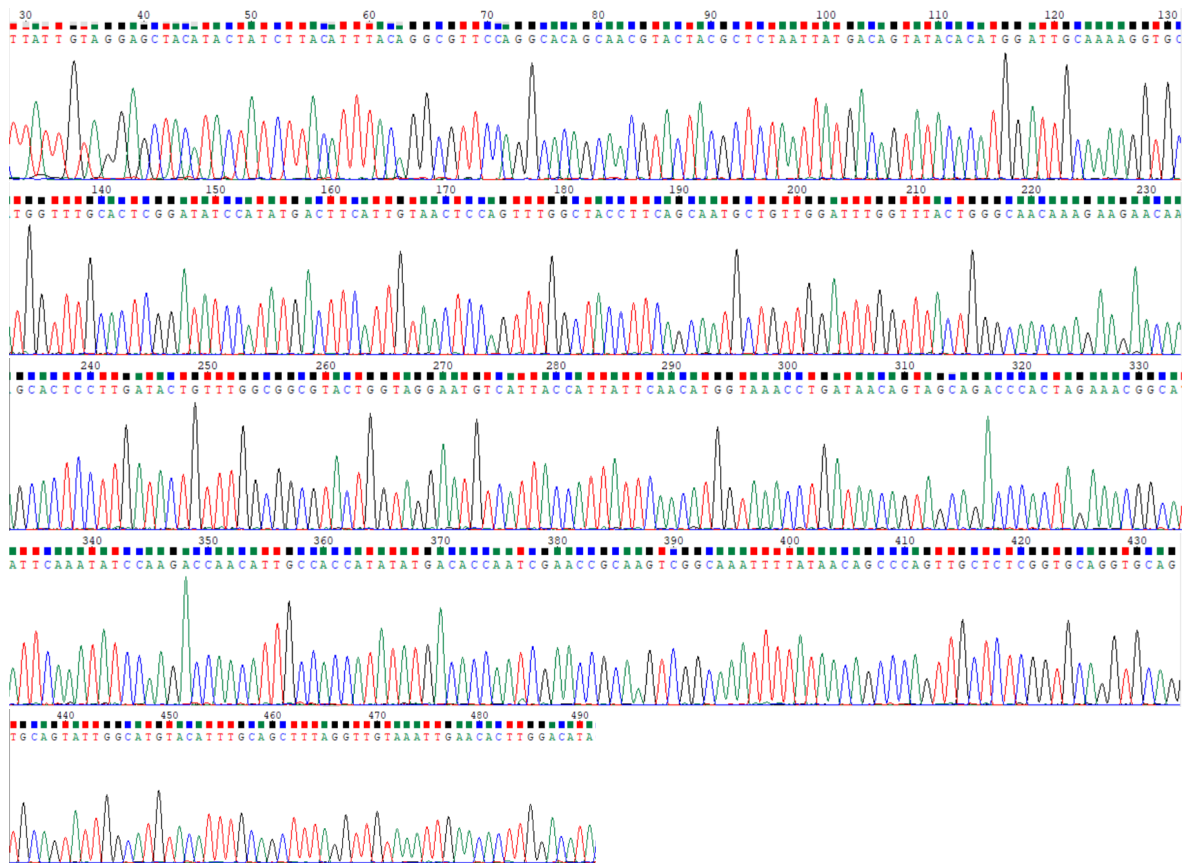
Figure 1 above shows that amoA gene in Archeal community is diverse. However, through inspection of the Illumina 16S metabarcoding data (Figure 3.8), *Nitrosopumilus* was the only previously known nitrifier taxa in the Archaeal domain; therefore, the qPCR primers were designed narrowed down to amplify amoA gene of this taxa. The pipeline of primer

design is described in Persson et al. (2022). The design process followed the extensive guideline in Rodríguez et al. (2015). Afterward, the candidate primers were checked for secondary structures, i.e., self-dimer, cross-dimer, and hairpin structure. The specificity of the candidate primers was evaluated through alignment (BLAST) to the largest NCBI Nucleotide database.
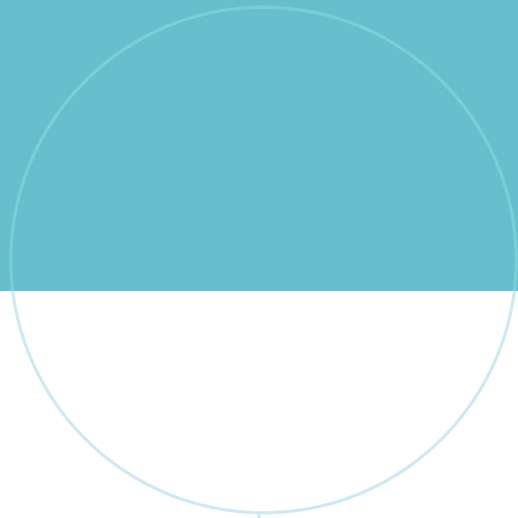
References

Arp, D. J., Sayavedra-Soto, L. A., & Hommes, N. G. (2002). Molecular biology and biochemistry of ammonia oxidation by Nitrosomonas europaea. *Archives of Microbiology*, *178*(4), 250–255. https://doi.org/10.1007/s00203-002-0452-0

Bartelme, R. P., McLellan, S. L., & Newton, R. J. (2017). Freshwater Recirculating Aquaculture System Operations Drive Biofilter Bacterial Community Shifts around a Stable Nitrifying Consortium of Ammonia-Oxidizing Archaea and Comammox Nitrospira. *Frontiers in Microbiology*, *8*, 101. https://doi.org/10.3389/fmicb.2017.00101

Hu, L., Dong, Z., Wang, Z., Xiao, L., & Zhu, B. (2022). The contributions of ammonia oxidizing bacteria and archaea to nitrification-dependent N2O emission in alkaline and neutral purple soils. *Scientific Reports*, *12*(1), 19928. https://doi.org/10.1038/s41598-022-23084-1

Persson, S., Larsson, C., Simonsson, M., & Ellström, P. (2022). rprimer: An R/bioconductor package for design of degenerate oligos for sequence variable viruses. *BMC Bioinformatics*, *23*(1), 239. https://doi.org/10.1186/s12859-022-04781-0

Rodríguez, A., Rodríguez, M., Córdoba, J. J., & Andrade, M. J. (2015). Design of Primers and Probes for Quantitative Real-Time PCR Methods. In C. Basu (Ed.), *PCR Primer Design* (pp. 31–56). Springer. https://doi.org/10.1007/978-1-4939-2365-6_3

Winter D.J. (2017). "rentrez: an R package for the NCBI eUtils API." *The R Journal*, **9**, 520–526.

G Chromatograms from Sanger sequencing

Figure G.1: Chromatogram of the archaeal *amoA* gene sequenced with Sanger sequencing.

Figure G.2: Chromatogram of the *amoA* gene in *Nitrosopumilus* sequenced with Sanger sequencing.