Master's thesis

NTNU
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Start-up of a nitrifying MBBR biofilter for a pilot-RAS operated at high salinity for grow-out salmon at Havlandet

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Ingrid Bakke
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Acknowledgments

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Abstract

The increasing demand for food produced in environmental friendly ways can partly be solved with fish production in land-based recirculating aquaculture systems (RAS). The water in RAS is mechanical and microbiological treated before it is reused for production of fish, in a way that results in better utilization of the resources compared to traditional flow-through systems. In the Norwegian aquaculture industry, smolt is commonly produced in freshwater RAS, before relocation for further production in seacages. Lately, it has been increasing interest for production of salmon at bigger size in RAS to limit the problems with fishlice and -escape in the seacages. Industrial challenges are linked to the function of nitrifying biofilters in RAS operated at high salinities, and several strategies for start-up is therefore used in marine RAS.

In this master thesis, two strategies for start-up of a nitrifying MBBR biofilter for use in a pilot-RAS at high salinity (25 ppm) for production of grow-out salmon at Havlandet RAS Pilot AS were investigated. In the first strategy, a liquid, commercial inoculum and clean biofilm carriers were used in a MBBR biofilter which was fed with ammonium in a period on 137 days (the Maturation period). The salinity was varying between 15 and 25 ppm. During the Maturation period it was observed some ammonium oxidization, but also some nitrite accumulation into inhibitory levels. Since the Maturation period did not result in a successful nitrifying biofilter, a new strategy was tested. A MBBR biofilter was inoculated with 1.5 m^3 biofilm carriers from another RAS operated at high salinity for post-smolt production at Erko Seafood. Over a period on approximately 15 days (the Poding period) new biofilm carriers were added to a total volume of 81.5 m^3 and the salinity was increased from 15 ppm to 25 ppm. It was achieved successful nitrification only a few days after the biofilm inoculum was mixed with the clean biofilm carriers. A major finding in this thesis was that the use of already adapted biofilm carriers to high salinity as inoculum is a effective start-up strategy of nitrifying biofilters for marine RAS.

The Illumina sequencing of amplicons encompassing variabel regions 3 and 4 of the *16S rRNA* gene showed that one zOTU, representing *Nitrospira*, had a relative abundance of 51 % of the total reads for the biofilm inoculum sample from Erko. This zOTU was related to *Nitrospira salsa*. In addition, a low abundance of ammonium oxidizing bacteria was found in the Poding filter samples, which can indicate that comammox *Nitrospira* or AOAs were contributing to the ammonium oxidization in the Poding filter. Sanger sequencing of PCR products for AOA *amoA* genes indicated the presence of the AOA *Nitrosopumilus oxyclinae*. qPCR of *amoA* genes showed a higher abundance of AOBs than AOAs in the Poding filter. It was concluded that AOAs were probably not the major ammonium oxidizer in the biofilm community of the Poding filter. PCR and qPCR for samples from a well-functioning, marine biofilter for cod production at Havlandet, indicated that AOBs were present in the biofilm, but that AOAs were absent or very rare. There were no indications of comammox *Nitrospira* in any of the biofilm samples.

There were found a significant difference ($p=0.0007$) in the microbial communities in two successful marine, nitrifying biofilters; the Poding filter and the well-functioning biofilter for production of cod at Havlandet. Additionally, the relative abundance of zOTUs representing nitrifiers in the biofilm communities in the Poding filter was much higher (up to 77 % of the total reads) than in the well-functioning biofilter for cod production (up to 4.2 %). This indicates that biofilm communities of highly different composition can perform nitrification at high salinity.

Sammendrag

Den økende etterspørselen etter mat produsert på en miljøvennlig måte kan delvis bli løst med fiskeproduksjon i landbaserte resirkulerende akvakulturelle system (RAS). Vannet i RAS blir mekanisk og mikrobiologisk renset før det blir brukt om igjen i fiskeproduksjonen, noe som fører til bedre utnyttelse av ressursene i forhold til tradisjonelle gjennomstrømmingsanlegg ("flow-through systems", FTS). I den norske oppdrettsnæringen blir smolt som oftest produsert i fersksvanns-RAS før fisken blir flyttet til merder i sjøen. I det siste har det vært en økende interesse rundt produksjon av større laks i RAS for å begrense problemene med fiskelus og rømming i merdene. Det er industrielle utfordringer knyttet til nitrifiserende biofiltre i RAS ved høy salinitet, og forskjellige strategier for oppstart er derfor blitt brukt.

I denne masteroppgaven har to strategier for oppstart av nitrifiserende MBBR biofilter til bruk i en pilot-RAS ved en høy salinitet (25 ppm) for produksjon av slakteklar laks ved Havlandet RAS Pilot AS blitt undersøkt. Den første strategien ble startet med et flytende, kommersielt inokulum og rene biofilmbærere i et MBBR biofilter som ble matet med ammonium i en periode på 137 dager (Modningsperioden). Saliniteten varierte mellom 15 og 25 ppm. Det ble observert noe ammoniumoksidering, men også nitritt akkumulering til inhiberende nivå. Siden Modningsperioden ikke oppnådde vellykket nitrifisering, ble en ny strategi testet. Et MBBR biofilter ble inokulert med 1,5 m^3 biofilmbærere fra et annet RAS-anlegg adaptert til en salinitet på 15 ppm ved Erko Seafood. I løpet av 15 dager (Podeperioden) ble nye biofilmbærere tilsatt til et totalt volum på 81,5 m^3, og saliniteten ble økt fra 15 ppm til 25 ppm. Vellykket nitrifisering ble oppnådd etter bare noen få dager etter at biofilm inokulumet ble blandet med de nye biofilmbærerne. Et hovudfunn var at å bruke biofilmbærere som allerede var adaptert til høy salinitet som inokulum kan være en effektiv oppstartsstrategi for nitrifiserende biofilter i marine RAS.

Illumina sekvenseringen av amplikon variabel region 3 og 4 i *16S rRNA* genet viste at en zOTU som representerer *Nitrospira*, hadde en forekomst på opp til 51 % av de totale avlesningene i biofilm inokulumet fra Erko. Denne zOTUen var beslektet med *Nitrospira salsa*. I tillegg ble det funnet en lav forekomst av ammoniumoksiderende bakterier i Podefilteret, noe som kan indikere at ammoniumoksiderende arker eller en comammox *Nitrospira* bidro til ammoniumoksideringen. Sanger sekvensering av et PCR-produkt av AOA *amoA* gener indikerte en tilstedeværelse av en AOA klassifisert som *Nitrosopumilus oxyclinae*. qPCR av *amoA* gener estimerte en høyere forekomst av AOB enn AOA i Podefilteret. Det ble konkludert med at AOA mest sannsynlig ikke bidrar vesentlig til ammoniumoksideringen i Podefilteret. PCR og qPCR av prøver fra det vellfungerende biofilteret for torskeproduksjon ved Havlandet indikerte at AOB var tilstede i biofilmen, men at det var en svært lav forekomst av AOA. Det var ikke indikasjoner på forekomst av comammox *Nitrospira* i noen av biofilm prøvene.

Det var en signifikant forskjell (p=0,0007) mellom de mikrobielle samfunnene i de to vellykkede nitrifiserende biofiltrene ved høy salinitet; Podefilteret og det vellfungerende biofilteret for torskeproduksjon ved Havlandet. Likevel var forekomsten av zOTUer som representerer nitrifiserende bakterier i biofilm samfunnene mye høyere for Podefilteret (opp til 77 % av de totale avlesningene) i forhold til det vellfungerende biofilteret for torskeproduksjon (opp til 4,2 %). Dette indikerer at biofilm samfunn med svært forskjellig sammensetning kan utføre nitrifisering ved høy salinitet.

List of abbreviations

AmoA - ammonia monooxygenase
AOA - Ammonia oxidizing archaea
AOB - Ammonia oxidizing bacteria
B - Biofilm
Comammox - Complete ammonia oxidizer
C_t - cycle threshold
D - Day
DNA - Deoxyribonucleic acid
DO - dissolved oxygen
E - Amplification efficiency
Er - Erko
FTS - Flow-through systems
Ha - Havlandet
HTS - High-throughput seqencing
In - Inoculum
MBBR - Moving Bed Biofilm Reactor
MBR - Membrane bioreactor
MF - Maturation filter
MP - Maturation period
NGS - Next-generation sequencing
NOB - Nitrite oxidizing bacteria
NSC - Norwegian sequencing center
NTC - Non Template Control
nxr - nitrite oxidoreductase
PCR - Polymerase chain reaction
PcOA - Principal coordinate analysis
PF - Poding filter
PP - Poding period
ppm - parts per million
qPCR - real-time polymerase chain reaction
RAS - Recirculating aquaculture systems
RDP - Ribosomal Database Project
RNA - Ribonucleic acid
rRNA - ribosomal ribonucleic acid
SE - Standard Error
TAN - Total Ammonium Nitrogen
U - units
v3 - Variabel region 3
v4 - Variabel region 4
W - Water
zOTU - zero-ratio operational taxonomic units

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1 Introduction

The rapid human population growth [14] increases the demand for environmentally friendly food. A relative new and innovative way of fish production is called recirculating aquaculture system, RAS. These land-based systems reuse the water after mechanical and microbiological water treatment. RAS gives many advantages. Primarily, it is made an environment where the fish farmer has good control of the rearing conditions as the water quality. Among other things, a constant and optimal temperature could be kept through the year resulting in a higher fish production. There is no risk of fishlouse or -escape, something that has been challenging in the Norwegian aquaculture industry in traditional seacages [39]. It has also been shown higher survival in RAS compared with traditional flow-through systems, FTS, for cod larvae [1]. RAS in Norway is mostly used in production of smolt and post-smolt [17], but the use of RAS for production of different fish species up to grow-out size is increasing [33] [62] [60].

From an environmental point of view, RAS is desirable in comparison with both land-based flow-through systems, FTS, and production of fish in cages in the sea [47]. The waste from RAS could be utilized in several ways. It could for example be used as fertilizer [7] or for biogas production [12]. In comparison with FTS the concentrations of pollutants is much higher in the waste from RAS [41], something that makes further utilization easier. This gives possibilities for other utilization methods such as aquaponics. In comparison, waste from open aquaculture farms including residues from chemicals is directly released into the environment. In addition the low consumption of water gives a lower environmental impact. This will also give economic arguments for the use of RAS up to grow-out size, no matter if the investment costs are high [47].

For the microbiological water treatment in a RAS, a biofilter is necessary. The biofilter consists of a large surface area for optimal bacteria growth [63]. Before insertion of fish into a RAS the biofilter has to be matured. This is to achieve the desired convertion of the toxic compound ammonium into the less toxic compound nitrate. This convertion is performed by nitrifying bacteria. The start-up process to achieve the desired capacity can be challenging in environments with high salinity [43], and it is found to be more time consuming in marine environments compared with freshwater systems [51]. This project is a continuation of an earlier student project where the nitrification rate during the maturation was investigated at different salinities [58]. The biofilter in a pilot-RAS was matured for operating at 25 ppm salinity at Havlandet RAS AS. In this master thesis the microbial communities during two strategies of start-up of the marine biofilter in the pilot-RAS was further investigated.

1.1 Biological life cycle of Atlantic salmon

Atlantic salmon, *Salmo salar*, is an anadromous species which means that it lives in two stages; A freshwater stage and a seawater stage. The freshwater stage starts with eggs in a freshwater river [61] before the fish goes through several life stages: Alevins, fry, parr, smolt and grown-up salmon (Figure 1.1) [61]. The Atlantic salmon lives its first one to eight years in a freshwater river before it undergoes smoltification and migrates to seawater. After about two to five years in the sea the salmon is sexually mature and swims back to the freshwater river to spawn. Some salmonids spawns several times [27].

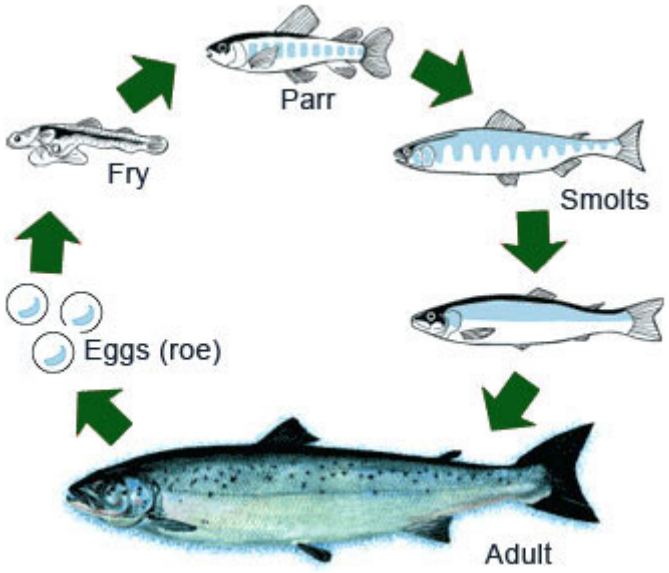

Figure 1.1: An overview of the biological life cycle of Atlantic salmon. The salmon lives in freshwater until it smoltifies and migrates to seawater at the smolt stage. The figure is found in [40].

1.2 Fishproduction in RAS

The Norwegian aquaculture industry accounts for a large share of exports from Norway. In 2019 seafood was exported for 107.3 billion NOK [59]. Atlantic salmon accounted for about 94 % of the total amount of seafood produced in Norway [56]. The industry has problems with both fishlouse and -escape [39]. These problems could be neglected if RAS is used all the way to fish of grow-out size. Traditionally the salmonids is produced in land based fresh-water systems until smoltification. The fish is thereafter moved to open cages in the sea for production of grow-out fish [17].

It is two types of land-based aquaculture; Flow-through systems, FTS, and recirculating aquaculture systems, RAS (Figure 1.2). In FTS the water is only used one time, but in RAS it is recycled in varying degree [30]. This gives a higher water consumption in FTS relative to RAS. The low water consumption in RAS entail treatment of the water. The most important part of the water treatment in RAS is the microbiological treatment in the biofilter [2]. Recently there has been seen an increase in use of recycling technology for production of smolt and post-smolt, but there is also made and planned systems for production of fish up to grow-out size [33] [62] [60]. Today almost all new fish production units is based on recycling technology [17]. An illustration of a flow-through system and a recirculating aquaculture system is shown in Figure 1.2.

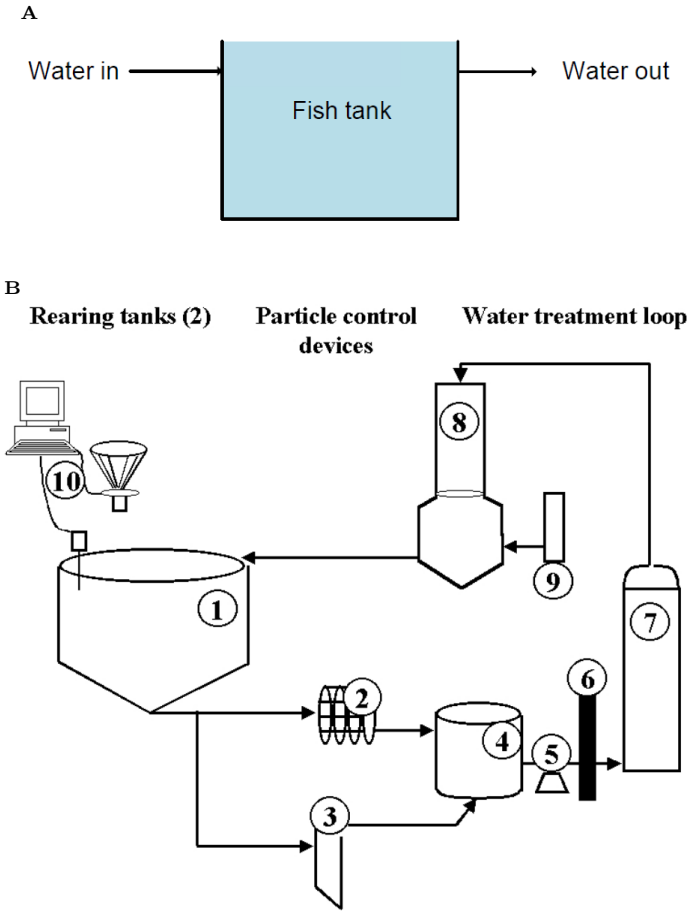

Figure 1.2: A simplified flow chart over (A) a flow-through system and (B) a recirculating aquaculture system found in [4]. In the flow-through system (A) the water is used one time before it goes out of the system. In the recirculating aquaculture system (B) the water is going through a water treatment before it is reused in the fish tank (1). The water treatment consists of a pumping tank where the pH and temperature is regulated (4), a pump (5), UV-disinfection (6), microbiological treatment in the biofilter (7), a CO_2-degasser (8) for removal of CO_2 and oxygenation (9). The RAS illustration also consists of a drum screen filter (2), a particle trap (3) and a self-feeding system (10). However, different RAS units are designed in different ways, and deviations from this illustration will take place in the industry.

1.3 Water quality and water treatment in RAS

Reuse of water in RAS requires treatment of water to maintain good water quality. The water quality in RAS is important for fish welfare. RAS provides the opportunity to control the water quality, resulting in the optimal conditions for fish growth. Some important water quality parameters are dissolved oxygen (DO), concentration of ammonium, nitrite, carbon dioxide, alkalinity and dissolved solids [63].

One of the most important water quality parameters in RAS is the concentration of total ammonium nitrogen, TAN [63]. Ammonium, NH_4^+, is the main excrete from the fish and it is in equilibrium with the much more toxic compound ammonia, NH_3 (Equation 1.1) [13].

$$NH_3 + H^+ \rightleftharpoons NH_4^+ \tag{1.1}$$

The equilibrium (Equation 1.1) depends on the pH. A higher pH gives a higher fraction of ammonia, and increases drastically when the pH passes 8.5 [13]. A higher salinity or temperature

will similarly give a higher fraction with the very toxic compound ammonia [63]. Another nitrogenous compound which is toxic is nitrite. Nitrite can cause low ability to take oxygen up in the blood of the fish [13]. It is formed in uncomplete nitrification in the microbiological treatment in RAS. It is therefore important to achieve a matured biofilter with complete conversion of TAN into nitrate before insertion of fish into the RAS.

1.3.1 Microbiological treatment in RAS

Nitrifying bacteria in biofilm in biofilters is used in RAS to convert the toxic waste product ammonium to the less toxic compound nitrate, in a process called nitrification (Equation 1.2) [45]. Nitrifying bacteria are aerob, autotrophic bacteria that uses ammonium or nitrite as electron donor [63]. In nitrification ammonia is first converted to nitrite by ammonia oxidizing bacteria, AOB. Nitrite is thereafter converted to nitrate by nitrite oxidizing bacteria, NOB (Equation 1.2).

$$NH_4{}^+(\text{toxic}) \xrightarrow{AOB} NO_2{}^-(\text{toxic}) \xrightarrow{NOB} NO_3{}^-(\text{not toxic}) \tag{1.2}$$

Examples of AOBs are *Nitrosomonas*, *Nitrosococcus* and *Nitrosovibrio* [63]. These bacteria oxidizes ammonium into nitrite by using O_2 as electron acceptor (Equation 1.3). It has also been discovered microbes from the kingdom *Archaea* performing ammonia oxidation (Equation 1.3). These microbes are called ammonium oxidizing archaea, AOA [71].

$$NH_4{}^+ + 1.5\ O_2 -> NO_2{}^- + 2\ H^+ + H_2O \tag{1.3}$$

Further convertion of nitrite to nitrate is performed by NOBs by using oxygen (Equation 1.4). Examples of NOBs are *Nitrobacter*, *Nitrococcus* and *Nitrospira* [63].

$$NO_2{}^- + 0.5\ O_2 -> NO_3{}^- \tag{1.4}$$

The total nitrification therefore needs oxygen and will produce acidity (Equation 1.5).

$$NH_4{}^+ + 2\ O_2 -> NO_3{}^- + 2\ H^+ + H_2O \tag{1.5}$$

To achieve optimal growth conditions for the nitrifying bacteria the environment has to meet the needs of the bacteria. Equation 1.6 is derived by balancing the equation for nitrification, and take the production of biomass into account [63]. Bicarbonate is added to the nitrifying biofilter to prevent a rapid decrease in pH.

$$NH_4{}^+ + 1.83\ O_2 + 1.97\ HCO_3{}^- -> 0.0244\ C_5H_7NO_2 + 0.976\ NO_3{}^- + 2.90\ H_2O + 1.86\ CO_2 \tag{1.6}$$

In the nature, nitrifying bacteria are found in both fresh- and marine-environments [46] as soils, water, wastewater and in the ocean [34]. In biofilters in RAS nitrifying bacteria grows in biofilm in competition with heterotrophic bacteria, competing for oxygen and space [54]. The heterotrophic bacteria is generally fast-growing compared to the nitrifying bacteria, and will therefore have a considerable abundance in the biofilm [63]. The heterotrophic bacteria uses organic carbon as a energy-source, and it will be favorable with as little organic carbon as possible to select for the nitrifying bacteria [72]. In this way the concentration of organic substances has a negative impact on the nitrification [21].

Several other factors affects the nitrification [63]. Among other factors, the concentration of substrate, the temperature [69], the oxygen concentration, pH and the salinity has an impact on the nitrification [30]. Optimal temperature for nitrification is around 30 °C, and a temperature under 5 °C will give a low growth [30]. The optimal pH value for nitrification is between 8 and 9 [22], but since the proportion of ammonia increases with increasing pH the pH is usually kept below 8 in RAS [13]. The access to substrate is one of the main parameters for nitrification, but a high concentration of TAN or nitrite can also inhibit the nitrification [8]. In biofilters in RAS the nitrification has to be effective at low substrate concentrations, especially for nitrite

since the concentrations has to be very low to ensure good fish welfare [25].

An increase in salinity has a negative impact on the nitrification rate [63]. There is found a clearer negative effect for the nitrite oxidation than the ammonium oxidation [43]. A salinity over 10 ppm on cells not adapted for the saline conditions will give loss of microbial activity [24]. In addition, a salinity change of 5 ppm or more will give the nitrifying bacteria shock, and therefore decrease the nitrification rate [18]. It is found up to 60 % lower nitrification rates, and a longer time for biofilter start-up in seawater than for fresh water [43] [51]. Simultaneously, a biofilter adapted to the salinity of 32 ppm is not found to have higher nitrification effectivity at any other salinities [18]. Recent studies show that a fresh-water biofilter can be adapted to saline conditions [18], and even to a high salinity [43]. It is limited knowledge about how salinity affects the microbial communities in RAS [16], and most studies performs lab-scale reactor experiments to investigate nitrification. More knowledge about the microbial communities in successful marine biofilters is needed. This can improve the process of adapting nitrifying biofilters in RAS to a high salinity.

1.4 Biofilters in RAS

As mentioned earlier, nitrifying bacteria grows in biofilm in the biofilter in RAS [54]. To facilitate for biofilm formation the biofilter should have a large specific surface area [30]. This can be achieved in several ways [63]. The two most commonly used biofilters in RAS are called moving bed biofilm reactor (Figure 1.3A), MBBR, and fixed bed biofilter (Figure 1.3B) [13]. The MBBR consists of loose biofilm carriers with a large surface. They will scratch against each other and therefore not create a thick biofilm. This makes MBBR self-cleaning [13]. The fixed bed biofilter consists of a growth-material which is fixed in the reactor [53]. The fixed growth material gives the possibility for the bacteria to grow in a thick biofilm, something that makes washing of the biofilter necessary [13]. To meet the conditions of the nitrifying bacteria both MBBRs and fixed bed biofilters are aerated [13]. The MBBR is usually designed with a filling degree of 70 % biofilm carriers [30].

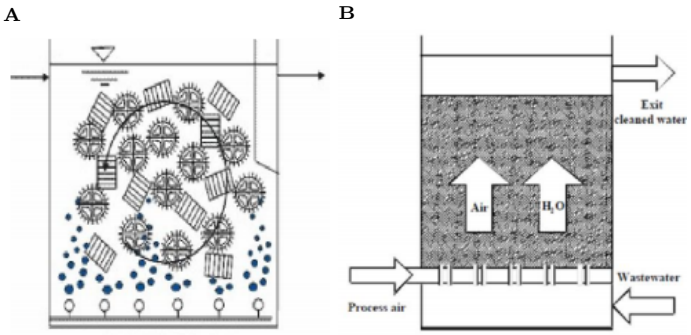

Figure 1.3: Two common constructions of biofilters in recirculating aquaculture systems. The main purpose for the biofilter is to achieve optimal conditions for the nitrifying bacteria to convert ammonium into nitrate via nitrite. Illustration of **(A)** a moving bed biofilm reactor from [44] and **(B)** a fixed bed biofilter from [53].

1.4.1 Start-up of biofilters in RAS

The biofilter has to be activated properly before start-up of the RAS. The convertion of the desired amount of ammonium into nitrate has to be initiated, and the bacteria has to be adapted to the relevant conditions. There are several procedures for start-up of new biofilters to adapt it to the desired environments [13]. A MBBR biofilter could be started with new, clean biofilm carriers regularly fed with ammonium chlorid. In addition a commercial, liquid inoculum adapted to the relevant salinity could be added. Another strategy is to add already matured biofilm carriers from another RAS as biofilm inoculum. River sand could also be used as inoculum in the start-up of a new biofilter [13]. Usually new biofilters is started up with addition of a liquid inoculum [50].

Addition of ammonium chloride during a time period will feed the ammonia oxidizing bacteria. This is expected to lead to an increase in the concentration of nitrite (Figure 1.4). Nitrite is thereafter converted to the less toxic compound nitrate by NOBs. A high concentration of nitrite can inhibit both the AOBs and the NOBs [8], something that can lead to delays in the maturation of the nitrifying biofilter. A faster start-up of the biofilter can be achieved if both nitrite and ammonium is fed to the biofilter simultanously [13]. NOBs is then activated simultaneously as the AOBs. It has been found that a longer time for maturation is needed at higher salinities compared with maturation in fresh water [51].

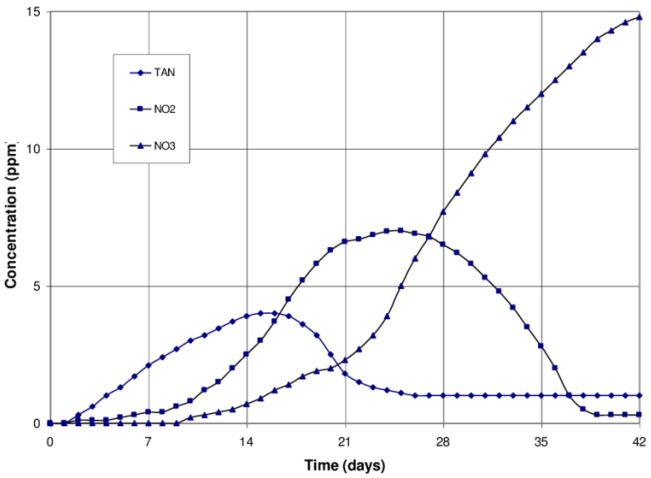

Figure 1.4: Grafical illustration of the concentration of TAN, nitrite and nitrate (From [63]). The biofilter is fed with ammonium, and the concentration of the total nitrogenous components is increasing. It can be seen an increase of nitrite during the maturation. The concentration of nitrite will decrease when NOBs are activated. This gives formation of nitrate, which is less toxic compared to TAN and nitrite.

1.5 Microbial communities in biofilters of marine RAS

As earlier mentioned, it is limited knowledge about the microbial communities in biofilters in marine RAS. The salinity effect on the microbial communities needs further investigation. Most studies are performed in lab-scale, and may not reflect the diversity of the microbial communities in biofilters in marine RAS.

Recently, it was found that one single bacterial species is able to perform the convertion of ammonium all the way to nitrate. This is more energetically favored for the bacteria compared with performation of one of the nitrification oxidations [64]. These bacteria has been called complete ammonia oxidizers, comammox, and belongs to the genus *Nitrospira* [64]. Comammox *Nitrospira* was first found in biofilters in engineered, fresh-water systems [64]. Later, comammox *Nitrospira* was found in fresh-water RAS [3]. Marine comammox species is not characterized per 2021. However, the AOB:NOB ratio in marine RAS is found to be low, something that indicates presence of comammox in these systems [16]. It is found a highly diverse group of comammox bacteria in mangrove ecosystems in southeastern China. A study shows that comammox bacteria exists in high-salinity environments [32]. It is therefore not unthinkable that comammox is present in marine RAS biofilters. The presence and effects of comammox *Nitrospira* in marine environments should be further investigated [55].

It is also found microbes in the kingdom *Archaea* which converts ammonia to nitrite (Equation 1.3). AOAs are previously found in a biofilter in RAS for shrimp production [6]. It was found higher abundance of AOAs than AOBs in the study, and it was found along with the nitrite oxidizing species *Nitrospira* in the biofilter [6]. However, other studies give a indication that AOA does not contribute considerably to ammonium oxidization in RAS [15]. There are not many studies focusing on AOAs contribution on the ammonium oxidation in RAS biofilters.

Seawater recirculating aquacultural systems have earlier been investigated through 16S rDNA amplicon sequencing [29]. Nitrifiers in water from biofilters at higher salinity (20 - 32.5 ppm) was associated with *Nitrosomonas*, *Nitrospira* and *Nitrospina*. It have been found a relative abundance of the complete bacterial communities up to 16 % in the water samples from different marine biofilters [29]. Other analysis from RAS biofilters is done by sequencing the *16S rRNA*- and *amoA*-genes. These studies show presence of the AOBs *Nitrosomonas* sp. Nm143-lineage and *Nitrosomonas marina*. The most abundant NOB found was *Nitrospira marina*, but other NOBs as *Crenarchaeota* was also found [15].

Start-up of marine biofilters using already matured biofilm carriers as inoculum has been compared with the use of a commercial, liquid inoculum [48]. The results shows an earlier formation of nitrite and nitrate when using biofilm inoculum from another RAS. Sequencing (Figure 1.5) showed a relative abundance of 63.7 % nitrifying microorganisms in the liquid inoculum, where the main part was the nitrifying archea *Thaumarchaeota*. In comparison the biofilm inoculum had a relative abundance of 15.2 % nitrifiers, where the main parts were classified as bacteria. The main nitrifiers on the biofilm carriers were *Nitrospira*, *Nitrosomonas* and *Nitrosococcus* [48]. Despite the lower abundance of nitrifiers on the biofilm inoculum compared with the liquid inoculum, the start-up with biofilm inoculum was more successful [48].

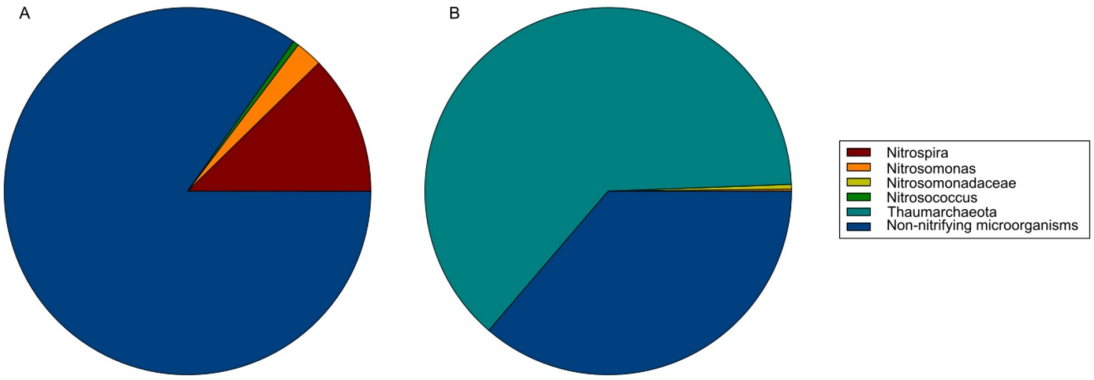

Figure 1.5: Comparison of microorganisms in two different inoculums consisting of (A) already matured biofilm and (B) a commercial, liquid inoculum. The figure is from [48].

1.6 Methods to study microbial communities

Microorganisms usually lives in complex microbial communities. The microbes are dependent on each other and the environment around them. They interact with each other and the surroundings [35]. These complex microbial communities can be challenging to study. Microbiology was traditionally studied by cultivation and isolation of single strains. Sanger sequencing was the only sequencing method until 1980 [52]. Sanger sequencing can only be used to sequence one DNA-template at the time [9]. This gives limited possibilities to study the complex microbial communities. Some microbes can be challenging and even impossible to cultivate. It is for example difficult to isolate and cultivate NOBs, and especially *Nitrospira* [11]. One strain of *Nitrospira* took up to twelve years to isolate [28]. It has been estimated by environmental microbiologists that less than 2 % of bacteria can be cultivated in the laboratory [66]. These challenges have been a part of the motivation for the development of new methods to study microbial communities. It has been a revolutionary development of methods to study microbial communities since the 1980s. Next generation sequencing, NGS, can be applied to sequence millions of DNA fragments at the same time, and is based on a prepared DNA library [9].

The 16S ribosomal RNA gene is a marker gene for microbial diversity, and can be used for taxonomic identification [70]. It is found in all prokaryotes [67]. The *16S rRNA* gene consists of nine variabel regions (v1-v9) [19] and nine conserved regions [68]. The *16S rRNA* gene variable sequences is found to be unique for every species and could therefore be used for taxonomic identification, diversity and phylogeny analysis [36]. The conserved regions provides the opportunity to design universal PCR primers for amplification of parts of the *16S rRNA* gene in all members in a microbial community.

After PCR amplification of the *16S rRNA* gene the samples will consist of many different DNA sequences. Massive parallel sequencing is a valuable method in this case. In comparison, Sanger sequencing, would not work because it only sequences one DNA template at a time. NGS can be used to characterize the microbial diversity in these microbial communities. NGS gives the possibilities to study bacterial species that are challenging to cultivate, and has low abundances in a microbial community. Today, the most common NGS technology used in studies are Illumina sequencing [9].

Illumina sequencing is a type of high-throughput sequencing, HTS, and can therefore read several sequences simultaneously [10]. Sanger sequencing is in comparison a low-throughput sequencing, and will only read one genome at a time [10]. Illumina sequencing is based on the sequencing-by-synthesis approach, and uses one fluorescent labeled nucleotide per cycle [10]. The sequencing starts with the preparation of an amplicon library. DNA extracts from the samples is targeted with primers which is marked with unique primers in each end. Thereafter cluster generation is performed, and the DNA is bound to oligonucleotides on the surface. The 3' end is denatured, and the complementary strand is replicated. The sequencing is then performed by adding a single fluorescent labeled nucleotide which is CCD-pictured and cleavaged. The cleavage opens up for the next nucleotide to be added [10] [9].

1.7 The adaptation of a nitrifying biofilter for a pilot-scale RAS operated at 25 ppm salinity at Havlandet RAS pilot AS

Havlandet RAS pilot was building a RAS at pilot-scale at higher salinity (25 ppm) for production of grow-out Atlantic salmon. The aim with the pilot-RAS at Havlandet was to achieve knowledge for later building of a RAS at commercial scale. The pilot-RAS was dimensioned for production of 200 tonnes grow-out salmon yearly, with a MBBR biofilter with 123 m^3 biofilm carriers[58]. The maturation was at this point performed with new, clean biofilm carriers with addition of a commercial, liquid inoculum made for a higher salinity. The filter was fed with ammonium chloride. This biofilter is hereafter called the Maturation filter.

The nitrification has been investigated at different salinities during the start-up of the Maturation filter. The student project[58] was based on production data from Havlandet and lab-scale batch experiments with biofilm carriers from Havlandet. The lab-scale batch experiments were performed with biofilm carriers from day 24, 52 and 68 after start-up of the MBBR biofilter. Biofilm carriers from a well-functioning, marine biofilter in a RAS for production of cod at Havlandet were also investigated in a lab-scale batch experiment. At day 52 and 68 after start-up there was found a higher nitrification capacity for biofilm carriers from the Maturation filter at salinity 10 ppm compared with 20 ppm. At day 68 after start-up the nitrification capacity was twice as high at 10 ppm salinity than at 20 ppm salinity. This indicates that the biofilter was not adapted to the higher salinity after 68 days of start-up. The nitrification capacity was higher for biofilm carriers from the well-functioning, marine biofilter for cod production at 20 ppm salinity than for the lab-scale batch experiments with biofilm carriers from the Maturation filter at any point[58].

The nitrification rate in pilot-scale in the Maturation filter was analyzed based on data delivered from Havlandet. The salinity, concentration of TAN, nitrite and nitrate was reported. The salinity was varying between 10 ppm to 26 ppm. The concentration of TAN was falling at the start, but remained constant after day 56 after start-up. It was reported a high concentration of nitrite (26 mg L^{-1}) after 60 days of start-up. The nitrification rate during the start-up of the Maturation filter was also calculated as converted ammonium relative to time (Figure 1.6). It was observed a marked increase in the nitrification rate in the first 35 days of start-up. The nitrification rate stagnated, something that can indicate an uncomplete nitrification into nitrite. The nitrification rate thereafter increased at maturation day 55, but decreased after that. The nitrification rate was varying in the Maturation filter. This could be because of varying salinity and nitrite inhibition of AOBs and NOBs.

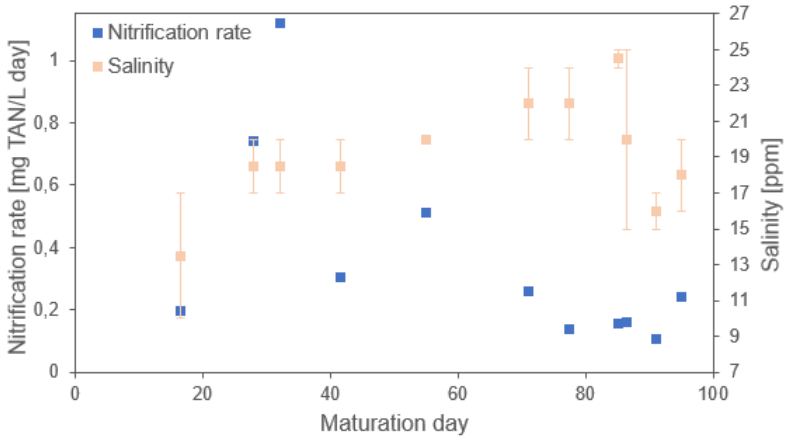

Figure 1.6: Calculated nitrification rate as converted ammonium relative to time during start-up of the biofilter at Havlandet. (From[58]). The values on the salinity is shown on the secondary axis at the right.

1.8 Aims

Start-up of biofilters in marine RAS can be challenging and time consuming. Several strategies for start-up of biofilters have been used by the industry and in studies. Still, there is limited knowledge of how the microbial commmunities are affected by changes in the salinity. More knowledge is therefore needed to evaluate the start-up strategies for marine biofilters. Such knowledge may contribute to decreased start-up time of marine biofilters in the aquaculture industry.

This master thesis is a continuation of an earlier student project in collaboration with Havlandet RAS Pilot, where the nitrification rate was investigated during adaptation of a nitrifying biofilter to increased salinity [58]. In this thesis, two different strategies for start-up of marine biofilters for a pilot-RAS are investigated. The main aims for this master thesis are to

1. Characterize the bacterial communities, in the biofilm and water, in the MBBR biofilter at Havlandet RAS pilot AS during adaptation to higher salinity.
2. Evaluate two strategies for start-up of a marine, nitrifying biofilter based on respectively a liquid, commercial inoculum and a biofilm inoculum.
3. Identify the potential ammonia and nitrite oxidizing members in the biofilm communities during these start-up strategies of the marine biofilters.

2 Materials and method

This master thesis is a project in collaboration with Havlandet RAS Pilot AS. Havlandet RAS Pilot is operating a recently started pilot-scale RAS for production of 200 tonnes grow-out salmon yearly. Havlandet RAS Pilot wanted to gain experience from this pilot-scale production with RAS before start-up of a RAS at bigger scale, specifically 10 000 tonn fish yearly [23]. Havlandet RAS Pilot partly funded this project, and provided production data and material for microbial analyses. The moving bed biofilm reactor, MBBR, was started up for 171 days before the salmon was moved into the pilot-RAS. The start-up of the MBBR was divided into two periods; A 137 days long Maturation period where the MBBR was started with liquid, commercial inoculum, which is followed by a 34 days long Poding period where the MBBR was started with a biofilm inoculum from another producer. These periods are described in more detail below.

2.1 Description of the start-up of the MBBR biofilter at Havlandet

At Havlandet, a well-functioning marine biofilter was already operated in another RAS for production of cod. This well-functioning biofilter was not used as poding material as it contained of too few biofilm carriers and concerns for transfer of undesirable microbes from RAS with cod. Samples from this biofilter was included in the microbial analysis as a positive control for a well-functioning, marine biofilter. Havlandet was going to start-up a new biofilter to use in the pilot-RAS at marine conditions using new, clean biofilm carriers and a liquid, commercial inoculum.

Havlandet started the first trial of start-up of the MBBR biofilter with new, clean biofilm carriers without any biofilm in a smaller maturation container on 25 m^3 (Day 0, Figure 2.1A). The biofilter was regularly fed with ammonium chloride and a liquid, commercial inoculum. This period is hereafter called the Maturation period (MP) and lasted from day 0 until day 137 in the Maturation filter (MF). At day 68, the MF was moved from the smaller maturation container and into the bioreactor in the pilot-RAS. The biofilm carriers (10 m^3) was at this point diluted with 100 m^3 clean biofilm carriers (Day 68, Figure 2.1A). At day 99 an accident occured and the bioreactor in the marine pilot-RAS collapsed (Day 99, Figure 2.1A). Most of the biofilm carriers were lost, but some were captured, and an attempt of continuing the MP was performed. Unfortunately, Havlandet did not observe any nitrification activity, and they therefore wanted to try a new strategy to achieve a successful biofilter before the fish was moved into the system.

Havlandet was getting already matured biofilm carriers from a successful marine RAS (15 ppm salinity) for post-smolt production at Erko Seafood. These biofilm carriers was used as biofilm inoculum in the Poding period (PP). At day 137, 1.5 m^3 inoculum biofilm carriers from Erko (In-PP, Figure 2.1B) was mixed with 5 m^3 clean biofilm carriers. This filter is from now on called the Poding filter (PF). The biofilm carriers from Erko had a different look than the biofilm carriers used at Havlandet. This gave the opportunity to characterize the change in the microbial biofilm composition on the new, clean biofilm carriers from Havlandet after mixing with the biofilm inoculum from Erko. It was added approximately 25 m^3 clean biofilm carriers gradually between day 140 and 144. Thereafter it was added approximately 50 m^3 new, clean biofilm carriers at day 151. This resulted in a total of 81.5 m^3 biofilm carriers in the MBBR biofilter at day 151. After 171 days of start-up in the MF and the PF the fish was moved into the pilot-RAS.

2.1.1 Sampling of water and biofilm samples during start-up

It was sampled a total of 75 biofilm (B) and water (W) samples for microbial analysis. In the MP the liquid, commercial inoculum (In-MP-W) was sampled in addition to the biofilm carriers (MF-BHa) and the water samples (MF-W). Before addition of the matured biofilm from Erko Seafood into the PF it was sampled from the inoculum biofilm (In-PP, Figure 2.1B). The first sample of the new biofilm carriers (BHa) was sampled already 3 days after the biofilm inoculum was added, at day 140 (PF-BHa-D140, Figure 2.1B). The sample days, sample types and number of samples taken from the MF and the PF are specified in Figure 2.1. Sampling was also performed from the marine well-functioning biofilter at Havlandet (Wellf-BHa, Wellf-WHa) from the cod RAS as a positive control.

The biofilm samples were gently dripped of on a paper towel and stored in zip-lock plastic bags. For the water samples, 150 mL water from the biofilter was collected in 0.22 μm Sterivex filter (Millipore) with Omnifix ® syringes. The samples were stored in a freezer at -20 °C before and after shipment to NTNU until the analysis was performed.

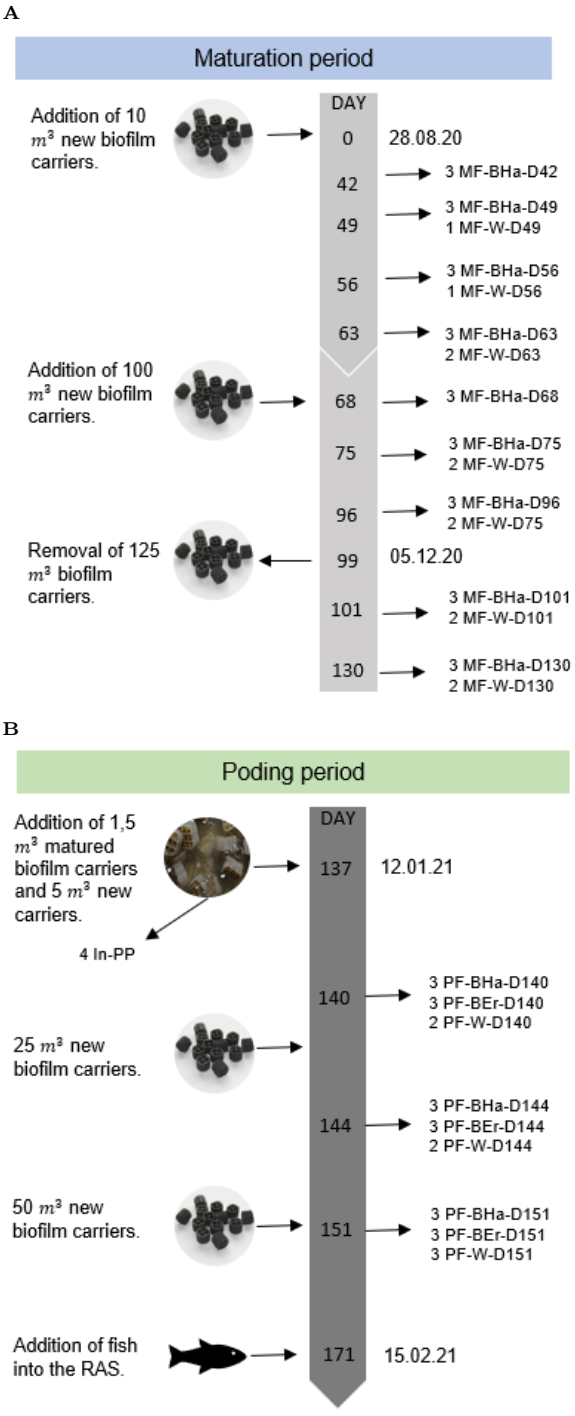

Figure 2.1: A timeline with the sampling points during (A) the Maturation period and (B) the Poding period at Havlandet RAS pilot during start-up of the marine moving bed biofilm reactor. The first 68 days was performed in the Maturation container with new biofilm carriers and a liquid, commercial inoculum. At day 68 the biofilm carriers was moved to the bioreactor and diluted with new biofilm carriers in the pilot-RAS without fish. At day 99 an accident resulted in loss of biofilm carriers. One attempt of continuing the Maturation period was performed, but with little signs of nitrification. The start-up was restarted in a new period, the Poding period. In the Poding period already matured biofilm carriers from Erko Seafood was used as biofilm inoculum (In-PP). The black arrows indicates biofilm carriers added to the system as well as samples taken out for microbial community analysis. The samples is named after the biofilters; The Maturation filter (MF) or the Poding filter (PF), sample type; Biofilm (B) or water (W), type of carrier; From Havlandet (Ha) or from Erko Seafood (Er) and day (D) number after start-up of the Maturation filter.

2.2 Characterization of microbial communities by Illumina Sequencing of 16S rDNA amplicons

2.2.1 DNA Extraction

DNA was extracted from the 75 samples (See section 2.1.1) by using Powersoil® DNA Isolation Kit (Qiagen). For the biofilm samples approximately $\frac{1}{4}$ of the biofilm carriers was added to the beads in the kit as start material. The protocol (Appendix C.1) was followed. Several methods was used to reduce the DNA contamination. Firstly, all the tubes was UV-radiated 30 minutes before use. The biofilm carriers was cut up in sterile petri dishes, and the equipment was decontaminated with etanol before use. The DNA extractions resulted in 75 DNA extracts.

2.2.2 PCR

The samples from the DNA extraction was used as template in PCR to amplify variabel region 3 and 4 of the *16S rRNA* gene by using universal bacterial primers. It was made 25 μL PCR reaction for each sample. The mastermix consisted of the final concentrations of 1x phusion buffer HF, 0.3 μM of both of the primers ill341F_Kl/805R, 200 μM of each dNTP, 0.5 mM $MgCl_2$ and 0.02 units μL^{-1} Phusion Hot Start DNA polymerase. DNA free water was added to achieve the desired amount. 1 μL DNA extract was added as template for each PCR reaction. Some of the DNA extracts were diluted 1:10 before it was used as template.

It was made 16S rDNA amplicons of all the 75 DNA extracts. The universal primers Ill338F and Ill805R was used to amplify a PCR product on 467 basepairs for each sample. The PCR machine T100^{TM} Thermal Cycler (BioRad) was used for the temperature cycling steps as shown in Table 2.1. It was used 38 cycles on most of the samples. The 16S rDNA amplicons were stored at 4 °C until further analysis. It was achieved PCR results for all of the DNA extracts at expected lengths (467 bp). A none template control (NTC) was included in every run of PCR reactions.

Table 2.1: The PCR program for amplification of bacterial 16S rDNA variabel region 3 and 4.

Step	Temperature [°C]	Time	Cycles
Denaturation	98	2 min	
Denaturation	98	15 sec	
Annealing	55	20 sec	x 36-38
Elongation	72	20 sec	
Final elongation	72	5 min	

2.2.3 Agarose Gel Electrophoresis

The PCR amplicons was analyzed using agarose gel electrophoresis. The agarose gel (1 %) was made by dissolving agarose in TEA buffer (1 %) by heating it in a microwave. The agarose solution (1 %) was cooled in room temperature until it was 60 °C before use. It was made 1.5 % agarose gel for some applications. The gel was made by mixing 50 mL of agarose solution (1 %) with GelRed®(Biotium) resulting in the final concentration of 50 mM of GelRed. The solution was put in a gelation chamber with a gel comb at the end of the gel. After the gel was polymerized, the PCR products (4 μL) was mixed with 1 μL 6x DNA Loading dye (Thermo Scientific) on a parafilm and added to the chambers. To indicate size GeneRuler^{TM} 1 kb Plus DNA Ladder (Thermo Scientific) was added to one of the chambers. The gel electrophoresis was performed by adding a voltage of 110 Volt for 1 hour. After 1 hour the gel was visualized and photographed in G:Box HR Geldoc (Syngene). The fragment sizes was observed by comparison with the GeneRuler.

2.2.4 Preparation of the amplicon library for Illumina sequencing

The 16S rDNA amplicons was normalized by using the SequalPrep Normalization Plate Kit. The protocol (Appendix C.2) was followed. After the purification and normlization of the PCR amplicons, indexing PCR was performed to mark the 75 samples with a unique combination of two indexes. The indexing PCR was done by using a mastermix with the final concentrations of 1x phusion buffer HF, 0.2 mM of each dNTP and 0.015 units μL^{-1} Phusion Hot Start DNA polymerase. An unique combination of two indexes (2.5 μL) from the Nextera XT DNA Library Preparation Kit was added. 2.5 μL of the normalized PCR product was added as template. The temperature cycling steps was performed in the PCR machine as shown in Table 2.2. The 16S rDNA amplicons with little results in traditional PCR (Section 2.2.2) was performed at 12 cycles and the 16S rDNA amplicons with clear PCR results was performed with 10 cycles. After the indexing PCR the samples was stored at 4 °C until further analysis. It was performed agarose gel electrophoresis on all of the samples, and all the samples achieved positive indexing PCR results.

Table 2.2: The temperature cycling steps for indexing PCR in preparation of the Illumina library.

Step	**Temperature [°C]**	**Time**	**Cycles**
Denaturation	98	2 min	
Denaturation	98	15 sec	
Annealing	50	20 sec	x 10-12
Elongation	72	20 sec	
Final elongation	75	5 min	

After indexing PCR on all the 75 samples, purification and normalization was performed with the SequalPrep Normalization Kit. The protocol was followed (Appendix C.2). The 75 samples was thereafter mixed in one tube. The sample was concentrated by using a AmiconUltra 0.5 centrifugal filter. The protocol (Appendix C.3) for the AmiconUltra 0.5 centrifugal filter was followed. At step 4 the sample was washed with sterile filtrated 1xTE buffer. The sample was centrifuged at 14 000 x g for 10 minutes. The washing was repeated two times. The protocol was further followed. The concentrated sample was applied on a agarose (1 %) gel. The sample was measured in NanoDrop with satisfying result before sending to the Norwegian sequencing center, NSC, for Illumina sequencing. It was stored at a temperature of -20 °C until shipping.

2.3 Ammonium oxidizing members in biofilm samples

2.3.1 PCR amplification of the *amoA* gene

The results after Illumina amplicon sequencing was analyzed, and a dominance of nitrite oxidizing bacteria was found in the samples representing the Poding filter. The potential presence of microbial species performing ammonium oxidation was therefore further investigated in the biofilm samples. Samples from the three biofilters were investigated: The Poding filter, the Maturation filter and the well-functioning biofilter for cod production. PCR with specific primers was performed on the following samples: PF-BEr-D151-R1, PF-BEr-D151-R3, Wellf-BHa-R1, Wellf-BHa-R2, MF-BHa-D68-R1 and MF-BHa-D68-R3. The specific primers (Table 2.3) targeted the ammonium oxidizing gene, *amoA*, in AOAs, AOBs and two clades of comammox *Nitrospira*.

Table 2.3: Primer pairs used for PCR amplification of the *amoA* gene in biofilm samples. The primers was found in Sun et al [57].

Primer name	Target	Primer sequence 5'-3'
CamoA-19F	*amoA* in AOA	ATGGTCTGGCTWAGACG
CamoA-616R		GCCATCCATCTGTATGTCCA
AmoA-1F	*amoA* in AOB	GGGGTTTCTACTGGTGGT
AmoA-2R		CCCCTCKGSAAAGCCTTCTTC
ComaAF	*amoA* in comammox clade A	TGCGGIGACTGGGAYTTC
ComaAR		AGATCATAGTGCTRTGICC
ComaB-244F	*amoA* in comammox clade B	TAYTTCTGGACGTTCTA
ComaB-659R		TAYTTCTGGACATTCTA

It was made one mastermix for each primerpair (Table 2.3). The mastermixes (24 μL per reaction) contained the final concentrations of 1xphusion buffer HF, 200 μM dNTP, 0.02 units μL^{-1} phusion hot start DNA polymerase and 0.3 mM of each primer. The volume was adjusted with DNA free water. DNA extract (1 μL) was added as template to the mastermix in different tubes. The PCR was performed with the temperature cycling steps as shown in Table 2.4. The PCR amplicons was investigated using agarose gel electrophoresis with agarose gel (1.5 %) as described in Section 2.2.3.

Table 2.4: Temperature cycling steps for PCR with specific primers targeting *amoA* gene in **(A)** AOA and AOB, and **(B)** comammox *Nitrospira* clade A and clade B.

A

Step	Temp	Time	Cycles
Denaturation	98	2 min	
Denaturtion	98	30 sec	
Annealing	53	30 sec	x 37
Elongation	72	20 sec	
Final elongation	75	5 min	

B

Step	Temp	Time	Cycles
Denaturation	98	2 min	
Denaturtion	98	30 sec	
Annealing	52	30 sec	x 37
Elongation	72	20 sec	
Final elongation	75	5 min	

2.3.2 Sanger sequencing of *amoA* gene PCR products

Some of the *amoA* PCR amplicons with clear PCR results at the expected lengths was sent to Sanger sequencing to identify ammonium oxidizers in the samples. QIAquick® PCR purification Kit (Qiagen) was used to purify the PCR amplicons by following the protocol (Appendix C.4). Purified PCR product (5 μL) and 5 μL primer (5μM) was mixed before sending to Eurofins Genomics for Sanger Sequencing. The same primers as used in PCR of the *amoA* genes was used as sequencing primers (see Table 2.3). It resulted in two sequencing reactions per PCR product, one for the forward primer and one for the revers primer. The results were analyzed using Chromas (Technelysium DNA Sequencing Software) and the NCBI Nucleotide Blast function.

2.3.3 qPCR for quantification of *amoA* gene copies

To quantify DNA in a sample qPCR can be performed. In qPCR the DNA is labeled with the fluoresecent dye; SybrGreen, which is binding double-stranded DNA. The amount of fluorescent signal therefore correlates with the amount of target DNA early in the amplification. In traditional PCR the amount of PCR product will not correlate with the amount of target DNA in the template. The amount of PCR product will correlate with the amount of target DNA in the template early in the exponential phase of the amplification. qPCR can therefore measure the cycle treshold, C_T, and correlate this to the C_T of templates with known concentrations. This is done by measuring the fluorescent signal after each qPCR cycle.

qPCR was performed to quantify the copies of the *amoA* gene from AOAs and AOBs in the biofilm samples. The qPCR was performed on two samples representing the biofilm inoculum from Erko in the Poding filter at day 151 (PF-BEr-D151), two samples from the well-functioning, marine biofilter for cod production at Havlandet (Wellf-BHa) and two samples representing the Maturation filter at day 68 (MF-BHa-D68). DNA extracts from the 6 samples was used as template in qPCR of the samples.

The same primers (Table 2.3) as used in traditional PCR of the *amoA* genes, as described in Section 2.3.1, were used in qPCR. qPCR with these primers is earlier described by Sun et al[57]. It was used a standard curve approach, and broad-coverage 16S rDNA primers were used to target all of the microbial community for normalization. The primers RT-966 (5'-GCAACGCGMRGAACCTTA- CCTA) and RT-1089 (5'-CSGGACTTAACCSAACATYTCA) was used for this purpose.

Standard curves were made by using the same primers (Table 2.3) and conditions as in the traditional PCR of the *amoA* genes (Section 2.3.1). The templates used for the standard curves was made by traditional end-point PCR. For *amoA* in AOB a DNA extract from Wellf-BHa-R2 was used as template in the PCR, while a DNA extract from PF-BEr-R1 was used for the AOA *amoA* gene. For the 16S rDNA amplicon standard curve a template was made by PCR of the *16S rRNA* gene with the primerpair RT-966/-1089 as earlier described (Section 2.2.2), but without addition of $MgCl_2$ in the mastermix. *Yersinia* was used as template. The PCR products were purified by using the QIAquick® PCR purification Kit (Qiagen) (Appendix C.4) before it was used as templates for the standard curves in qPCR. The purified PCR products was determined in NanoDrop, and diluted in series with concentrations from 0.1 ng μ L^{-1} DNA to 0.0000001 ng μL^{-1} DNA.

The qPCR reactions was setup in triplicates in two 96-wells plates as illustrated in Table 2.5. Two standard curves were included on each plate; one for the *16S rRNA* gene and one for the *amoA* gene in either AOB or AOA. It was made one mastermix for each of the 3 primerpairs with SYBR®Green master mix (Thermo Scientific), DNA free water and two primers with the final concentrations at 0.3 mM of each. Each of the mastermixes for the *16S rRNA* gene and for the *amoA* gene in AOB was distributed to 39 wells each in the first plate. Similarly, in the second plate, the mastermixes for the *16S rRNA* gene and for the *amoA* gene in AOA was distributed to 39 wells each. Purified and diluted PCR products were used as templates for the standard curves as earlier described. DNA extracts representing the biofilm were used as templates for the samples. A non template control was included with primers for both the *16S rRNA* gene and the *amoA* gene. The 6 samples, the samples for the standard curves and the non template controls for each primerpair resulted in 78 samples in each of the two 96-wells plates with primers for amplification of the *amoA* in respectively AOB and AOA. All the qPCR reactions were setup in triplicates.

Table 2.5: The setup of the two plates in the qPCR. All the qPCR reactions included in the two plates was setup in triplicates. Diluted and purified PCR products was used as templates for the standard curves. DNA extracts representing the samples was used as templates for the samples. The difference between the two plates was the primers. In the first plate primers for amplification of *amoA* gene in AOB was used (the AOB-plate), and in the second plate primers for amplification of *amoA* in AOA was used (the AOA-plate).

Standard curve 16S rRNA C_{DNA}	**Samples 16S rRNA**	**Standard curve *amoA* in AOA or AOB C_{DNA}**	**Samples *amoA* in AOA or AOB**
0.1 ng μ L^{-1}	PF-BEr-D151-R1	0.1 μ L^{-1}	PF-BEr-D151-R1
0.01 ng μ L^{-1}	PF-BEr-D151-R3	0.01 ng μ L^{-1}	PF-BEr-D151-R3
0.001 ng μ L^{-1}	Wellf-BHa-R1	0.001 ng μ L^{-1}	Wellf-BHa-R1
0.0001 ng μ L^{-1}	Wellf-BHa-R2	0.0001 ng μ L^{-1}	Wellf-BHa-R2
0.00001 ng μ L^{-1}	MF-BHa-D68-R1	0.00001 ng μ L^{-1}	MF-BHa-D68-R1
0.000001 ng μ L^{-1}	MF-BHa-D68-R3	0.000001 ng μ L^{-1}	MF-BHa-D68-R3
	NTC		NTC

qPCR was run in the qPCR instrument QuantStudio™ 5 Real-Time PCR System. It was performed with up to 40 cycles with the cycling temperatures as shown in Table 2.6. A melting point analysis was included. The same setup was used in a second qPCR run, but without melting point analysis. This was done to be able to analyze the qPCR products in an agarose gel (1.5 %).

Table 2.6: The temperature cycling steps for the qPCR.

Step	**Temperature [°C]**	**Time**	**Cycles**
Denaturation	95	10 min	
Denaturation	95	15 sec	
Annealing	53	30 sec	x 40
Elongation	72	30 sec	
Melt Curve	95	15 sec	
Melt Curve	60	1 min	
Dissociation	95	1 sec	

The qPCR data was analyzed by using the program QuantStudio^{TM} Design and Analysis Software v1.5.1 (AppliedBiosystems). The standard curves were made by calculating the copy number of the DNA in the templates according to Equation 2.1. The PCR products used as templates had a length of 123 basepairs for the *16S rRNA*[37], 491 basepairs for the AOB *amoA* and 629 basepairs for the AOA *amoA*[49]. The copy numbers were calculated according to Equation 2.1.

$$CN_{stock}[\frac{molecules}{\mu L}] = \frac{DNA_{conc}[\frac{g}{\mu L}] \cdot 6.022 \cdot 10^{23}[\frac{molecules}{mole}]}{DNA_{length} \cdot 660[\frac{g}{mole}]} \tag{2.1}$$

The C_t-values obtained for each qPCR for the standard curves were plotted as a function of the logaritmic copy number. The slope was determined by linear regression in Excel, and was used to calculated the amplification efficiency according to Equation 2.2.

$$\text{E } [\%] = (-1 + 10^{\frac{-1}{slope}}) \cdot 100 \tag{2.2}$$

3 Results

The microbial communities has been investigated during start-up of the marine biofilter at Havlandet RAS Pilot AS. The aim at Havlandet was to adapt the biofilter to higher salinity. Two strategies was performed to achieve this. Illumina sequencing of 16S rDNA amplicons was done during the two strategies of start-up and the nitrification was explored by manually inspection of the zOTUs classified as ammonium- or nitrite- oxidizers. To further investigate the ammonium oxidation in the biofilter, PCR with specific primers, Sanger sequencing and real-time PCR was performed.

3.1 Performance of the nitrification filter during start-up

Havlandet was starting a biofilter for use in a marine, pilot RAS with at 25 ppm salinity. The nitrifying biofilter had to adapt to higher salinity. To achieve the salinity adaptation, two strategies was performed. The first strategy performed is called the Maturation period (MP). The Maturation period was started with a commercial, liquid inoculum and new, clean biofilm carriers in the Maturation filter (MF). After the unsuccessful results from the Maturation period, the next strategy was performed. The next period, called the Poding period (PP), was started with addition of already matured biofilm carriers from Erko Seafood as biofilm inoculum. At Erko Seafood the biofilm inoculum was operated in a RAS biofilter at 15 ppm salinity. The biofilm inoculum was diluted with new, clean biofilm carriers at Havlandet in the Poding filter (PF).

Data for measured TAN, nitrite, nitrate and salinity in the Maturation filter and the Poding filter was delivered from Havlandet (Appendix A). The concentrations of nitrogenous products and salinity are shown in Figure 3.1. The Maturation period lasted from start-up at day 0 until day 137. As seen in Figure 3.1A it was a small transformation of the nitrogenous substances in the Maturation period. It was reported from Havlandet that the Maturation filter did almost not need addition of ammonium chloride. The unsuccessful Maturation period gave therefore rise to the Poding period. The production data until 99 days after start-up, as well as the nitrification rate, has earlier been presented and investigated in a student project [58]. After 68 days of start-up in the Maturation period it was concluded that the Maturation period was not adapted to higher salinity [58]. As seen in Figure 3.1 a high concentration of nitrite is observed in the Maturation filter (Day 115). This along with the low nitrification rate can indicate a inhibition of the nitrification.

After 137 days of start-up a new strategy was performed, the Poding period. Already matured biofilm carriers was mixed with new, clean biofilm carriers at Havlandet. The already matured biofilm carriers was used as biofilm inoculum in the Poding period. The biofilm inoculum was from a successful marine biofilter operated at 15 ppm salinity from another RAS facility at Erko Seafood. From Figure 3.1B one can see a rapid change in TAN in the Poding period. It was reported of a lot of feeding with ammonium chloride in the Poding filter to keep TAN higher than the lower reference area of the measuring instrument. This indicates a considerable higher nitrification rate in the Poding period relative to the Maturation period.

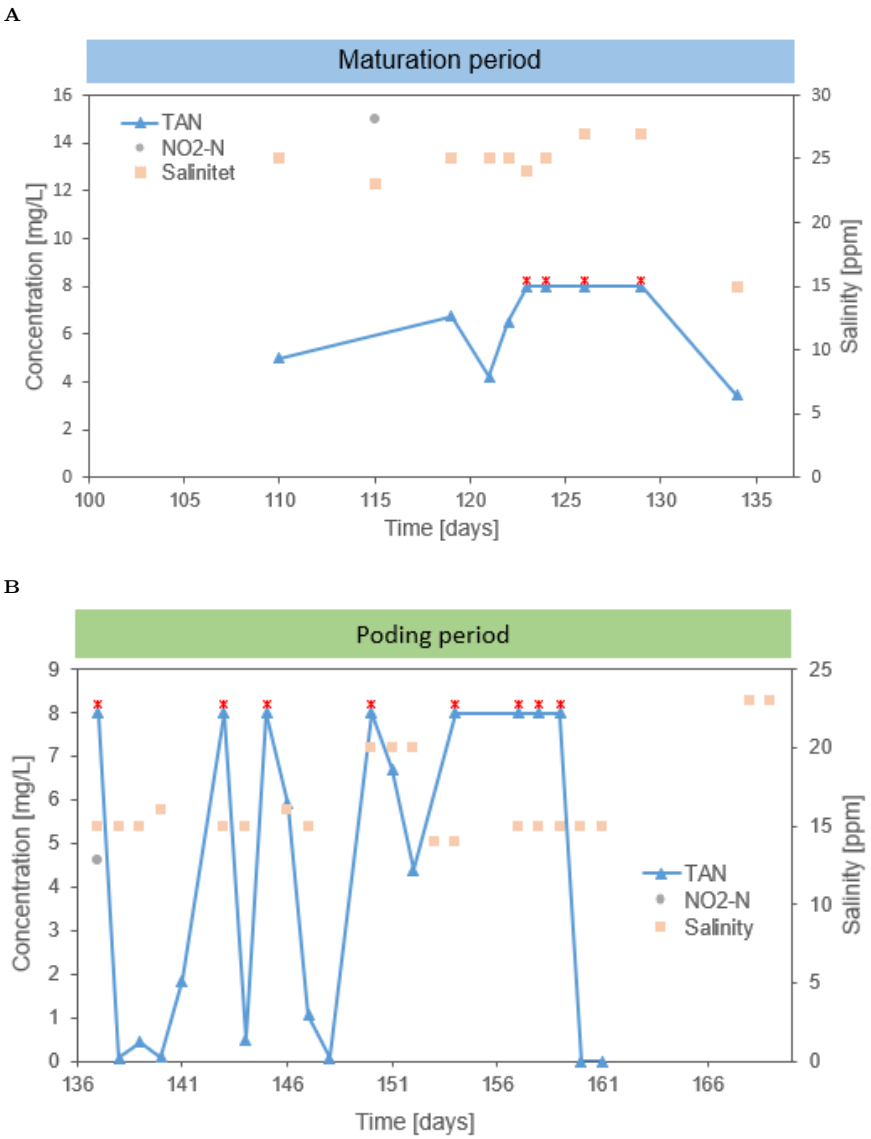

Figure 3.1: Measurements of TAN, nitrite and salinity in (A) the Maturation filter and (B) the Poding filter at Havlandet RAS Pilot during start-up. To adapt the biofilter to higher salinity (25 ppm) two different strategies were used, called the Maturation period and the Poding period. The Maturation period was started with a commercial, liquid inoculum and new, clean biofilm carriers at Havlandet. After lack of success in the Maturation period a new strategy for salinity adaptation was performed, the Poding period. The Poding period was started with already matured biofilm carriers from a successful RAS biofilter operating with 15 ppm salinity. The red marks indicates the upper reference area of the measuring instrument.

3.2 Microbial community analysis

During the Maturation period and the Poding period biofilm (B)- and water (W)-samples were collected for microbial community analysis (See Figure 2.1). It was also collected samples from a marine, well-functioning biofilter for cod production at Havlandet. This resulted in a total of 75 samples. The Illumina sequencing of the 16S rDNA amplicons resulted in a total of 4316 zOTUs and the zOTU-table was normalized to 19 000 reads per sample.

3.2.1 Alpha diversity

The alpha diversity was investigated by the observed zOTU richness, estimated zOTU richness (chao-1) and exponential Shannons index (Figure 3.2). The zOTU richness of the biofilm communities was higher in the Poding period compared to the Maturation period. The exponential Shannons index was approximately equal in the Poding period and in the Maturation period. The zOTU richness and the exponential Shannons diversity index was higher in the Poding period than in the Maturation period. The biofilm inoculum in the Poding period had a higher zOTU richness than the liquid inoculum in the Maturation period. Exponential Shannons diversity index was higher for the liquid inoculum compared to the biofilm inoculum. This indicates a lower evenness in the biofilm inoculum used in the Poding period relative to the liquid inoculum used in the Maturation period.

Both the well-functioning marine biofilter for cod production and the Poding filter were reported to have achieved successful nitrification (Section 3.1). The biofilm carriers from the well-functioning biofilter for cod production had a considerable lower zOTU richness and exponential Shannons diversity index than the biofilm carriers in the Poding filter.

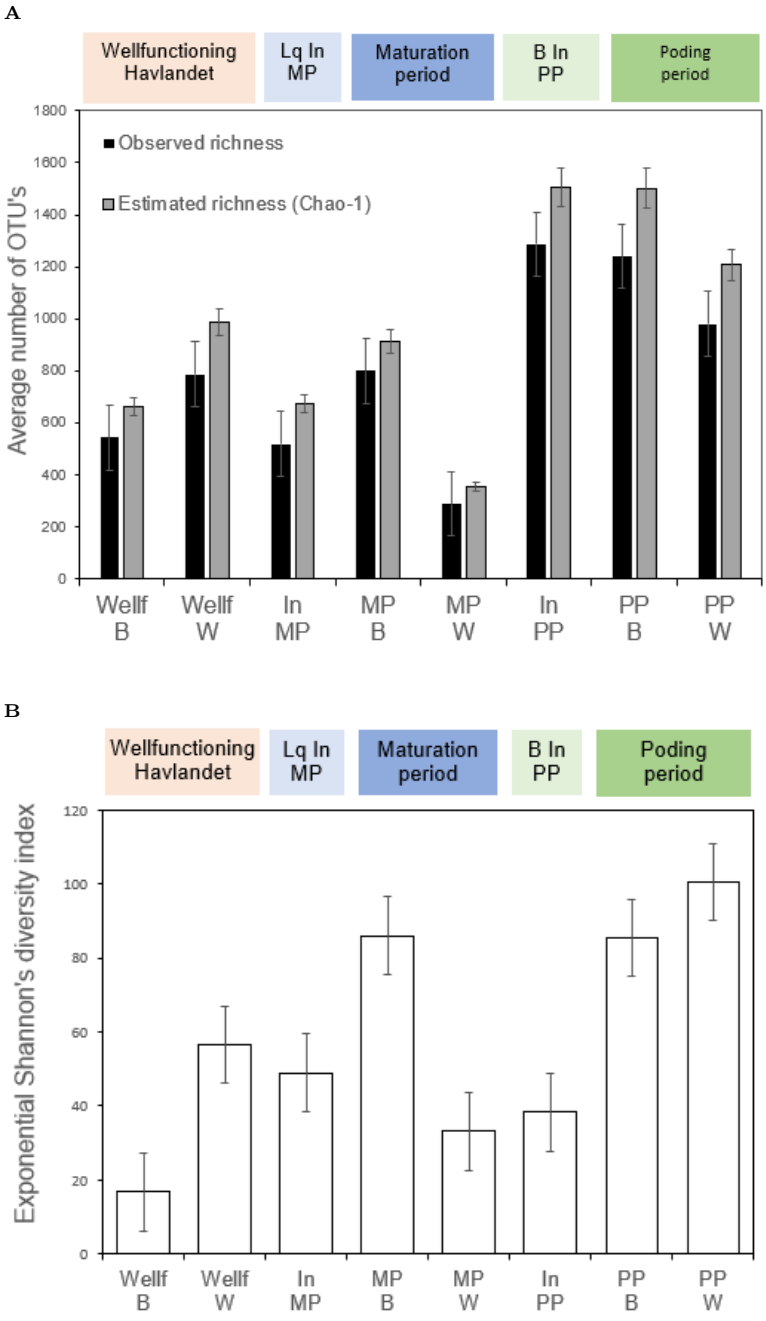

Figure 3.2: Average alpha diversity indices for biofilm and water samples: (A) Observed and estimated richness (Chao-1) and (B) exponential shannon's diversity index. The Maturation period lasted until day 137, and was started with clean biofilm carriers and a liquid, commercial inoculum. The Poding period started at day 137 when already matured biofilm carriers was diluted with clean biofilm carriers as biofilm inoculum. The alpha diversity of the inoculums and the well-functioning biofilter for cod production is also included. MP = Maturation period, PP = Poding period, B = biofilm, W = water, Lq = liquid, In = inoculum, Ha = Havlandet, Er = Erko and Wellf = well-functioning (for cod production).

3.2.2 Composition of bacterial communities

The bacterial community compositions at order level was determined for the 75 water- and biofilm-samples (Figure 3.3). There was a considerable difference in the microbial communities composition in the Maturation period relative to the Poding period. The most dominant orders through the Maturation period and the Poding period was *Sphingomonodales* (Up to 54 %), *Alteromonodales* (Up to 88 %), *Rhodobacterales* (Up to 75 %) and *Nitrospirales* (Up to 57 %).

In the Maturation filter including the liquid inoculum, with unsuccessful nitrification, there was not found any *Nitrospirales*. It was found a low abundance of the order *Nitrosomonodales* (Up to 5 %). The biofilm communities on the biofilm carriers in the Maturation period was highly distinct from the biofilm carriers in the Poding period. The microbial communities of the biofilm carriers in the Poding filter, both from Havlandet and the inoculum biofilm carriers from Erko, had a especially high abundance of the order *Nitrospirales*. The clean biofilm carriers from Havlandet got already after 3 days biofilm communities similar to the communities on the biofilm inoculum from Erko (Figure 3.3).

Both the well-functioning biofilter for cod production and the Poding filter has earlier been reported as successful nitrifying biofilters at higher salinity (Section 3.1). The microbial biofilm communities at the biofilm carriers from the two successful marine biofilters is highly distinct. The highly abundant order *Nitrospirales* in the Poding filter is not found in the well-functioning biofilter for cod production. It was found presence of the order *Nitrosomonodales* in both the Poding filter and the well-functioning biofilter for cod production.

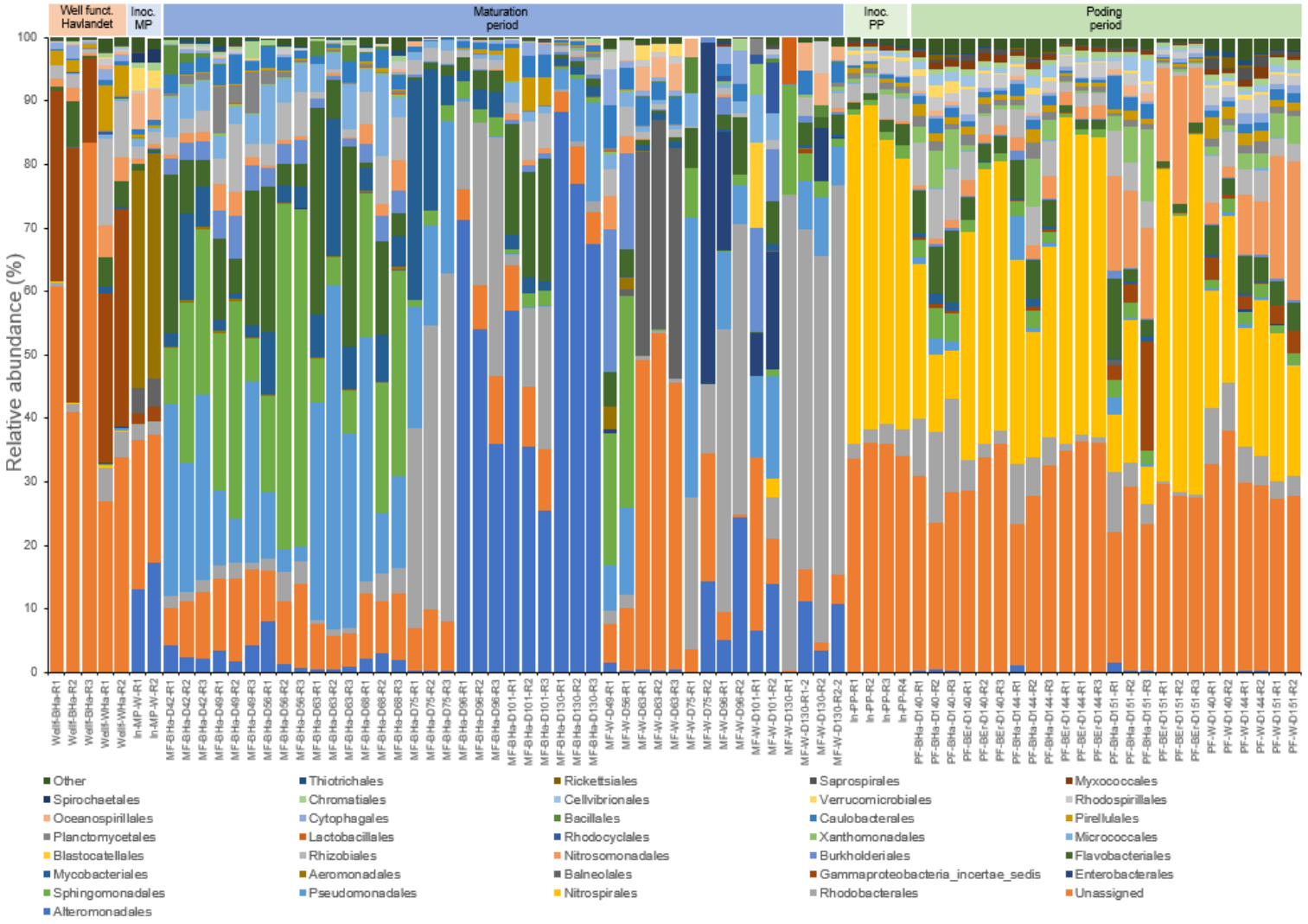

Figure 3.3: Microbial community composition at order level for biofilm (B)- and water(W)-samples from the Maturation filter (MF), the Poding filter (PF), the inoculum (In), the well-functioning biofilter for cod production (Wellf) at Havlandet (Ha) and the biofilm inoculum from Erko (Er) Seafood. It was taken biofilm-samples from Erko both before and after addition to the Poding filter. The biofilm from Havlandet was used both in the Maturation period and in the Poding period. At day (D) 137 already matured biofilm carriers was poded into clean biofilm carriers from Havlandet and started the Poding period. Most of the samples was analyzed with three replicates (R). Orders which contributed less than 1 % was included under "other".

3.2.3 Comparison of microbial communities in the Maturation period and the Poding period

To compare the microbial communities in water- and biofilm-samples from the Maturation period and the Poding period, the Bray-Curtis similarities was calculated in PAST. A PCoA-plot based on the Bray-Curtis similarities was made with the biofilm- and water-samples (Figure 3.4). There was seen a considerable difference in the microbial communities in the Maturation period, the Poding period and the well-functioning biofilter for cod production at Havlandet. A Permanova test confirmed that the biofilm communities in samples from the Maturation period was significant different than the samples from the Poding period (p=0.0001). In the Poding period, it was already after 4 days seen a microbial community similar to the inoculum biofilm on the carriers on Havlandet. It was reported two successful biofilters at higher salinity: The Poding filter and the well-functioning biofilter for cod production. These two marine biofilters had significant different microbial communities in the biofilm (p=0.0007).

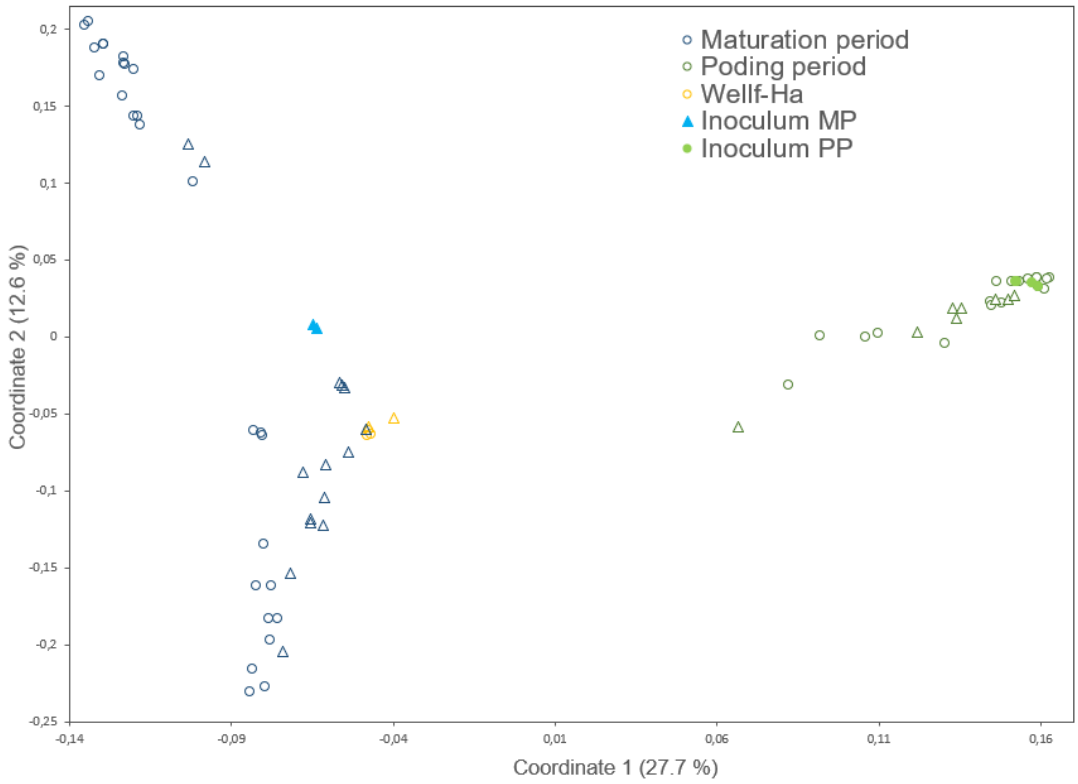

Figure 3.4: PcOA-plot based on the calculated Bray-Curtis similarities of biofilm- and water-communities during the Maturation period and the Poding period. The liquid inoculum used in the Maturation period (MP) and biofilm inoculum used in the Poding period (PP) is plotted as well. Samples from a well-functioning (Wellf), marine biofilter at Havlandet (Ha) is also plotted. △: Water-samples; ○: Biofilm-samples.

The Bray-Curtis similarities in the Poding period was calculated to further investigate the biofilm communities in the Poding period (Figure 3.5). At day 130 the biofilm carriers from the Maturation filter was compared with those from the Poding filter. The biofilm carriers at day 137 was represented by the biofilm inoculum used in the Poding period. The biofilm communities at the biofilm carriers from Havlandet changed more with time than the biofilm carriers from Erko Seafood (Figure 3.5A).

The microbial communities at Havlandet changed more from day 144 to 151 than from day 140 to 144 (Figure 3.5A). This is probably because of the longer time interval. Generally the biofilm carriers from Havlandet had a larger change in the microbial communities than the biofilm carriers from Erko Seafood. This indicates a successful Poding period.

Figure 3.5B shows the Bray-Curtis similarity between the carriers from Havlandet relative to the carriers from Erko Seafood (PF-BHa vs PF-BEr) and the microbial similarity of the three different replicates at Havlandet (PF-BHa) or at Erko (PF-BEr). The microbial communities on the carriers from Havlandet relative to the carriers from Erko was completely different in the Maturation filter compared to the Poding filter (D130/D137, Figure 3.5B). The Bray-Curtis similarity of the biofilm carriers at Havlandet was decreasing from day 140 to 151. This could be because of evenly dilution of biofilm carriers from Havlandet (see Figure 2.1) to increase the biofilter capacity. The increasing Bray-Curtis similarity of the biofilm carriers from Havlandet versus Erko from day 140 to day 144 indicates that the microbial communities on the new biofilm carriers at Havlandet changes fast into microbial communities similar with the carriers from Erko (D140 to D144, Figure 3.5B).

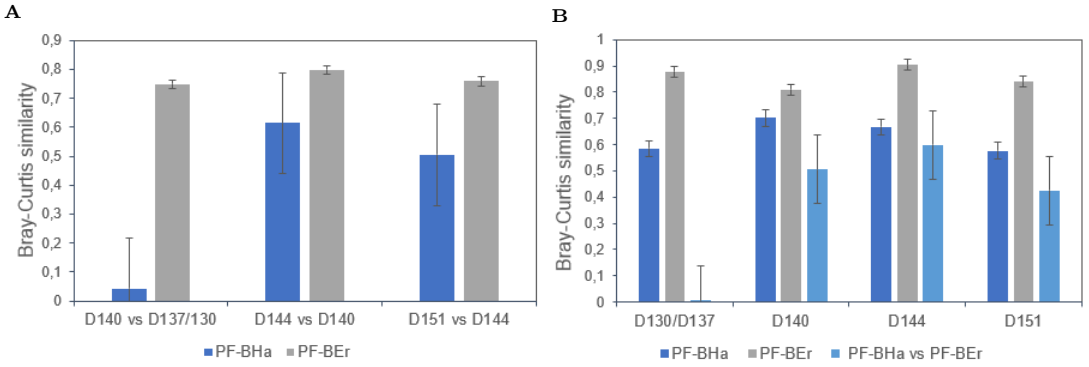

Figure 3.5: Bray-Curtis similarity in the Poding filter after addition of matured biofilm carriers from Erko at day 137. Comparison of microbial biofilm communities between sampling times for each of the carriers from Erko and Havlandet. **(A)** At different sampling points from Havlandet or Erko and **(B)** at same sampling points from Havlandet and/or Erko with comparison of the biofilm carriers from Havlandet relative to the biofilm carriers at Erko. The biofilm carriers at day 137 was the biofilm inoculum that was poded into the Poding filter. The biofilm carriers from day 130 was taken from the Maturation filter. D = day, PF = poding filter, B = biofilm, Ha = Havlandet and Er = Erko Seafood.

3.2.4 Abundance of nitrifying bacteria in biofilm communities

By manual inspection of the zOTU table, zOTUs classified as potential nitrifying bacteria using the MiDAS or RDP reference data set was investigated. The nitrifiers was classified on order or family level. The two successful biofilters, the well-functioning marine biofilter for cod production and the Poding filter, has considerable different microbial communities and different zOTUs is found (Figure 3.6). It is considerable more nitrifiers in the Poding filter than it is in both the well-functioning biofilter for cod production and in the Maturation filter. It was found low abundance of nitrifiers in the Maturation filter (Figure B.1, Appendix B). In the Poding filter it was found a high abundance of zOTUs classified as *Nitrospira*, and the occurence of zOTU1 was very high (Figure 3.6). It was found approximately 51 % of zOTU1 in the biofilm carriers from Erko at day 137 (PF-BEr-D137-R1/-R2). It was also found very low abundance of potential ammonium oxidizing bacteria in the Poding filter.

In the well-functioning marine biofilter for cod production there was found considerable less zOTUs classified as ammonium- or nitrite-oxidizers (Figure 3.6). It was also found different zOTUs in the well-functioning biofilter for cod production compared to the Poding filter. zOTU1 classified as *Nitrospira* was also found in the well-functioning marine biofilter for cod production, but in lower occurence. It was found more zOTUs classified as ammonium oxidizers in the well-functioning marine biofilter for cod production compared to the Poding filter (Figure 3.6).

The low abundance of zOTUs classified as ammonium oxidizers and the presence of *Nitrospira* in the Poding filter (Figure 3.6) can indicate the presence of a complete ammonium oxidizer, comammox. To further investigate this a phylogenetic analysis was performed in MEGA, where highly abundant *Nitrospira*-zOTUs was compared with well described, known comammox *Nitrospira*. A phylogenetic three was made (Figure 3.7). The three does not show near relationship with any known comammox. The highly abundant zOTU1 is most related to the species *Nitrospira salsa* KC706459. In addition all zOTUs classified as *Nitrospira* in the Poding filter is related to *Nitrospira* from marine environments.

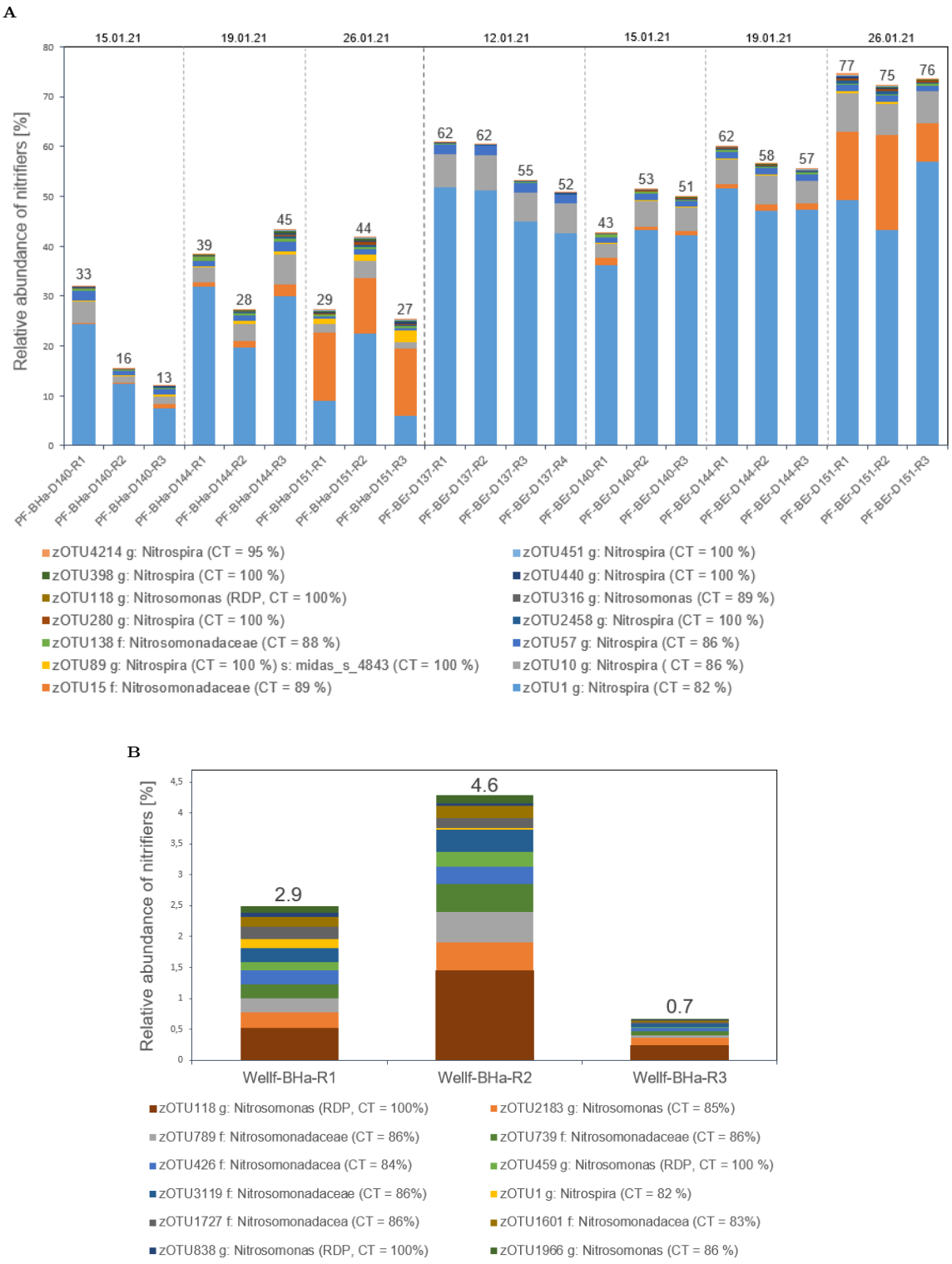

Figure 3.6: Relative abundance of zOTUs potentially representing nitrifiers in the biofilm samples. The biofilm nitrifiers in two different successful, marine biofilters; **(A)** The Poding filter and **(B)** the well-functioning biofilter for cod production. A taxonomy with a confidence treshold (CT) of 80 % or higher at family- (f) or genus- (g) level is included. All the samples is given with minimum 2 replicates. The zOTUs is classified using the two databases MiDAS and RDPv18. The highest achieved confidence treshold is represented, and RDP is marked for the cases where RDPv18 database is used. PF = Poding filter, B = biofilm, Ha = Havlandet, R = replicate, D = day, W = water, Er = Erko, Wellf = well-functioning (for cod production) and CT = confidence treshold.

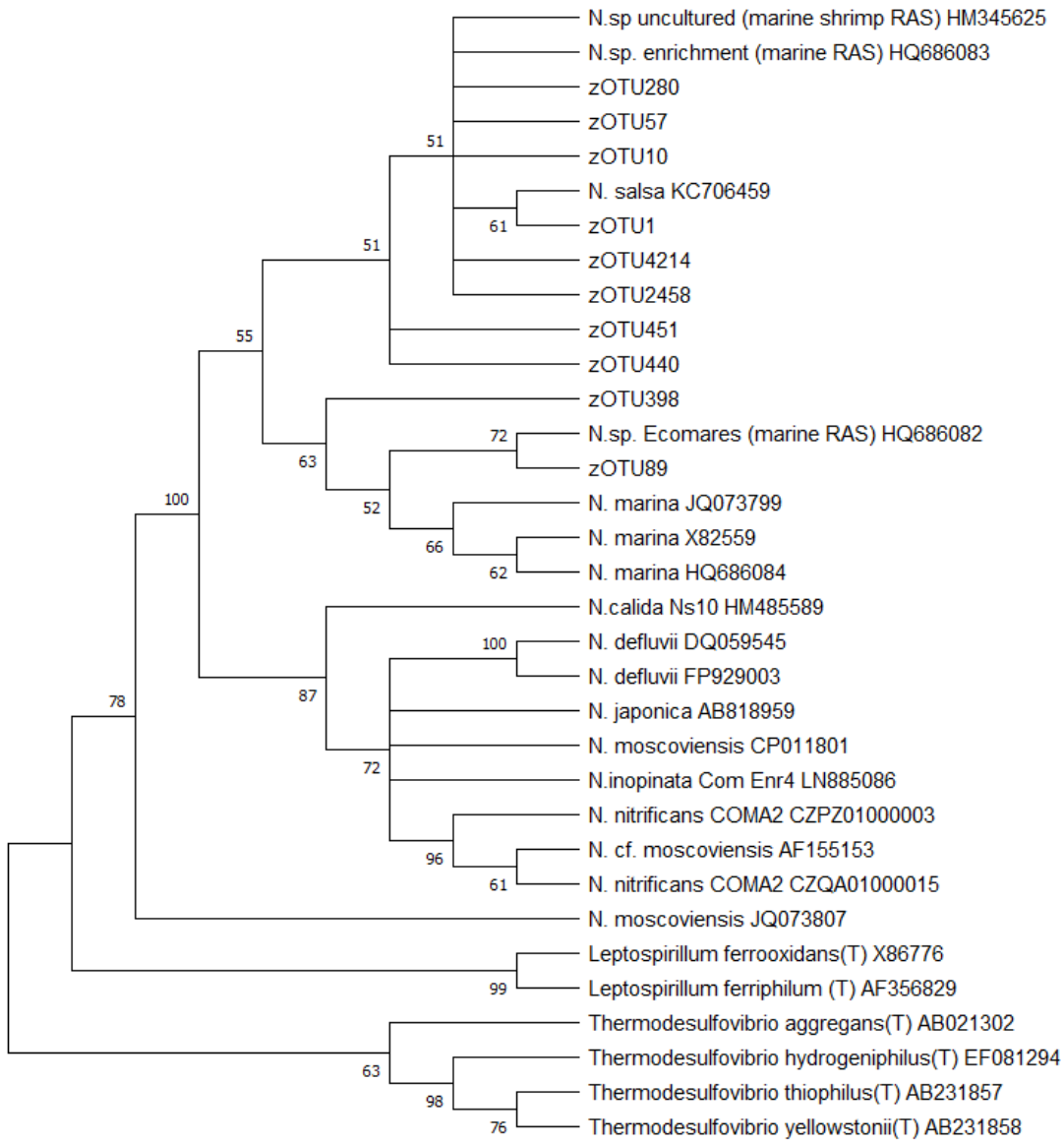

Figure 3.7: Maximum likelihood three for evolutionary analysis of zOTUs classified as *Nitrospira* and *16S rRNA* gene sequences from previously described *Nitrospira*, representing both comammox and marine *Nitrospira*. Sequences were retrieved from RDP database or the NCBI Genbank. Accession numbers are specified for with the species names. Type strains are indicated by a (T). The analysis was performed in MEGA-X version 10.2.4. First, the sequences were aligned using ClustalW with the default parameters. Next, maximum likelihood analysis was performed with 1000 bootstrap replicates and the Tamura-Nei model for sequence evolution. The three was condensed with 50 % cut-off value, and bootstrap support values are shown at the nodes, and a indicate reliability of clusters descending from that node. The three includes representatives for the other genera included in *Nitrospiraceae* familiy (*Thermodesulfovibrio* and *Leptospirillum*). The three is rooted at the *Thermodesulfovibrio* node.

3.3 Ammonium oxidizers during start-up of the marine nitrifying biofilter

To investigate what kind of microbes who performed the ammonium oxidation in the biofilters PCR with *amoA* specific primers, Sanger sequencing and qPCR was performed (See Section 2.3) on a selection of samples representing; Biofilm carriers from Erko in the Poding filter at day 151 (PF-BEr-D151), biofilm carriers from the well-functioning biofilter for cod production at Havlandet (Wellf-BHa) and biofilm carriers from the Maturation filter at day 68 (MF-BHa-D68).

3.3.1 PCR amplification of the *amoA* gene

PCR with specific primers for the *amoA* gene (Table 2.3) was performed to investigate the potential presence of AOB, AOA and comammox in the biofilm communities (Figure 3.8). The PCRs indicated presence of both AOA and AOB in the samples from the biofilm inoculum from Erko. It was also found presence of AOB and AOA in the well-functioning biofilter for production of cod at Havlandet. The results indicated that AOAs or AOBs were not present in the Maturation filter at day 68.

The PCR amplification with primers targeting the *amoA* gene in comammox did not give any product for the samples from the Maturation filter at day 68 or the well-functioning biofilter for production of cod. This indicated that comammox *Nitrospira* not was present in the samples. For the biofilm samples of the inoculum carriers in the Poding filter at day 151 it was achieved an unspecific PCR product of various sizes with the primers targetting comammox clade B *amoA* (PF-BEr-D151, Figure 3.8D). In addition to the unspecific products, a band at expected length (415 bp) was observed.

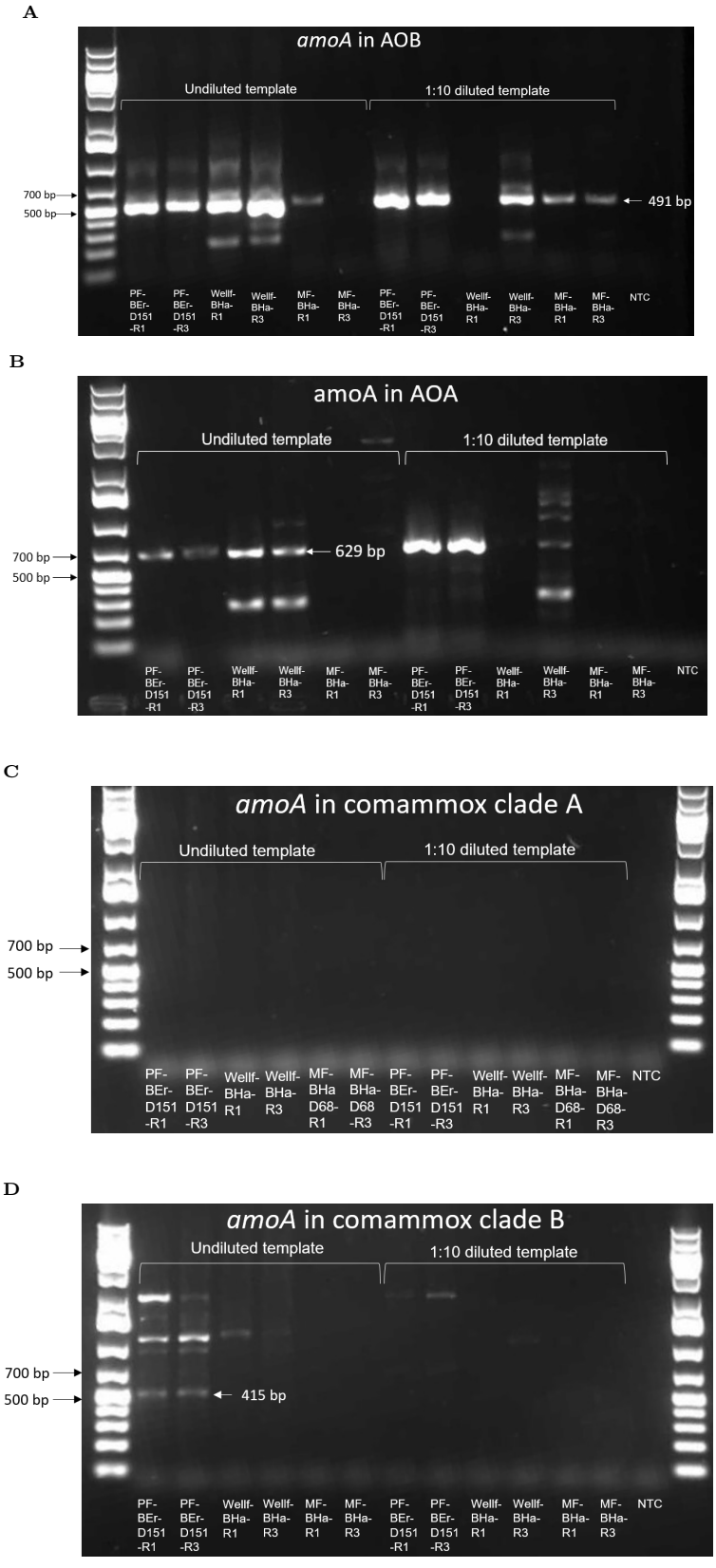

Figure 3.8: Agarosegel (1 %) with PCR products generated with primers targeting the *amoA* gene of AOBs (A), AOAs (B), comammox clade A (C) and comammox clade B (D). PF = Poding filter, B = Biofilm, Er = Erko Seafood, D = Day, R = replicate, Wellf = well-functioning (for cod production) and Ha = Havlandet.

The Sanger sequencing of the PCR product obtained with the AOA *amoA* primers for the biofilm samples (Erko) from the Poding filter resulted in high quality sequences (Figure D.1) and indicated that the template was dominated by a single DNA sequence. A Nucleotide Blast search (NCBI) showed that the sequence was 100 % identical to the *amoA* gene of *Nitrosopumilus oxyclinae* that has been classified as AOA. The Sanger sequences for the PCR products obtained with the AOB *amoA* primers for the Poding filter sample resulted in a messy chromatogram (Figure D.2), indicating that the PCR product consisted of several *amoA* sequences, and that there might be several different AOBs present in the biofilm.

The Sanger sequences obtained for the AOB PCR products obtained with the AOB *amoA* primers for the samples representing the well-functioning biofilter for cod production was of relative good quality, but in some positions there where double signals (Figure D.3), which might indicate that there was several AOBs present. The parts of the sequence that was most reliable was used in a Nucleotide Blast search which showed that the sequence had 98 % identity to the *amoA* sequence of a cultured strain of *Nitrosomonas*. This corroborates the ammonium oxidizers found by manual inspection of zOTUs representing nitrifiers in the well-functioning biofilter for cod production (Figure 3.6B).

3.3.2 Quantification of the *amoA* gene by using real-time PCR

qPCR was performed for further quantification of the *amoA* gene in AOBs and AOAs in samples representing the Poding filter (PF), the well-functioning marine biofilter for cod production (Wellf-Ha) and the Maturation filter (MF). qPCR was performed in two plates; one for AOA *amoA* and one for AOB *amoA*. Standard curves were made with primers targeting the *16S rRNA* gene and the *amoA* genes in each plate to determine the relationship between C_T-values and copy numbers of the target DNA. The standard curves for 16S rDNA on the AOB-plate and on the AOA-plate is shown in Figure 3.9A and Figure 3.9C, respectively. The standard curves made for the PCR amplicon of the *amoA* gene from AOB and AOA are shown in Figure 3.9B and Figure 3.9C, respectively.

Based on the linear regressions, the amplification efficiencies were calculated by Equation 2.2. The calculated amplification efficiencies is shown in Table 3.1. The amplification efficiencies for the *16S rRNA* qPCR product was found to be 79 % and 78 % for the AOB- and AOA-plate, respectively. This is lower than expected, and can come from to high template concentrations. The amplification efficiencies for the *amoA* qPCR product was found to be 91 % and 84 %, for AOB and AOA respectively. This is lower than expected as well. The low amplification efficiencies indicates not optimal PCR conditions. The amplification curves is shown in Figure E.1.

Table 3.1: Amplification efficiencies of the standard curves during real-time PCR.

Plate	PCR amplification of *16S rRNA* gene	PCR amplification of *amoA* gene
AOB	79 %	91 %
AOA	78 %	84 %

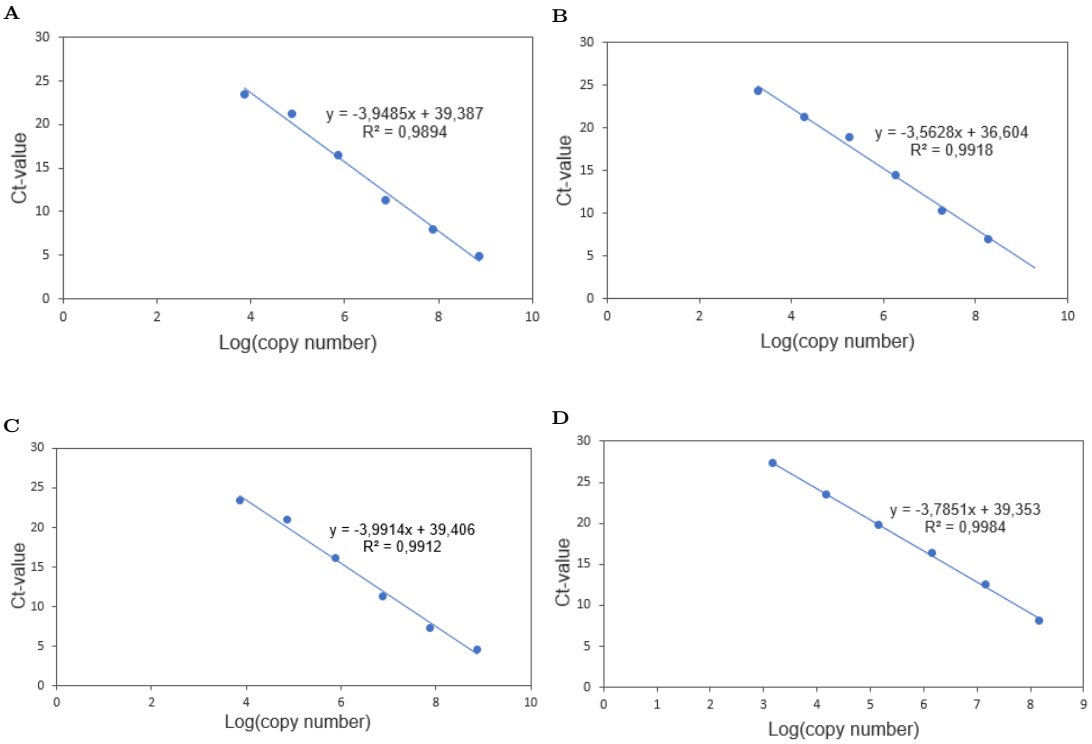

Figure 3.9: Standard curves based on two plates with primers targeting *16S rRNA* gene in the AOB-plate (A) and in the AOA-plate (C), and standard curves with specific primers targeting the *amoA* gene in AOB (B) and AOA (D). The copy number was calculated using the known DNA concentrations, and the logaritmic copy number was plotted against the measured C_t-value. The *16S rRNA* standard curves were based on a dilution series of a 123 basepair long PCR product of known concentrations of the *16S rRNA* gene using a *Yersinia* strain as template in qPCR. The standard curves for *amoA* qPCR products were based on PCR products from specific DNA extracts.

The standard curves were used to determine the copy number of the PCR amplicons of the *16S rRNA* and *amoA* genes. The copy numbers of the *amoA* gene was thereafter normalized by using copy numbers for the *16S rRNA*. It was assumed a average of 4.2 copies of the *16S rRNA* gene per microbe cell[65]. Using this approach, the copy numbers of the *amoA* genes from AOA and AOB per microbe cell was estimated (Table 3.2). Generally, it was found lower abundance of the AOA *amoA* gene than the AOB *amoA* gene. The highest abundance of AOA *amoA* gene was found in the samples from the Poding filter with a maximum abundance of 0.026 per cell (Table 3.2). There was found a considerable higher abundance of the AOB *amoA* gene in one of the samples (Wellf-BHa-R1) from the well-functioning biofilter for cod production than in any other of the samples (Table 3.2).

Table 3.2: The estimated number of AOA and AOB gene copies per microbial cell in biofilm samples. It is assumed 4.2 copies per cell of 16S rDNA [65]. PF = Poding filter, Er = Erko, D = Day, R = replicate, Wellf = well functioning, B = biofilm, Ha = Havlandet and MF = Maturation filter.

Sample	**Copy number per cell** AOA	**Copy number per cell** AOB
PF-Er-D151-R1	0.02620	0.1907
PF-Er-D151-R3	0.00487	0.1638
Wellf-BHa-R1	$1 \cdot 10^{-4}$	0.3352
Wellf-BHa-R2	$3 \cdot 10^{-5}$	0.1104
MF-BHa-D68-R1	-	0.0122
MF-BHa-D68-R3	0.0015	0.0483

The products after the qPCR was analyzed either by melting point analysis or agarose gel electrophoresis. The melting point analysis (Figure 3.10) indicates generally a specific PCR amplification with some exceptions. The melting curves for the 16S rDNA amplicons from samples representing the Poding filter indicates that two PCR products were generated (Figure 3.10C and 3.10A) in both of the two plates. The melting point curves from the amplification of *amoA* gene in AOB (Figure 3.10B) has a clear peak for the samples representing the Poding filter and the well-functioning biofilter for cod production. This indicates a PCR amplification resulting in one specific product. The melting point analysis for the AOB *amoA* product from samples representing the Maturation filter indicated an unspecific PCR amplification because of the formation of more than one peak (Figure 3.10B). The AOA *amoA* qPCR product for the samples from the Poding filter shows a clear, specific PCR amplification unlike the samples from the well-functioning biofilter for cod production (Figure 3.10D). The specific qPCR product further indicates that the product represents *amoA* AOA.

The qPCR products were analyzed by agarose gel electrophoresis (Figure 3.11). The qPCR products were of the expected lengths. This corroborates the results from the melting point curves. However, the amplification of the *amoA* gene in AOA in the samples representing the well-functioning biofilter for cod production had two weak results at different DNA lengths (Wellf-BHa-R1, Figure 3.11B). This indicates some unspecific amplification. An unspecific amplification of the *amoA* gene in AOA in the wellfunctioning biofilter for cod production was also observed from the melting point analysis after the qPCR (Figure 3.10D).

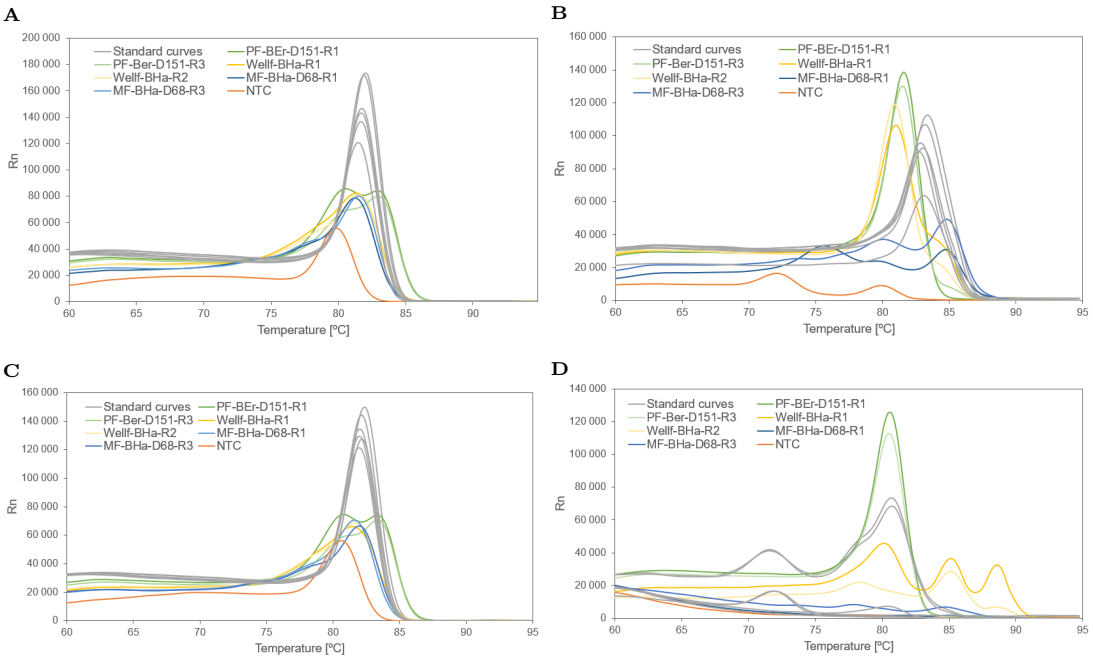

Figure 3.10: Melting point curves after qPCR of the *16S rRNA* gene on the AOB-plate (A) and the AOA-plate (C), and the *amoA* gene in AOB (B) and AOA (D). The average of three parallels for each sample is used. PF = Poding filter, Er = Erko Seafood, B = biofilm, D = day, Ha = Havlandet, R = replicate and Wellf = well-functioning (for cod production).

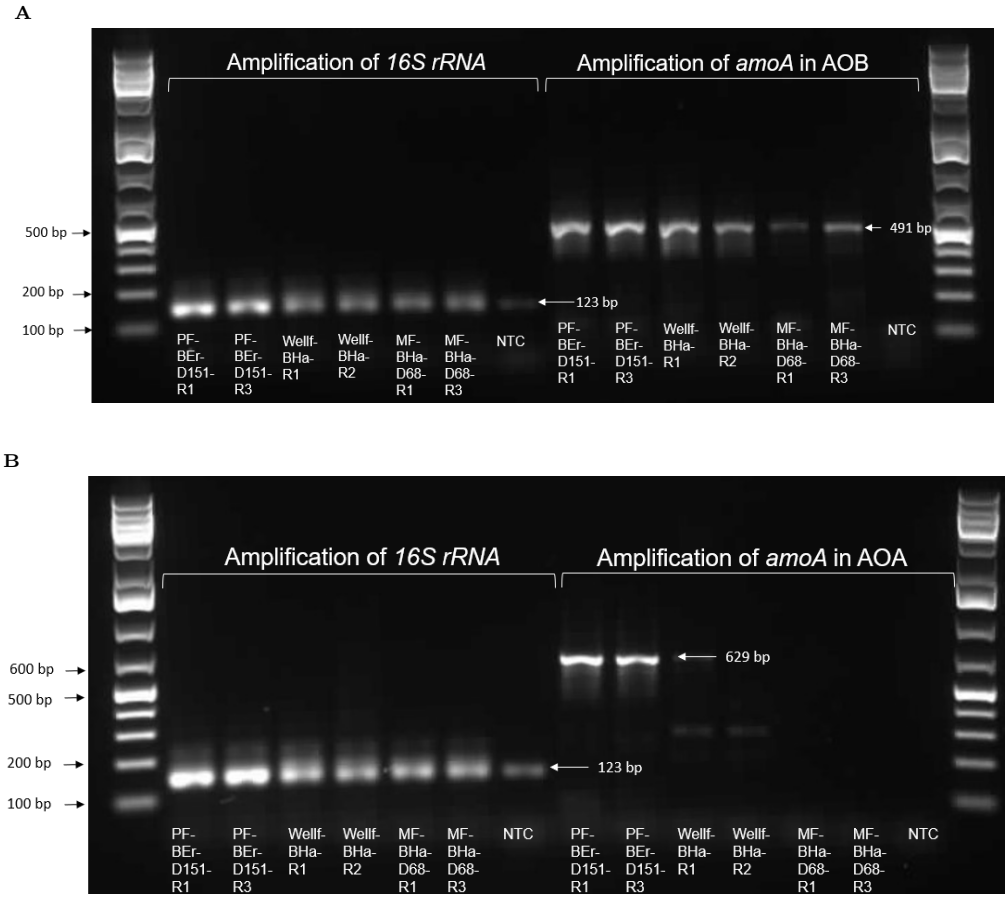

Figure 3.11: Agarosegel (1.5 %) with qPCR products generated with primers targeting the *16S rRNA* and the *amoA*-gene in AOB (A) and AOA (B). PF = Poding filter, B = Biofilm, Er = Erko Seafood, D = Day, R = replicate, Ha = Havlandet and Wellf = well-functioning (for cod production).

4 Discussion

4.1 Nitrification activity in the Maturation period and in the Poding period

The data delivered from Havlandet indicated that the nitrification activity was higher in the Poding filter compared with the Maturation filter (Figure 3.1). Firstly, the Maturation period, with start-up with clean biofilm carriers and a liquid inoculum, did not achieve nitrification and a low nitrite oxidation was reported from Havlandet. The Maturation filter could only convert low levels of ammonium chloride (personal communication, Fredrik Grahl-Jacobsen at Havlandet). The concentration of TAN was increasing with the addition of ammonium chloride (Day 110-119, Figure 3.1A). The concentration of TAN was decreasing 130 days after start-up (Day 135, Figure 3.1A). This can indicate a better nitrification later in the Maturation period. The nitrite concentration was measured only at day 115, and was as high as 15 mg L^{-1}. This can lead to nitrite inhibition of both the AOBs and NOBs [8]. It has earlier been found high levels of ammonium oxidizing genes compared with nitrite oxidizing genes in a nitrifying bioreactor started with commercial inoculum meant for RAS biofilters [31]. In another study it was found that the nitrifying inoculum had no effect on the nitrification rate [38], and that most of the nitrifying bacteria in a marine, nitrifying biofilter originated from environmental seawater and not from the commercial inoculum [5]. Anyway, the low nitrification activity in the Maturation period corroborates with the earlier lab-scale batch experiments performed with biofilm carriers from the Maturation filter [58].

The nitrifying biofilter in the Poding period at Havlandet, inoculated with biofilm inoculum, had high nitrification efficiency already at the start. Although the biofilm inoculum (1.5 m^3) was added to a larger volume of clean biofilm carriers (5 m^3), the biofilter had a high nitrification efficiency already from the first day in the Poding filter. The Poding filter was able to transform much more ammonium (personal communication, Fredrik Grahl-Jacobsen at Havlandet). TAN was quickly transformed after addition (Figure 3.1B). This indicates high nitrification activity where the nitrifying bacteria was active as long as the substrate was available. This strategy for fast start-up of a marine biofilter may have a huge potential. Compared to earlier studies where it took 60 days for adaptation of a freshwater reactor into saline conditions [42], it could be time saving for the aquaculture industry to start-up marine biofilters using already matured biofilm carriers as inoculum. The use of commercial inoculums is used a lot in the industry, but several studies have shown both failure and success with this start-up strategy of biofilters at higher salinity [26] [31]. This strategy by poding in biofilm inoculum in the biofilter can increase the success rate of start-up of marine biofilters.

The biofilm carriers from Erko was poded into the Poding filter at day 137. The Erko biofilm originated from a successful biofilter operated at 15 ppm salinity. The salinity was kept relative constant at 15-16 ppm in the first period in the Poding filter (Day 137-147, Figure 3.1B). Thereafter the salinity was increased to approximately 20 ppm at day 149. The TAN concentration was high at day 155 to day 158 (Day 155-158, Figure 3.1B). This indicates less convertion of TAN, and less nitrification. The sudden increase in salinity can give the cells shock [18], and the cells consequently needed time for adaptation to the new salinity [51]. The biofilm adapted in this case from 15 ppm to 20 ppm salinity in three days, so the biofilm adapted quickly to the change in salinity. To summarize, the Poding filter had a high nitrification efficiency already a day after addition of the biofilm inoculum, and the Poding filter adapted fast to the new conditions at higher salinity.

4.2 Microbial community analysis

The results from the Illumina sequencing indicates considerable different microbial communities in the three biofilters investigated: The well-functioning, marine biofilter for cod production, the Maturation filter and the Poding filter. The alpha diversities (Figure 3.2) indicates a higher diversity in the Poding filter than in the two other biofilters. A PcOA plot (Figure 3.4) and the microbial compositions (Figure 3.3) also shows considerable differences in the community compositions between the three biofilters.

The significant difference in the microbial communities representing the Poding filter and the well-functioning biofilter for cod production ($p=0.0007$) showed in the PcOA plot (Figure 3.4) indicates that the microbial communities of marine, nitrifying biofilters can be highly different, but still be successful. The PcOA plot also indicates that the biofilm communities in the well-functioning biofilter for cod production has a more similar microbial community as the Maturation filter. However, the microbial compositions at order level (Figure 3.3) shows a different picture, and the microbial communities seems to be very different in all of the three biofilters. The zOTUs potentially representing nitrifiers (Figure 3.6) in the Poding filter and the well-functioning biofilter for cod production also shows a considerable difference in the relative abundances. In the well-functioning biofilter for cod production the abundances of zOTUs representing nitrifiers was much lower, and it was seen a overweight of ammonium oxidizers. In comparison, samples representing the Poding filter (Figure 3.6A) had a much higher abundance of nitrifiers. The alpha diversities corroborates the differences between the two successful biofilters, and the well-functioning, marine biofilter for cod production has low exponential Shannons diversity index and richness compared to the Poding filter (Figure 3.2).

As mentioned earlier, nitrification was not achieved during the Maturation period and the period was therefore considered as unsuccessful (Figure 3.1A). The alpha diversities from the biofilm samples in the Maturation filter indicates a high evenness in these samples (MP-B, Figure 3.2). The populations has thus quite similar abundance in the biofilm microbial communities. This corroborates with the observation of approximately equal abundances at order level (Figure 3.3) in the biofilm until day 96 in the Maturation filter. It was observed a change in the microbial community at this point, and the abundance of the order *Alteromonadales* increased. This can explain the especially low nitrification activity reported at increasing TAN concentrations at 110 days after start-up (Day 110, Figure 3.1A). It was also reported a low abundance of zOTUs potentially representing ammonium or nitrite oxidizers in the Maturation period (Figure B.1). The higher abundance of zOTUs representing ammonium oxidizers than nitrite oxidizers corroborates with earlier observations indicating nitrite inhibitions in the same period [58]. It was not observed any zOTUs representing nitrite oxidizers in the liquid, commercial inoculum used in the Maturation period. Therefore, the liquid inoculum which was used for the Maturation filter did probably not contribute with nitrite oxidizing bacteria. This can lead to nitrite inhibition [8]. The most abundant nitrifying zOTU in the Maturation filter was zOTU37, classified as *Nitrosomonas*, with a relative abundance on up to 3.7 % of the biofilm. This ammonium oxidizing bacteria had increasing abundance in the start of the Maturation period (Figure B.1), but the abundances decreased later in the Maturation period. This can be because of nitrite accumulation, which probably led to nitrite inhibition. The nitrifying zOTUs from the inoculum is not seen after 75 days of start-up in the Maturation filter. This agrees with an earlier study, where a nitrifying biofilter started up with seawater and a commercial, liquid inoculum was investigated [5]. The active nitrifying bacteria in the biofilter was from the environmental seawater, and not from the commercial inoculum [5]. In another study regarding start-up of a marine biofilter, it was found earlier formation of nitrate when biofilm inoculum was used compared with liquid inoculum [48].

The Poding period, which was inoculated with biofilm inoculum from another RAS operated at 15 ppm salinity, achieved successful nitrification after mixing of biofilm inoculum with the clean biofilm carriers. The microbial communities of the biofilm inoculum had a low exponential Shannons diversity and a high richness (Figure 3.2). This indicates a low evenness, and therefore a high abundance of a few populations in the biofilm community. In an earlier study a MBBR with brackish water had lower diversity than a freshwater MBBR [42]. The low evenness was further observed for the community composition at the order level where the order *Nitrospirales* was highly abundant (Figure 3.3). This is also seen at zOTU-level where zOTU1 (*Nitrospira*) had a surprisingly high abundance of up to 51 % of the total reads. This zOTU is further discussed in Section 4.3. The beta diversities in the Poding filter (Figure 3.5) indicates that the microbial communities on the carriers from Havlandet already after only a few days developed a biofilm community similar as the one on the biofilm inoculum from Erko. An earlier study of start-up of marine biofilters in RAS shows that addition of biofilm inoculum was the best method for fast and efficient nitrification [48].

The relative abundance of nitrifiers in the well-functioning biofilter for cod production on up to 4.6 % was considerable lower than the relative abundance of nitrifiers in the Poding filter which was up to 77 %. Anyway, both of the nitrifying biofilters were reported as successful at Havlandet. So, it appears that a successful nitrifying biofilter in RAS do not need to have high abundances of nitrifiers to achieve an effective nitrification. It could be interesting to compare the nitrification capacity of these two biofilters. It can be conceivable that the Poding filter has a higher nitrification capacity than the well-functioning biofilter for cod production.

It should also be mentioned that the relative abundance of nitrifiers on the carriers from Erko used at biofilm inoculum in the Poding filter was increasing from approximately 60 % at day 144 to approximately 76 % at day 151 (Figure 3.6A). The main contributor to this increase was zOTU15 (the AOB *Nitrosomonadaceae*) which had a relative abundance on approximately 1 % at day 144 and 14 % at day 151. The salinity was increased from 15 ppm to 20 ppm salinity at around day 148. In an earlier study on a nitrifying biofilter in a RAS with smoltified salmon the salinity was increased to investigate the salinity impact on the microbial communities in the biofilter [16]. The salinity increase from 6 to 18 ppm gave a relative abundance increase of a OTU representing *Nitrosomonas* from around 3 % to around 8 % [16]. However, the relative abundance of the same OTU (*Nitrosomonas*) was low in a RAS operated at 28 ppm salinity constantly [16]. In both of the RAS units the *Nitrosomonas* was in a community with OTUs representing *Nitrospira* [16]. Another study investigates the microbial communities in the Dutch coastal North Sea water [20]. It was found indications of halophilic or halotolerant *Nitrosomonas* lived in communities with *Nitrospira* [20]. The highly abundant zOTU1 in the Poding filter is closely related (Figure 3.7) to the strain of *Nitrospira* in this study [20]. It is therefore conceivable that zOTU15 is halophilic or halotolerant, and closely related to *Nitrosomonas* found in the dutch coastal North Sea water [20].

4.3 Nitrospira

One specific zOTU, representing *Nitrospira*, dominated the biofilm inoculum and the samples representing the Poding filter generally (Figure 3.6A). zOTU1 constituted up to 51 % of the Poding filter biofilm comunities on the biofilm inoculum from Erko (Figure 3.6A). zOTU1 was highly abundant on the biofilm on the carriers from Havlandet after only a few days after addition to the Poding filter. This indicates a *Nitrospira* strain able to quickly form biofilm even when the concentration of nitrite is very low, but a higher concentration of TAN. It was also seen a low relative abundance of zOTUs representing ammonium oxidizers in the Poding filter (Figure 3.6A). One possible explanation of this observation can be that zOTU1 represents a commamox *Nitrospira*, and therefore performes the whole oxidation from ammonium to nitrate. In an earlier study, the abundance of comammox *Nitrospira* is found to negative correlate with high concentrations of Nitrite and TAN [57]. Another study about marine biofilters in RAS for post-smolt production also observed a low AOB:NOB ratio [16]. This could indi-

cate the presence of comammox bacteria[16]. Comammox *Nitrospira* has earlier been found in fresh-water RAS[3], and can possibly be found in marine RAS as well.

The phylogenetic three (Figure 3.7) indicates close relationships between zOTUs representing *Nitrospira* in the Poding filter and previously described marine *Nitrospira*, but none of these are known as comammox. The most abundant zOTU in the Poding filter, zOTU1, was closely related to *Nitrospira salsa KC706459*. This strain was found in the Dutch coastal North Sea water, and performs nitrite oxidation in marine environments[20]. *Nitrospira* representatives closely related with *N. salsa* has previously been found in marine RAS[20]. The phylogenetic three (Figure 3.7) included *Nitrospira* comammox species from fresh water systems, and other *Nitrospira* species from marine systems. So far, no marine comammox is known. However, comammox *amoA* genes has been found in marine environments[32]. zOTU1 could therefore still be related to marine comammox species.

The surprisingly high abundance of *Nitrospira* in the Poding filter (Figure 3.6A) stands out compared to earlier studies reporting biofilm communities with abundances of nitrifiers in the range 0-20 % in RAS biofilters operated at higher salinities[48][16]. Navada et al.[42] has reported the relative abundance of nitrifiers at under 30 % during start-up of marine RAS biofilters. The relative abundances of potential zOTUs representing nitrifiers in the Poding filter was around 75-77 % at day 151 of the inoculum from Erko. An earlier study on the microbial community during salinity change in a RAS for production of post-smolt, had a relative abundance of one OTU representing *Nitrospira* at around 10 % at 18 ppm salinity[16]. It was an increasing relative abundance of nitrifiers on these biofilm carriers used as inoculum after the salinity was increased from 15 ppm to 20 ppm salinity at day 147 (Figure 3.6A). No matter if the relative abundance of nitrifiers increased when the salinity increased in the Poding filter, it seems like the relative abundance of zOTUs representing *Nitrospira* does not contribute much to this change. It is earlier seen a decrease of an OTU representing *Nitrospira* when the salinity increases[16].

The PCR amplification of *amoA* genes in comammox clade A (Figure 3.8C) and comammox clade B (Figure 3.8D) gave no product for most of the samples. However, the samples from the Poding filter gave PCR results for comammox clade B *amoA*, but at unexpected lengths (Figure 3.8D). All in all, there is no indications that comammox *amoA* genes is found in the samples. However, one can not be sure that these primers can be used on all comammox *amoA* genes. The primers was used in a study where comammox species was adapted from fresh water into more saline environments[32]. The phylogenetic analysis showed that zOTU1 in the Poding filter was related to *N. salsa* found in the dutch coastal North Sea water[20]. This can be a part of the explanation of why these primers do not target the potential comammox *amoA* genes in the samples representing the Poding filter.

4.4 The presence of AOBs and AOAs in the biofilm samples

qPCR was performed with low amplification efficiencies (Table 3.1) indicating non-optimal PCR conditions. Inhibitors in the template can influence the amplification efficiency at high concentrations, and the qPCR could therefore be started at a lower concentrations of the templates to achieve a higher amplification efficiency. One can also observe from the amplification curves (Figure E.1) that the standard curves for *16S rRNA* has a steeper curve than the standard curves of the *amoA* gene. Anyhow, the low amplification efficiencies may have affected the quantification, but all samples would be affected in the same way.

The high relative abundance of zOTUs representing nitrite oxidizers (around 60 %) and much lower relative abundance of ammonium oxidizers (around 14 %) (Figure 3.6A) indicates the presence of other ammonium oxidizing microbes such as comammox *Nitrospira* or AOA in the Poding filter biofilm. Firstly, PCR amplification using primers targeting the *amoA* gene in AOA (Figure 3.8B) indicates presence of AOA in the Poding filter. The PCR products from samples representing the Poding filter has the highest calculated copy number per cell of AOA *amoA* (Table 3.2). The melting point analysis of the qPCR product from the Poding filter samples shows a specific product (PF-BEr-D151, Figure 3.10D). The results after Sanger sequencing corroborates this result, and most likely consists of one or a few AOA strains closely related to *Nitrosopumilus oxyclinae*. This AOA species was found in biofilm communities with *Nitrospira* in marine RAS for production of shrimp [6]. The quantification of *amoA* genes showed a higher quantity of AOBs than AOAs in the biofilm community in the Poding filter (Table 3.2). This indicates that AOAs does not contribute essentially for the ammonium oxidation in the biofilm. This has been observed in a study where it was concluded that AOAs does not contribute considerably to the ammonium oxidation in a marine RAS [15].

The well-functioning biofilter for cod production (Wellf-BHa, Figure 3.8B) achieved more than one PCR product for amplification of AOA *amoA*. The melting point analysis of the qPCR product corroborates this (Wellf-BHa, Figure 3.10D). The low relative abundance of AOA in the well-functioning biofilter for cod production was confirmed by the quantification of AOA *amoA* gene in the samples from the well-functioning biofilter at Havlandet (Table 3.2). The relative abundance of nitrifiers (Figure 3.6B) shows a high abundance of ammonium oxidizing bacteria. This was confirmed in the qPCR (Table 3.2).

4.5 Future work

This master thesis showed that a strain of *Nitrospira* was highly abundant in a biofilm community in a marine biofilter in RAS during start-up with up to 51 % relative abundance of zOTU1 in the biofilm inoculum. This strain could be further investigated by trying to enrich this *Nitrospira*. As mentioned earlier, *Nitrospira* has proven to be very difficult to isolate, and one strain of *Nitrospira* took up to twelve years to isolate[28]. The high abundance of the strain can give higher probability for success of enrichment of this strain. The enrichment of this *Nitrospira* would give the possibility to sequence the whole genome. The genes for nitrification in the bacterium could be identified, and this could confirm whether the strain have genes for both ammonium- and nitrite- oxidization and therefore is a comammox *Nitrospira*. If this was the case, this strain would be the first comammox *Nitrospira* enriched from marine environments.

16S rDNA amplicon sequencing could be performed with primers targeting both *Archea* and *Bacteria*. One could from this investigate the ratio of ammonium oxidizing microbes relative to nitrite oxidizing microbes. This would give a better estimate of the AOA:AOB ratio. The ratio between ammonium oxidizing microbes and nitrite oxidizing bacteria could further indicate the potential presence of a comammox *Nitrospira*. Specific primers for the *amoA* and *nxr* gene could also been used in Illumina amplicon sequencing to investigate the origin of the nitrifying genes. One challenge with this would be to find primers targeting the *amoA* and *nxr* genes in all nitrifying microbes.

In this master thesis, a huge difference in the relative abundance of nitrifiers was observed in two successful marine biofilters for respectively production of grow-out salmon and cod. The Poding filter had a relative abundance reaching as high as 77 % nitrifiers, while the well-functioning biofilter for cod production had a relative abundance of nitrifiers of only 4.2 % nitrifiers. It would be interesting to perform lab-scale batch experiments on biofilm carriers from these two biofilters to see if the nitrification capacity is higher for the Poding filter compared to the well-functioning biofilter for cod production. The tolerance for salinity variations in the two biofilters could be compared in the experiments.

5 Conclusion

The biofilm and water communities in nitrifying biofilters were characterized by using Illumina sequencing of 16S rDNA amplicons. This characterization showed considerable differences in the microbial communities in the three biofilters investigated: The Maturation filter, the Poding filter and the well-functioning biofilter for production of Atlantic cod at Havlandet. The microbial communities in the two successful high-salinity biofilters; The Poding filter and the well-functioning biofilter for production of cod, were considerable different, and indicated that highly different microbial communities can perform nitrification at high salinity.

The performance of the Maturation filter and the Poding filter, which used two different strategies for start-up, were very different. The Maturation filter, started with clean biofilm carriers and a commercial, liquid inoculum, showed a low convertion of ammonium, but nitrite accumulated. The high concentration of nitrite probably led to inhibition of the AOBs and NOBs. The other strategy, performed for the Poding filter, was based on a biofilm inoculum from another RAS for post-smolt production. The Poding filter showed a fast conversion of ammonium to nitrate already a day after the biofilm inoculum was mixed with the clean biofilm carriers. All in all, the Poding filter achieved successful nitrification, while the Maturation filter did not. This strategy could therefore be a good choice for the aquaculture industry.

The performance of the biofilters can be seen in context with the microbial communities in the samples representing the biofilm in the biofilters. There was found a relative abundance of around 4 % of zOTUs representing nitrifiers in the biofilm samples of the Maturation filter, and the nitrifiers were primarily ammonium oxidizing bacteria. In comparison, the samples from the Poding filter had a surprisingly high abundance of zOTUs representing nitrifiers, constituting up to 77 % of the total bacterial community. The high abundance of *Nitrospira*, and especially zOTU1, with a relative abundance on up to 51 % of the bacterial community in the biofilm inoculum, could indicate the presence of a comammox *Nitrospira* or AOAs. zOTU1 (*Nitrospira*) was not closely related to any known comammox. It should be mentioned that the abundance of the AOB *Nitrosomonas* increased to around 15 % when the salinity was increased from 15 ppm to 20 ppm in the Poding filter. This could indicate the presence of halotolerant or halophilic ammonium oxidizing bacteria.

Traditional PCR using primers targetting the *amoA* genes in AOAs, AOBs and comammox *Nitrospira* indicated presence of AOAs in the Poding filter. Further quantification by qPCR indicated a higher abundance of AOBs than AOAs in the Poding filter. Sanger sequencing indicated presence of one AOA representative, closely related to *Nitrosopumilus oxyclinae*, previously found to exist together with *Nitrospira* in biofilters of marine RAS. It could still be concluded that AOAs were probably not the major ammonium oxidizer in the biofilm community of the Poding filter.

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Appendix

A Production data at Havlandet RAS Pilot

The production data from 100 to 169 days after start-up is given in Table A.1.

Table A.1: Overview of measurments in the biofilters during start-up. The Poding filter starts at day 137.

Day	TAN [mg L^{-1}]	NO_2^--N [mg L^{-1}]	NO_3^--N [mg L^{-1}]	Salinity [‰]	pH	T [°C]
100	-	-	-	-	-	-
110	4.99	-	-	25	7.9	-
115	-	15	-	23	7.62	-
119	6.75	-	-	25	7.94	16
121	4.2	-	-	25	8.06	16
122	6.51	-	-	25	8.15	16
123	8	-	-	24	8.13	15
124	8	-	-	25	8.1	15
126	8	-	-	27	8	15
129	8	-	-	27	7.87	15
134	3.45	-	-	15	7.66	15
137	8	4.6	-	15	7.8	14.5
138	0.08	-	-	15	7.59	15
139	0.43	-	-	15	7.44	15
140	0.1	-	-	16	7.43	15
141	1.84	-	-	-	7.93	-
142	-	-	-	-	7.46	-
143	8	-	-	15	-	15
144	0.5	-	-	15	7.14	15
145	8	-	-	-	6.7	-
146	5.92	-	-	16	7.45	16
147	1.05	-	-	15	7.5	16
148	0.07	-	-	-	8.11	-
149	-	-	-	-	7.3	-
150	8	-	-	20	7.3	15
151	6.7	-	-	20	7.22	15
152	4.4	-	-	20	7.02	-
153	-	-	-	14	6.5	15
154	8	-	-	14	7.7	15
155	-	-	-	-	6.8	-
156	-	-	-	-	8	15
157	8	-	-	15	6.26	15
158	8	-	-	15	7.24	15
159	8	-	-	15	8.2	16
160	0	-	-	15	7.2	15
161	0	-	-	15	7	15
163	-	-	-	-	7.3	-
164	-	-	-	-	7.51	-
165	-	-	-	-	7.2	-
168	-	-	-	23	7.47	13.6
169	-	-	-	23	7.75	14

B Nitrifiers in the maturation period

Realtive abundance of zOTUs representing nitrifiers in the Maturation period is shown in Figure B.1.

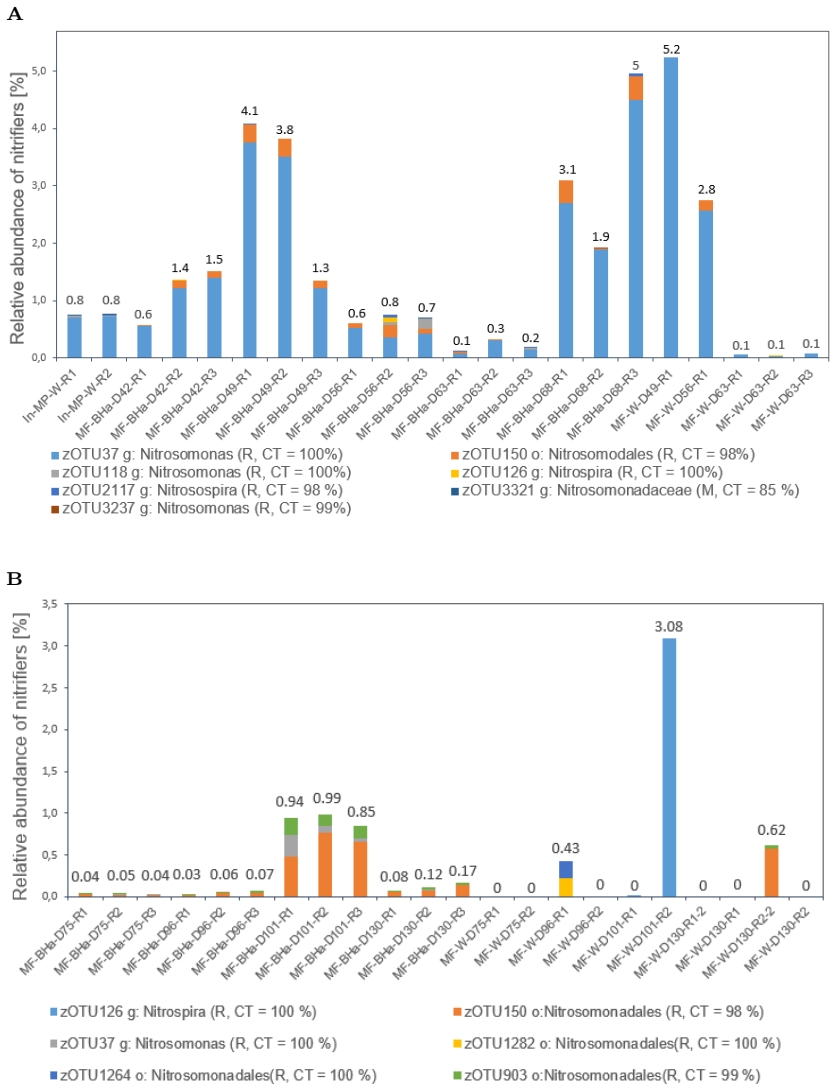


Figure B.1: Relative abundance of zOTUs characterized as nitrifiers in the Maturation period (A) from day 1 to 68 including the commercial inoculum and (B) from day 75 to 130. A taxonomy with a confidence treshold (CT) of 80 % or higher at family- (f) or genus- (g) level is included. The zOTUs is classified using the two databases MiDAS and RDPv18. The highest achieved confidence treshold is represented, and RDP is marked for the cases where RDPv18 database is used. The zOTUs is classified using the database MiDAS, but RDPv18 was used when it gave a higher confidence treshold. MF = Maturation filter, B = biofilm, Ha = Havlandet, R = replicate, D = day, W = water and CT = confidence treshold.

C Protocols

C.1 Powersoil® DNA Isolation Kit

EXPERIENCED USER PROTOCOL
PowerSoil® DNA Isolation Kit
Catalog No. 12888-50 & 12888-100

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

Note

If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION**: Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

Note

Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix **Solution C4** before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.

Note A total of three loads for each sample processed are required.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x g.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x g.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

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

C.2 SequalPrep Normalization Plate Kit

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 –20°C 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 –20°C 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).

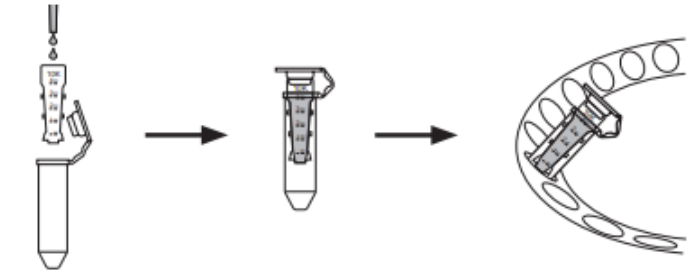
3. Incubate at room temperature for 5 minutes.

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.

C.3 Amicon® Ultra-0.5 Centrifugal Filter Devices

How to Use Amicon® Ultra-0.5 Centrifugal Filter Devices

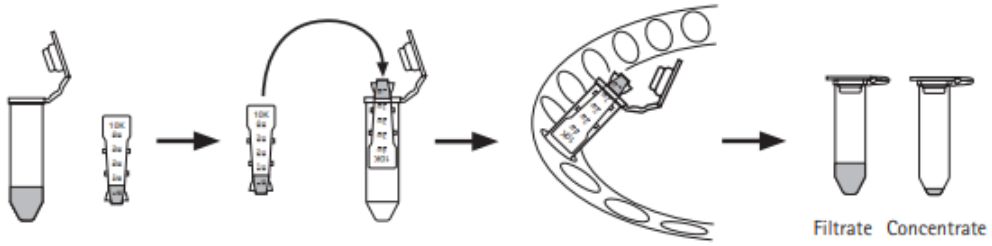
1. Insert the Amicon® Ultra-0.5 device into one of the provided microcentrifuge tubes.
2. Add up to 500 µL of sample to the Amicon® Ultra filter device and cap it.
3. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar 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 2 for typical spin times.



Add sample Cap Spin at 14,000 × g

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 microcentrifuge 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 from tube Turn device upside down in clean tube Spin at 1,000 × g Filtrate Concentrate

C.4 QIAquick® PCR purification Kit (Qiagen)

Quick-Start Protocol

QIAquick® PCR Purification Kit
QIAquick® PCR & Gel Cleanup Kit

The QIAquick PCR Purification Kit and the QIAquick PCR & Gel Cleanup Kit (cat. nos. 28104, 28106, 28506 and 28115) can be stored at room temperature (15–25°C) for up to 12 months if not otherwise stated on label.

Further information

- *QIAquick Spin Handbook*: **www.qiagen.com/HB-1196**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- This protocol is for the purification of up to 10 µg PCR products (100 bp to 10 kb in size).
- 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 table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH ≤7.5. The adsorption of DNA to the membrane is only efficient at pH ≤7.5. 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; do not add pH indicator I to buffer aliquots.
- Symbols: ● centrifuge processing; ▲ vacuum processing.

Sample to Insight

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ● a provided 2 ml collection tube or into ▲ a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ● centrifuge for 30–60 s or ▲ apply vacuum to the manifold until all the samples have passed through the column. ● Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 µl Buffer PE to the QIAquick column ● centrifuge for 30–60 s or ▲ apply vacuum. ● Discard flow-through and place the QIAquick column back into the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. 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. 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.
8. 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.

D Chromatograms from Sanger Sequencing

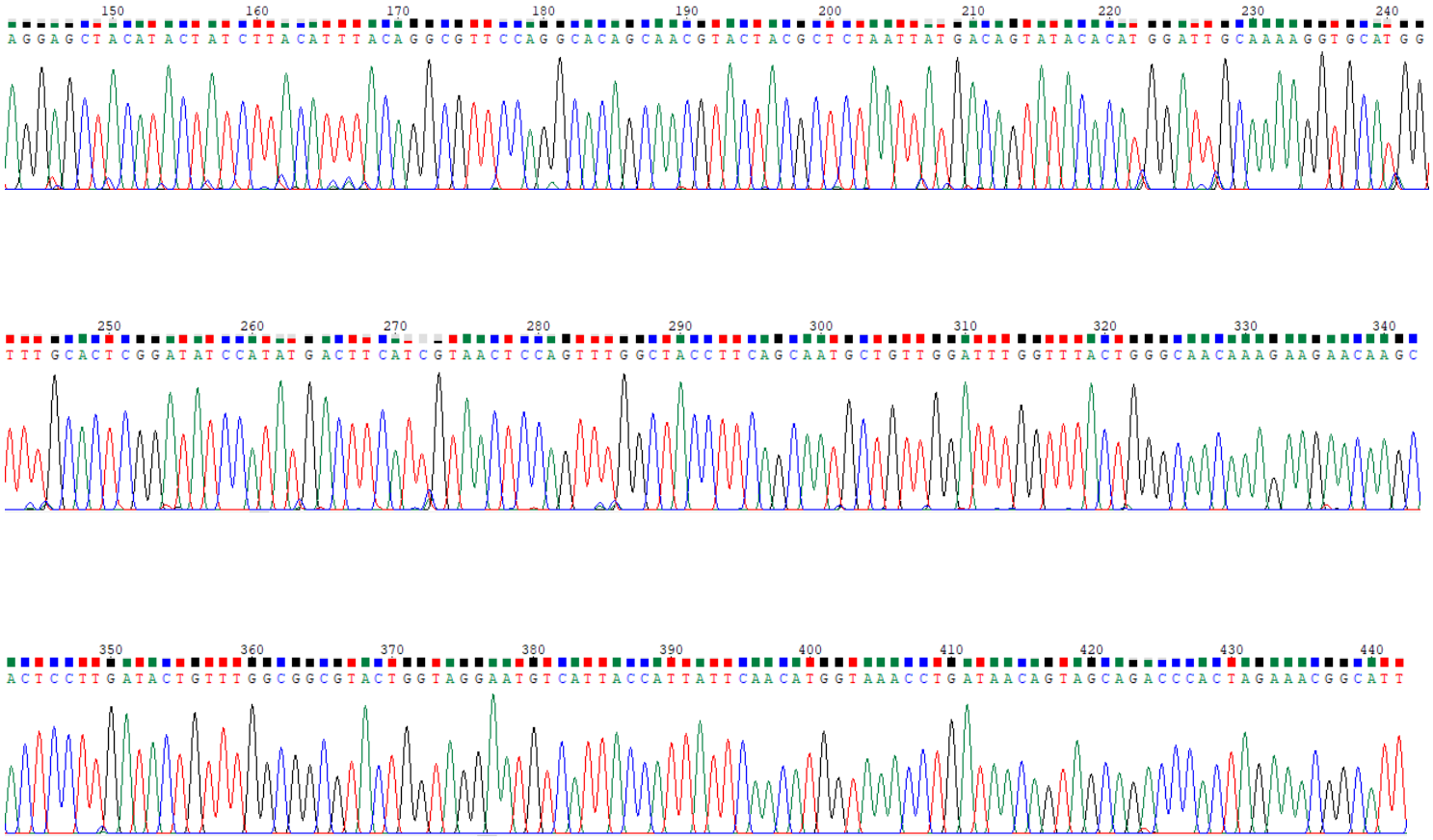
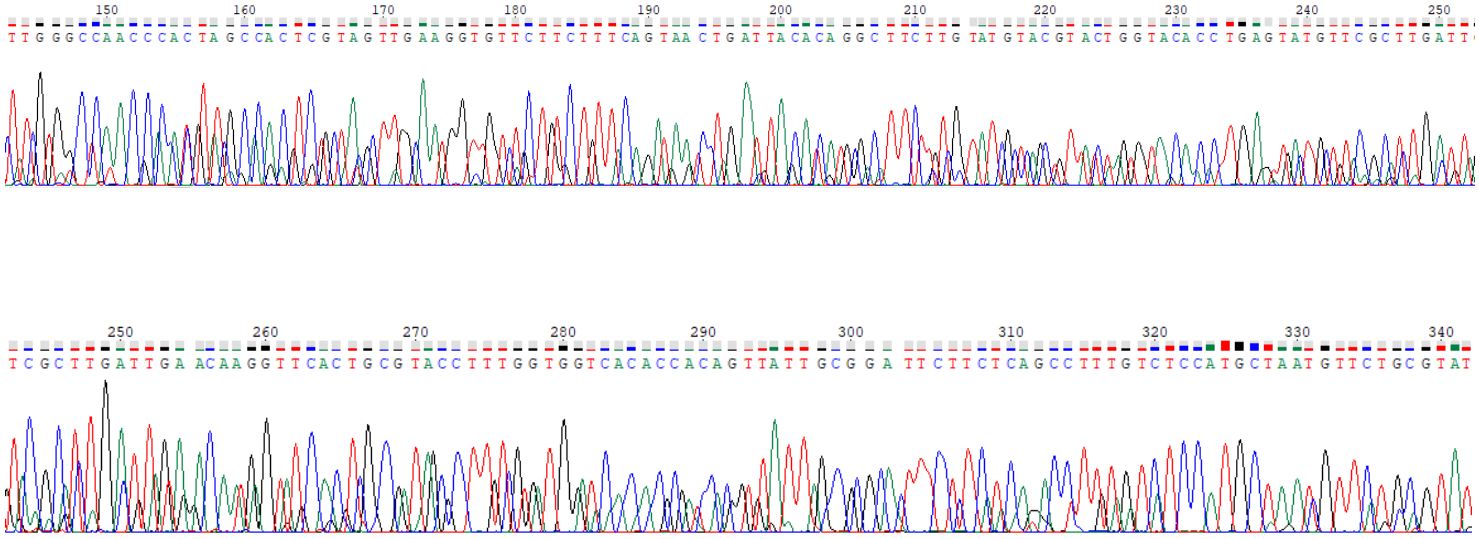


Figure D.1: Chromatogram for *amoA* genes in AOA sequenced with Sanger sequencing for PF-BEr-D151.



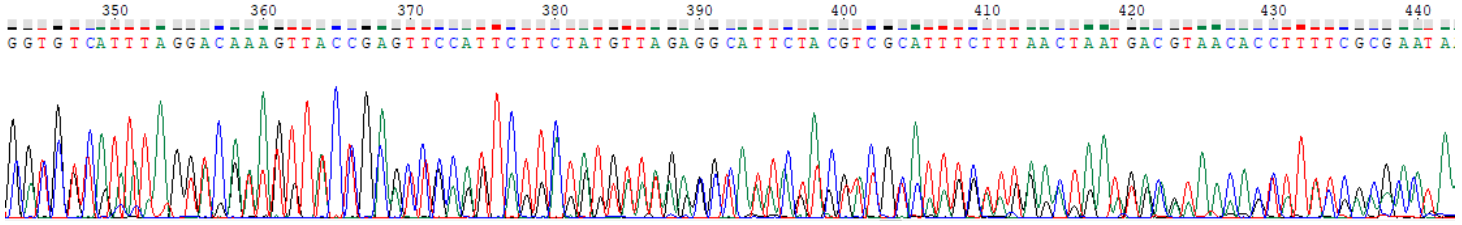


Figure D.2: Chromatogram for *amoA* genes in AOB sequenced with Sanger sequencing for PF-BEr-D151.

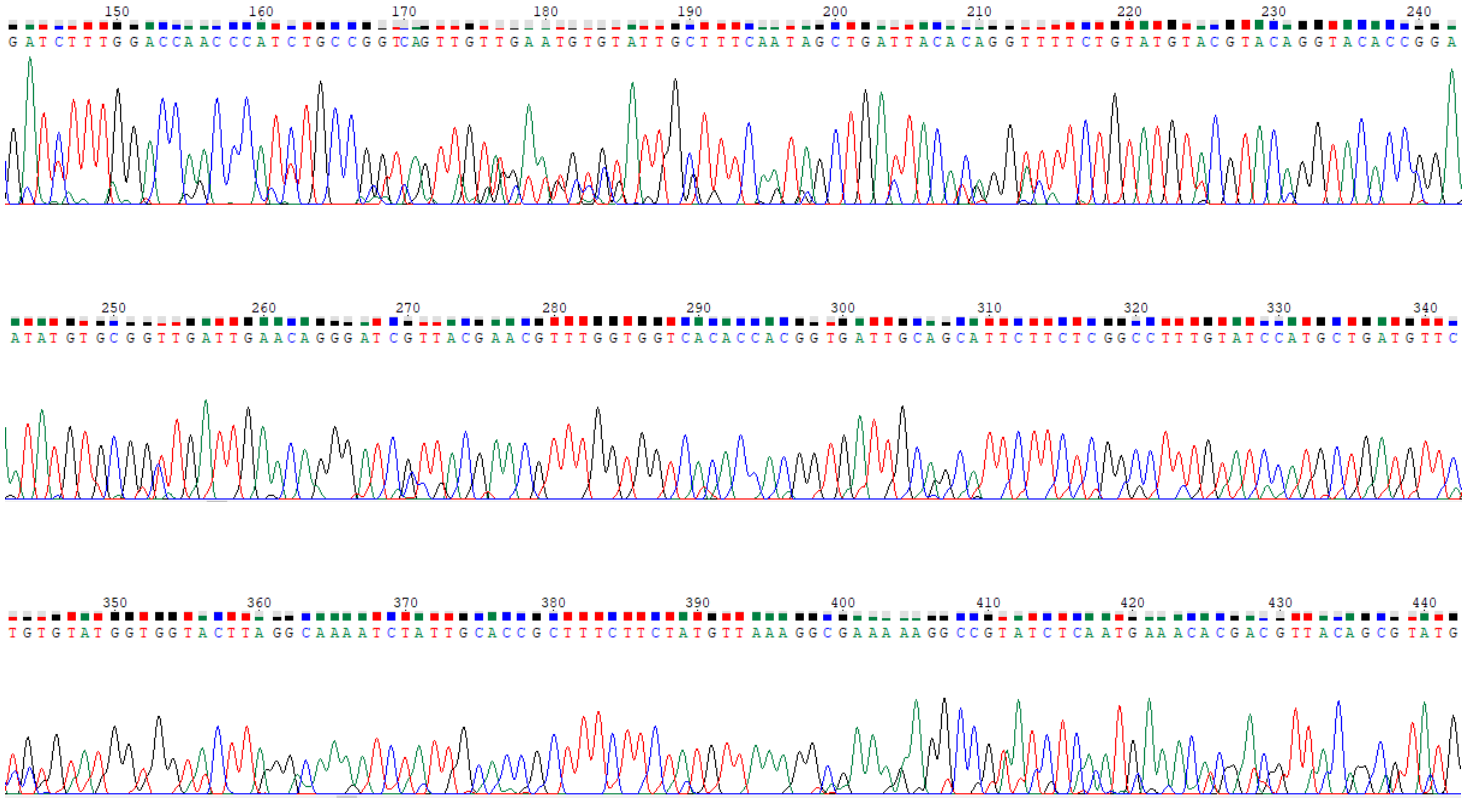


Figure D.3: Chromatogram for *amoA* genes in AOB sequenced with Sanger sequencing for Wellf-Ha-R1.

E Amplification curves

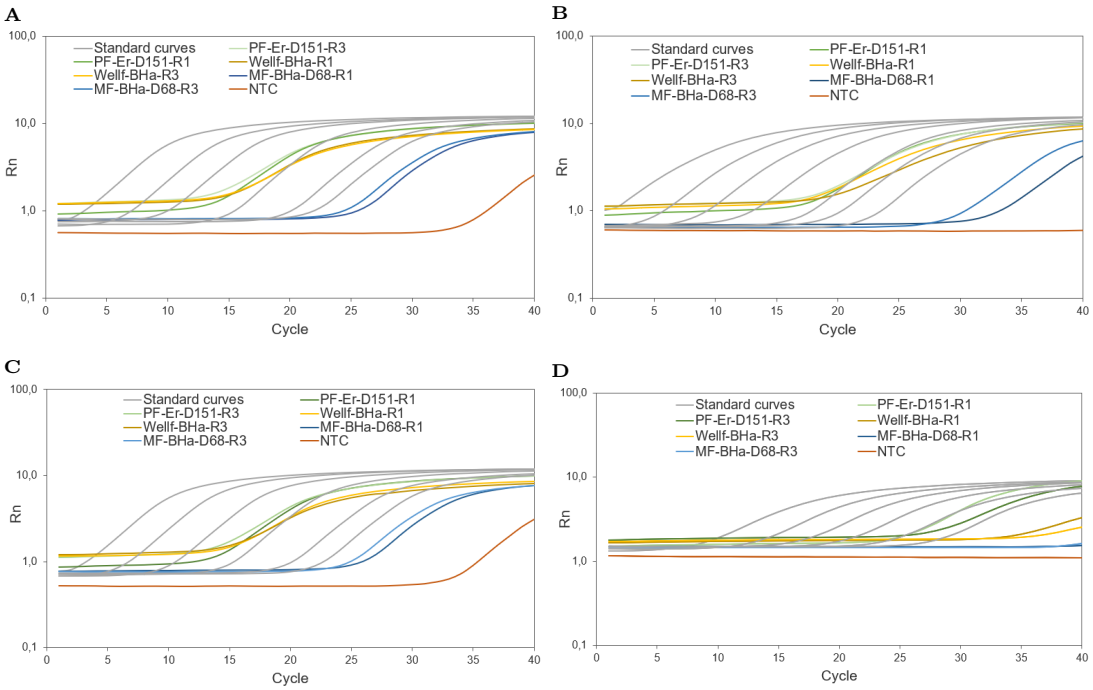


Figure E.1: Amplification curves for amplification of the *16S rRNA*-gene (A; AOB-plate, C; AOA-plate) and *amoA* gene in AOB (B) and AOA (D). The average of three parallels for each sample is plotted against the cycle number. The 6 standardcurves is made by samples with known DNA-concentrations measured in NanoDrop. PF = Poding filter, Er = Erko, B = biofilm, D = day, Ha = Havlandet and R = replicate.

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