Master's thesis

NTNU
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Biological nitrogen removal in recirculating aquaculture systems (RAS): Adaptation of a biological treatment process based on partial nitrification and anammox to RAS conditions

Master's thesis in Ocean Resources
Supervisor: Ingrid Bakke
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Lea Risnes

Abstract

Biological water treatment in recirculating aquaculture systems (RAS) is typically based on nitrification, a process for the conversion of the toxic waste product ammonium (NH_4^+). This process results in high nitrate concentrations in the RAS water and its effluents. Nitrate can be removed by denitrification, but this process is not typically used in commercial smolt production in Norway. Instead, the nitrate concentration in the rearing water is controlled by water exchange. However, more economic and environmental-friendly alternatives for nitrogen removal might be interesting.

Biological water treatment processes based on the anammox process have been applied to treat industrial wastewaters of high ammonium concentrations (high-strength wastewater). In these treatment processes, the anammox bacteria is utilized in combination with partial nitrification. The partial nitrification anammox process (PNA) consists of the partial nitrification of ammonium to nitrite by nitrifiers and the removal of ammonium and nitrite through anaerobic oxidation of ammonium by anammox bacteria. Currently, the PNA process is only applied in large-scale processes treating high-strength wastewater (500-2500 mg N/L), but it has also been applied to lower nitrogen concentrations of 20-50 mg/L in lab scale studies. In RAS, typical ammonium concentrations are 2 mg/L and in this study, we examined whether PNA and anammox processes could be adapted to lower TAN concentrations relevant to RAS conditions in one-stage, biocarrier MBBR reactors.

In this study, PNA carriers treating high-strength wastewater of 800 mg/L ammonium-N and 3000 mg/L COD at the wastewater facility "Nordre Follo" (Viken, Norway) were implemented in two MBBR 15L reactors to try to obtain anammox or PNA activity at low ammonium concentrations. One reactor (PNA reactor) was operated at 10 mg/L ammonium-N for 4 months, fed some acetate-carbon (C/N-ratio = 0.35) and operated with intermittent aeration. The other reactor (AMX reactor) was fed ammonium and nitrite and the ammonium-N and nitrite-N concentrations were lowered stepwise from 300 mg/L to 10 mg/L, respectively, over a 10-month period, and operated anaerobically by N_2 flushing of influent feeding water and the reactor content. Influent and effluent concentrations of nitrogen compounds were measured over the experimental periods of both reactors and K5 biocarriers with biofilm were sampled at regular intervals for Illumina sequencing of the 16S rRNA amplicons.

PNA activity was successfully achieved in the PNA reactor. A maximum nitrogen removal of 90% of the total influent nitrogen was observed and the average nitrogen removal during the final period of the three final days of operation was 74%. No accumulation of neither nitrite nor nitrate was observed during the experimental period. The bacterial community in the reactor showed high relative abundances and diversity of the Planctomycetes phylum. The two most abundant sequence variants (ASVs), representing the Brocadiaceae family and probably the phylum Chloroflexi, constituted in average around 40% of the total reads for the samples from the PNA reactor.

In the AMX reactor the nitrogen removal decreased over the experimental period, while nitrate production increased despite low oxygen concentration in the reactor. The microbial community in the AMX reactor also consisted of Planctomycetes and high abundances of the same two ASVs as in the PNA reactor, but the relative abundance of ASVs classified as Nitrospira increased over the experimental period and constituted in average 17% of the relative abundance in the last two biofilm samples. Based on the molar ratio of nitrite and oxygen for nitrite oxidation, there was not enough oxygen present for the nitrate production which was observed. However, nitrate production and Nitrospira abundance increased over the experimental period and one possible reason is that there were additional sources of oxygen which were unaccounted for in this study.

In conclusion, the strategy of directly exposing the biofilm carriers in the MBBR to low TAN concentrations was successful for PNA operation.

Sammendrag

Biologisk vannrensing i resirkulerende akvakultur systemer (RAS) består vanligvis av nitrifisering, en bakteriell prosess der ammonium, et giftig restprodukt som skilles ut av fisk, omdannes til nitrat. Nitrat kan fjernes fra vannet ved denitrifikasjon, men denne prosessen brukes ikke mye i kommersielle norske RAS. Isteden kontrolleres nitrat konsentrasjonen i RAS ved vannutskifting. Men andre prosesser for nitrogenfjerning kunne vært interessante.

Biologiske vannrensingsprosesser basert på anammox er i dag i bruk i renseanlegg for industrielle avløpsvann med høye ammonium konsentrasjoner (500-2500 mg N/L). I disse prosessene brukes anammox bakterien i kombinasjon med delvis nitrifisering. Delvis nitrifisering med anammox (PNA) består av en delvis nitrifisering av ammonium til nitritt utført av ammonium oksiderende bakterier og anaerob ammonium oksidasjon utført av anammox bakterier. Selv om prosessen kun er i bruk ved høye ammonium konsentrasjoner på stor skala, har den også vært anvendt ved lave ammonium konsentrasjoner (ned til 20 mg N/L) i labskala forsøk. Normale ammonium konsentrasjoner i RAS er 2mg N/L og i dette studiet ble det undersøkt om PNA prosessen kunne anvendes på lave ammonium-N konsentrasjoner som er relevante for RAS i ett-trinns reaktorer med biofilmbærere.

Biofilmbærere fra en PNA prosess med høye ammonium konsentrasjoner (800 mg N/L) ble brukt i to 15L reaktorer for å se om det var mulig å få PNA og anammox aktivitet ved lave ammonium konsentrasjoner. Den ene reaktoren (kalt PNA reaktor) fikk 10 mg/L ammonium-N i tillegg til noe organisk karbon i en periode på 4 måneder. I tillegg ble den luftet i korte perioder. Den andre reaktoren fikk ammonium og nitritt og konsentrasjonen ble senket gradvis fra 300 mg/L til 10 mg/L av henholdsvis ammonium-N og nitritt-N, over en periode på 10 måneder. Konsentrasjoner av ammonium, nitritt og nitrat ble målt regelmessig i innløpet og utløpet til reaktoren. I tillegg ble det tatt prøver av biofilmbærerne fra begge reaktorene for å analysere hvilke bakterier den besto av. Dette ble gjort ved hjelp av Illumina-sekvensering av 16S rRNA amplikon.

PNA prosessen fungerte godt i PNA reaktoren. Den høyeste andelen av fjernet nitrogen var 90%, men gjennomsnittlig andel av fjernet nitrogen var 74% mot slutten av den eksperimentelle perioden. Det var ingen akkumulering av nitritt og nitrat i løpet av forsøket. Bakteriesamfunnet i PNA reaktoren besto av en stor andel Planctomycetes, en bakterieorden som er kjent for anammox bakterier. To ASV-er skilte seg ut siden de utgjorde en stor del av samfunnet: familien Brocadiacaea og rekken Chloroflexi.

I AMX reaktoren avtok andelen med fjernet nitrogen, mens nitrat produksjonen økte over den eksperimentelle perioden. Det bakterielle samfunnet besto også av mange ASV-er knyttet til Planctomycetes rekken, men i tillegg utviklet det seg en stor populasjon av Nitrospira. I de to siste biofilm prøvene utgjorde Nitrospira i gjennomsnitt 17% i relativ forekomst. Basert på den molare ratioen mellom oksygen og nitrogenforbindelsene var det ikke nok oksygen tilgjengelig i reaktoren for den nitrifikasjonen vi observerte, men det kan tenkes at det fantes flere kilder til oksygen enn det vi målte i innløpsvannet.

For å konkludere fungerte det bra å eksponere biofilmbærerne direkte til en lav konsentrasjon av ammonium for å få PNA aktivitet i reaktoren.

Abbreviations

AOA = Ammonium oxidizing archaea
AOB = Ammonium oxidizing bacteria
C/N = Organic carbon / inorganic nitrogen
COD = chemical oxygen demand
D-COD = Dissolved chemical oxygen demand
DO = Dissolved oxygen
DOC = Dissolved organic carbon
DNA = Deoxyribonucleic acid
DNB = Denitrifying bacteria
HRT = Hydraulic retention time
IMA = intermittent aeration
MBBR = Moving Bed Biofilm Reactor
min = Minutes
N = Inorganic nitrogen
NRR = nitrogen removal rate
NOB = Nitrite oxidizing bacteria
PCoA =Principal Coordinate Analysis
PCR = Polymerase chain reaction
PNA = partial nitrification anammox
TAN = Total ammonia nitrogen ($NH_4^+ + NH_3$)
rRNA = Ribosomal RNA
RNA = Ribonucleic acid

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1 Introduction

1.1 Sustainable systems for future production

The global population increase has resulted in an increased fish demand for human consumption (FAO, 2016, p. 2). Wild fish catch has not increased since the 1990s, but aquaculture production has grown impressively over the last decades, resulting in providing over half of the fish for human consumption per year since 2014 (FAO, 2016, p. 2). Upholding the 2030 Agenda of sustainable development and meeting the ever-growing demand for fish will be challenging.

Aquaculture is the cultivation of aquatic organisms through controlled cultivation processes either in the sea, in freshwater ponds or in culturing tanks (Calixto et al., 2020). Among leading countries of aquaculture production are China, India, Indonesia, Norway and Chile (FAO, 2020, p. 32). The total aquaculture production in 2020 was 114.5 million tonnes, of which 82.1 million tonnes were aquatic animals (FAO, 2020, p. 21). Among the most produced organisms in the world are freshwater fish species such as carps and tilapia, seaweeds, mollusks, catfishes, marine shrimp and salmons (Cai et al., 2019). Within the salmon production, Norway is the biggest producer, followed by Chile, Scotland and Canada (Iversen et al., 2020).

Norwegian salmon production originated in the 1970s. The hatching and stages up until smolt have typically been in freshwater flow-through facilities on land, while the post-smolt on-growing stage traditionally have been in open net pens at sea. The production at sea include problems like sea and salmon lice welfare problems, the spreading of this lice to wild salmon populations through escapes, and the changing of wild populations genetics through interbreeding with escaped production salmon (Bjørndal & Tusvik, 2019; Misund, 2019). On-land production facilities have little or no problems with lice and escapes. A drawback of both on-land flow-through facilities and open production pens is that they offer no waste-control, but let aquaculture waste such as nitrogen and phosphorous freely into the environment, potentially causing eutrophication (Dauda et al., 2019).

In addition to the environmental concerns, the need for increased aquaculture production has led to increased competition for the basic needs of that production, such as area and water supply (Dauda et al., 2019). The challenge, it seems, is to make more out of less in a sustainable way. The intensification of aquaculture production is an alternative for that needed development. To intensify production, more inputs are required per area, especially feed (Henriksson et al., 2018). Since feed is the major source of waste production in aquaculture (Martins et al., 2010) intensification leads to higher waste production per production area. An effective waste treatment is therefore needed to meet the environmental concerns of the production, especially in areas where the recipient is vulnerable to nutrient pollution, such as rivers and lake recipients.

1.2 Recirculating aquaculture systems

Recirculating aquaculture systems (RAS) are intensive, land-based production systems which recycle the rearing water, thereby reducing its land and freshwater use (Martins et al., 2010). In contrast to the flow-through systems RAS offer control of the rearing conditions for fish, which is a major advantage since it gives predictable growth rate and thereby predictable production schedule throughout the year (Ebeling & Timmons, 2012). The RAS-system also provides the opportunity to treat its effluent more easily than flow-through systems or open sea cages, since the wastes from RAS are more concentrated.

Drawbacks of RAS include high energy requirements, high investment cost, and higher complexity in both system design and system operation (Badiola et al., 2012; Dalsgaard et al., 2013). The advantages seem to outweigh the drawbacks however, since these land-based farming systems have emerged in

several countries, including Norway, Denmark, Canada, the US and South Africa (Bjørndal & Tusvik, 2019). In Norway, salmon farmers are even converting their flow-through facilities to RAS and almost all new land-based systems are RAS (Dalsgaard et al., 2013). This increased focus on RAS for smolt production in Norway is due to limitations on freshwater resources during the summer, low water quality in the influent and the need for temperature-optimization, CO2-stripping and UV or ozone treatment of large influent water volumes (Dalsgaard et al., 2013; Kristensen et al., 2009).

The intensive production leads to high concentrations of waste compounds such as particles, organic material, nitrogen and phosphorous (Piedrahita, 2003) and since these wastes have negative consequences for fish welfare and recipients of effluents, the water must be treated. The extensive water treatment required is part of the reason for the high initial investment and operation cost.

1.2.1 Nitrogen and phosphorous wastes in RAS

Irrespective of species, fish require a high amount of protein (Dauda et al., 2019). The two main elements of fish feed are nitrogen and phosphorous (Dauda et al., 2019). Typically, less than 50% of these nutrients are taken up in the fish body (Piedrahita, 2003) and the rest is released back into the water column. Phosphorous is excreted mainly as particulate waste in faeces (Bureau & Cho, 1999; Dauda et al., 2019). The phosphorous itself is of no risk to fish health (Dauda et al., 2019), but the particles it is part of can cause gill blockage (Piedrahita, 2003). Particulate matter must therefore be removed quickly from the rearing water. Nitrogen is excreted as ammonia (NH_3), which is highly toxic to both fish. NH_3 exists in an equilibrium with the ionized form (NH_4^+; Figure 1-1). The un-ionized ammonia is the most toxic, but ionized ammonia also poses risk to cultured fish (Downing & Merkens, 1955). The ratio between them is determined mainly by pH and water temperature (Körner et al., 2001). RAS temperatures range from 10-13 °C and carbonate-compounds are used to adjust the pH to an optimal level to keep ammonia mainly existing on the ionized form. Together ammonia and ammonium are referred to as total ammonia nitrogen, or TAN (Dauda et al., 2019).

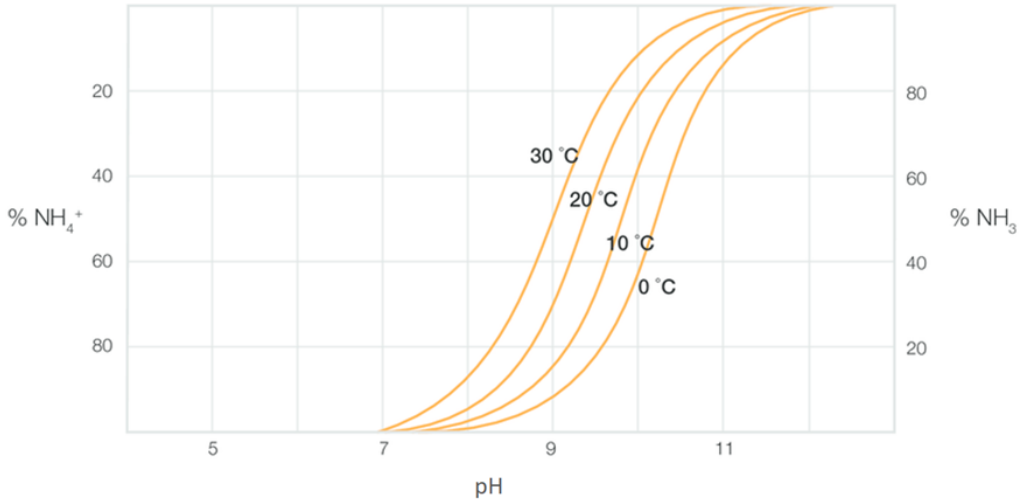

Figure 1-1. Ammonia-ammonium equilibrium as a function of temperature and pH. The figure is modified from Paul et al. (2016).

Ammonia is removed from the RAS rearing water by biological water treatment. This happens in the biofilters in RAS, where nitrifying bacteria convert ammonia to nitrate. Nitrate is far less toxic than ammonia (Dauda et al., 2019), but nitrite is a very toxic intermediate of this conversion. The recommended threshold concentrations of TAN and nitrite for Atlantic salmon (*Salmo salar*) are 2 mg/L and 0.1 mg/L, respectively (Fjellheim et al., 2016, p. 5). Exposure to above-limit ammonia and

nitrite can lead to both acute and long-term consequences for fish health. Higher ammonia concentrations can lead to respiration- and osmoregulation problems, while exposure to elevated nitrite-concentrations can lead to reduced growth rate and oxidation of haemoglobin, thereby reducing the blood capacity of transporting oxygen (Fjellheim et al., 2016, p. 6; Gutiérrez et al., 2019).

Nitrate is far less toxic than both ammonium and nitrite, and may accumulate to around 100 mg/L NO_3^--N without clear negative consequences to salmon (Davidson et al., 2017; Freitag et al., 2015). Consequences of elevated nitrate concentrations are thought to be effects on endocrine function and reproductive development (Good & Davidson, 2016), but an upper threshold limit of nitrate for Atlantic salmon has yet not been established. Although nitrate is not thought to render serious negative consequences on fish health, it still needs to be removed to not accumulate excessively. This removal can only be accomplished by water exchange.

Although nitrate is not toxic, it is still a nutrient pollutant, posing a eutrophication risk to the river and ocean ecosystems, since they are usually N-limited (Camargo & Alonso, 2006). The maximum nitrate level in RAS-effluents differ among facilities, dictated by water exchange rates and microbial activity (van Rijn et al., 2006). The allowed nitrogen effluents of land-based facilities in Norway are regulated by authorities after the state of the recipient (Lomnes et al., 2019). Particles with phosphorous in RAS-effluents can also cause eutrophication in the receiving water body, especially in combination with nitrogen-compounds (Ryther & Dunstan, 1971; Schindler et al., 2008).

Organic matter is also present in aquaculture water both as particles (solid organic matter) and dissolved organic carbon (DOC). High level of organic matter is not beneficial to either fish health nor biofilter operation. Normal DOC concentrations in aquaculture range from 10-35 mg/L (Aguilar-Alarcón et al., 2020). The concentration of organic compounds in water is also often measured as chemical oxygen demand (COD).

1.2.2 Water treatment in RAS

The treatment in RAS consists of several mechanical and biological water treatment steps, in addition to disinfection. Most of the bigger suspended solids from leftover feed and faeces are filtered out by mechanical filters, often drum filters. These filters are not as effective on fine solids (Piedrahita, 2003) and they often accumulate in RAS as a consequence. The common way to remove them is by dilution. Downstream from the particle removal is usually the biofilter. The biofilter is often in the form of a moving bed bioreactor (MBBR) or fixed bed biofilter (FBB). These biofilters are crucial for conversion of the toxic ammonia into the much less toxic nitrate by autotrophic nitrifying bacteria. Carbon dioxide (CO_2) is often removed from the water by trickling filters. Air is pumped through the water to strip it of CO_2. This degassing requires thorough ventilation of the room. Before the water can re-enter the fish tanks, it needs to be oxygenized and pH-adjusted by the adding of carbonate. Some RAS also include a disinfection step, such as UV or ozone-treatment, to keep the microorganisms at a low level in the rearing water (Fjellheim et al., 2016). These are usually placed after the biofilter, and before oxygenation. Figure 1-2 shows a typical design of the RAS treatment steps.

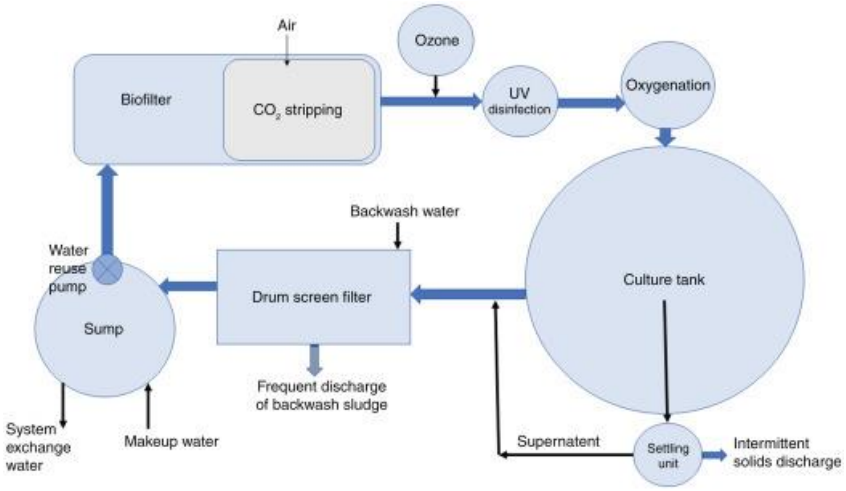

Figure 1-2. Schematic presentation of a recirculating aquaculture system with typical treatment steps. The figure is from Holan et al. (2020).

1.2.3 Nitrification in RAS biofilters

The biological biofilter is fundamental in the RAS water treatment. There are different kinds of biofilters, but they all have in common that it provides a substrate for microbial biofilm growth, such as stones or plastic carriers. Nitrifying bacteria in the biofilm convert toxic TAN to nitrate in a two-step process. Reaction equations 1.1 and 1.2 (Romano & Zeng, 2013) represent the overall energy metabolisms of the nitrogen conversions.

$$NH_4^+ + CO_2 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+ \quad (1.1)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (1.2)$$

In the first step (1.1), ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB), for example *Nitrosomonas*. In the second step (1.2), nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB), for example *Nitrospira*. Some archaeal microbes are also able of oxidizing ammonia and do so in natural environments (He et al., 2018), in RAS biofilters (Bartelme et al., 2017) and in aquarium biofilters (Bagchi et al., 2014) and some *Nitrospira* microbes have been found to be able to perform both steps of nitrification (Daims et al., 2015; van Kessel et al., 2015). This process is called Comammox, which is short for complete ammonia oxidation. The optimal pH for nitrification systems has been found to be approximately 7.8-9.0 (Amatya et al., 2011).

1.2.4 Denitrification for nitrogen removal in RAS

Denitrification is a biological a dissimilatory pathway involving nitrate reduction to nitrogen gas. In this process, anaerobic microorganisms use nitrate as an electron acceptor to oxidize organic material in the absence of oxygen. The reduction of nitrate happens in several enzymatic steps and the end product of complete denitrification is nitrogen gas, which then leaves the water to the atmosphere. Equation 1.3 shows the intermediates of denitrification (Matějů et al., 1992).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1.3)$$

Complete nitrogen removal includes nitrification and denitrification. The nitrification process requires a lot of oxygen to keep conditions aerobic, while denitrification is dependent on anoxic conditions and the presence of organic compounds as carbon source. Since the organic matter content in RAS is relatively low, denitrification operation in RAS usually requires an external carbon source, such as

methanol. In sum, the available technology for nitrogen-removal with nitrification and denitrification today is both energy-inefficient and costly (Kartal et al., 2010; Szatkowska & Paulsrud, 2014) and this process is not much used in Norwegian smolt production. However, advantages of removing nitrogen might include reduced water consumption, cleaner water for discharge and thereby a lower pressure on receiving water body and positive consequences for fish health and wellbeing.

1.3 Application of anammox bacteria for nitrogen removal in wastewater treatment

1.3.1 The anammox bacteria

In 1941, anaerobic ammonia oxidation was theorized to be a possible source of N_2 in the sea (Hamm & Thompson, 1941) and several observations of nitrogen-disappearance in anoxic marine environments were made (Cline & Richards, 1972; Murray et al., 1995). Since then, proof of the process of anaerobic ammonia oxidation were discovered in marine sediments (Thamdrup & Dalsgaard, 2002), the Arctic sea ice (Rysgaard & Glud, 2004), the coastal bay of Golfo Dulce in Costa Rica (Dalsgaard et al., 2003) and the Black Sea (Kuypers et al., 2003). Based on current estimates, the anammox bacteria might be responsible for about 50% of nitrogen turnover in marine environments (Dalsgaard et al., 2005).

The first evidence of anaerobic ammonium oxidation was found in a wastewater treatment facility in the Netherlands and was named "anammox" (Mulder et al., 1995). In 1998, the bacteria was grown successfully in sequencing batch reactors (Strous et al., 1998) and one year later the anammox bacterium was sequenced with 16S rRNA amplification and taxonomically placed within the Planctomycetes order (Strous et al., 1999a). Since then, anammox have been found in many wastewater plants (Kuenen, 2008) and today, five anammox bacterial genera have been reported: *Brocadia*, *Kuenenia*, *Anammoxoglobus*, *Jettenia* and *Scalindua* (Wen et al., 2020).

The anammox bacteria oxidizes ammonium with nitrite as electron acceptor, forming nitrogen gas. This process is possible due to a unique cell organelle found only in the anammox bacteria, called anammoxosome. This organelle retains the highly toxic intermediate of the process, hydrazine, to the cell, thus making the process possible (Kartal et al., 2010). Equation 1.4 shows the overall reaction of anammox and equations 1.5-1.7 show the half reactions with the intermediates nitric monoxide and hydrazine (Kartal et al., 2011).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1.4)$$

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (1.5)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad (1.6)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (1.7)$$

Anammox are autotrophic bacteria, so they use CO_2 as a carbon source. Nitrite works as the electron acceptor for ammonium oxidation, but is also the donor for reduction of CO_2 (Kuenen, 2008). The full metabolism of the anammox bacteria is shown in equation 1.8 (Strous et al., 1998). Equation 1.8 shows that anammox activity is associated with a small nitrate production.

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26 NO_3^- + 2.03 H_2O + .066 CH_2O_{0.5}N_{0.15} \quad (1.8)$$

The anammox bacteria are obligate anaerobic and are among the most slow-growing bacteria with a generation time of 10-12 days at 35 °C (Kartal et al., 2010). The anammox process has a pH range of 6.7 to 8.3 with an optimal pH of 8.0 (Strous et al., 1999b).

1.3.2 Water treatment processes based on the anammox process

1.3.2.1 High-strength wastewater treatment

Water treatment processes based on anammox currently in use are typically treating so-called high-strength ammonia waste streams (typically 500-2500 mg N/L; (Na et al., 2021; Ronan et al., 2021; Wang et al., 2018), such as industrial wastewater, landfill leachate and digestate from anaerobic treatment processes (Ma et al., 2020; Ronan et al., 2021).

In these applied processes, anammox is often used in combination with partial nitrification (Ronan et al., 2021). Partial nitrification is a term for the controlled process of allowing only the first step of nitrification, the ammonium oxidation to nitrite, to occur. Anammox requires approximately equal amounts of ammonium and nitrite to produce nitrogen-gas and therefore, a partial nitrification will provide these compounds. This process combining partial nitrification and anammox is called "Partial nitrification anammox" and can be abbreviated PNA. The PNA process for nitrogen removal has caught widespread interest as it saves up to 60% of energy on aeration (Szatkowska & Paulsrud, 2014), has a zero-CO_2 emission (Kartal et al., 2010; Ma et al., 2020; Szatkowska & Paulsrud, 2014), produces less biomass and requires no alkalinity compared to the nitrification-denitrification process (Szatkowska & Paulsrud, 2014). The PNA process also does not require an external carbon source for denitrification, since it uses CO_2 as carbon source (Kuenen, 2008; Strous et al., 1998).

Many configurations of PNA exists and they differ in design, growth media and reactor type (Ronan et al., 2021; Szatkowska & Paulsrud, 2014). The partial nitrification and anammox processes can be separated in a two-stage process or be combined in a one-stage process. Strategies for biomass retention include granular sludge, activated sludge and biofilm on carriers. Finally, reactor types typically used are moving bed bioreactors (MBBRs) and sequencing batch reactors (SBRs) (Szatkowska & Paulsrud, 2014). Several PNA-processes are used in full-scale water treatment systems of high-strength wastewater, for example the DEMON-Anammox (Wett, 2007) and the AnitaMox process (Lemaire & Christensson, 2021). The basic process strategy of PNA is implemented in around 200 full-scale facilities around the world (Podmirseg et al., 2022).

The AnitaMox-process is a one-stage carrier and sludge-based MBBR process (Lemaire & Christensson, 2021). It can work as a pure MBBR or as a combination between biofilm and flocs (integrated fixed film and activated sludge/IFAS). The principle is that partial nitrification happens in the aerobic layers of the biofilm and/or in the flocs, while anammox works in the anoxic layers of the biofilm on the biocarriers. The process meets the requirement of long retention of anammox through permanent biocarrier retention in the reactor. The Anita-MOX process was developed by Krüger Kaldnes and is currently in use at "Nordre Follo Renseanlegg" in the South-East of Norway.

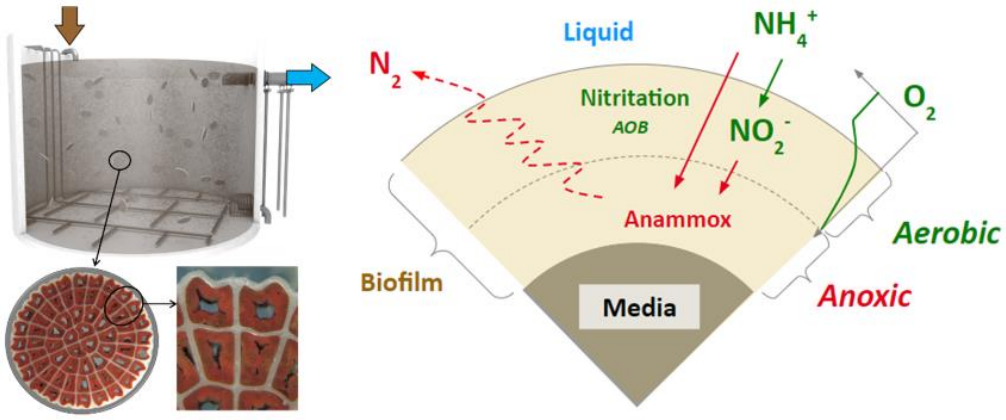

Figure 1-3. Schematic representation of biofilm layers on Anita-MOX carriers with aerobic and anoxic zones. NH_4^+ will diffuse into both layers; anoxic and aerobic. The AOB in the outer layer will use oxygen to oxidize NH_4^+ to NO_2^- and anammox, which sit protected against oxygen in the inner layers of the biofilm, perform the anoxic ammonium oxidation to make N_2 which diffuses out of the biofilm. The figure is from Lemaire and Christensson (2021).

Processes such as Anita-MOX are typically used to treat the high-strength industrial wastewater mentioned above, but they have also been applied to sidestreams of high-strength wastewater in municipal wastewater treatment systems (Trinh et al., 2021; Xu et al., 2015). Municipal mainstream wastewater has a much lower ammonia concentration than the high-strength wastewater, only up to 75 mg N/L (Ronan et al., 2021). However, municipal wastewater treatment usually includes anaerobic treatment of organic sludge in digester tanks and this process results in an effluent of high ammonia concentrations, low C/N-ratio and temperatures around 25-40 °C, which are optimal for anammox (Kartal et al., 2010; Kuenen, 2008; Szatkowska & Paulsrud, 2014). PNA can then be applied to this high-strength sidestream.

1.3.2.2 Low-strength wastewater treatment

Although anammox in nature performs at low concentrations (Dalsgaard & Thamdrup, 2002), applied anammox processes are still limited to high-strength wastewater such as sidestream processes in municipal wastewater or industrial wastewater treatment (Trinh et al., 2021; T. Tan & Shuai, 2015; Xu et al., 2015). One study reports successful use of the AnitaMox-process in a pilot plant treating mainstream wastewater, but with alternating feeding of sidestream (700-1000 mg N/L) and mainstream (40-60 mg N/L) wastewater (Lemaire et al., 2014). Research on applying anammox directly to the mainstream municipal wastewater of "low-strength" wastewater and lower temperatures is on-going. Challenges include high C/N-ratios leading to denitrifiers outcompeting Anammox bacteria, selection of the ammonia-oxidizing bacteria (AOB) over the nitrite-oxidizing bacteria (NOB), sufficient retention of Anammox-bacteria given their long generation time (Trinh et al., 2021; Xu et al., 2015) in addition to the low nitrogen concentrations and temperatures.

Some studies have examined the potential of applying the PNA process to low nitrogen concentrations. Hu et al. (2013) and Persson et al. (2017) lowered the ammonia stepwise from 630 to 70 mg N/L and 500-45 mg N/L, respectively. The first-mentioned dosed ammonium and nitrite, while the latter dosed only ammonium. Both studies reported successful PNA with a microbial community dominated by anammox and AOB, and some NOB and denitrifiers. Another study fed 20 mg NH_4^+-N/L for the whole experimental period and achieved stable ammonium- and total nitrogen removal efficiencies of 90% and 70%, respectively (Laureni et al., 2016). The amount of organic compounds in

the studies differed. Persson et al. (2017) and Laureni et al. (2016) had a C/N ratio between 0.6-1.1 and 3.5, respectively, while Hu et al. (2013) did not dose any carbon to the system. Anammox-performance is generally believed to be better under low C/N ratios (0.0-1.5; (Trinh et al., 2021) and it has been suggested that heterotroph denitrifiers may outcompete anammox at C/N ratios higher than 0.5 (Jenni et al., 2014).

To select for PNA activity, it is crucial to control the concentration of dissolved oxygen (DO). There must be enough oxygen for AOB to perform partial nitrification, but low enough to suppress NOB activity and enhance the anaerobic conditions for anammox. AOB are regarded as more competent oxygen competitors than NOB (Li et al., 2011; Qiu et al., 2019; Tappe et al., 1999). Therefore, partial nitrification can be selected for by controlling the aeration rate. For PNA systems a DO concentration of around 0.2 mg/L O_2 is preferable (Qiu et al., 2019). To select AOB and suppress NOB activity, intermittent aeration (IMA) has been suggested as a good strategy (Xu et al., 2015). IMA are aeration patterns with on and off-periods and it therefore provides low rates of aeration by introducing periods without oxygen supply. Several studies have shown the success of this strategy in keeping an appropriate DO concentration for PNA (Li et al., 2011; Qiu et al., 2019) and selecting for AOB over NOB (Xu et al., 2015). To remove DO, flushing the reactor with an inert gas, such as N_2, argon or CO_2 can be done (Hu et al., 2013). Another strategy is to dose some organic carbon to the system. This promotes the growth of heterotroph bacteria in the biofilm and since they consume DO, they contribute to lowering the total DO concentration in the system.

1.4 Methods to study microbial communities

Microbes cannot be studied in the same way as other perceptible species. Traditionally, microbes were studied under microscope and by culture-dependent methods. However, it is impossible to distinguish microorganisms only by morphological traits and only a small fraction (around 1%) of environmental microorganisms can be cultured with current techniques (Hahn et al., 2019). Therefore, the methods for studying microbial communities were limited. When the culture-independent molecular techniques to study microorganisms came along in the 1980s (Head et al., 1998), they revolutionized studies on microbial communities, microbial ecology and related fields.

The bacterial 16S rRNA gene encodes for the small subunit of ribosomal RNA in prokaryotes and has been particularly important for the development of the culture-independent molecular techniques for studying microbial communities. This gene consists of several variable regions (V1-V9) with highly conserved regions in between. The conserved regions are used as targets for broad-coverage primers in polymerase chain reaction (PCR) which makes it possible to amplify specific regions of the gene common for most of the populations in a bacterial community by using the total DNA extracted from the community as a template. The variable regions of the gene can be used to separate and taxonomically place different microbes and this makes the gene suitable for analyzing genetic diversity, taxonomy and phylogeny in addition to community structure (Bodor et al., 2020). The development of PCR was crucial for using the 16S rRNA as a marker gene for genetic analysis. Both DNA and RNA extracts can be used for microbial 16S rRNA analysis with PCR. DNA represents all bacteria in a sample, including rare or dormant taxa in addition to dead microbes, while RNA represents the actively growing bacteria in the community or the active part of the community. RNA extracts can be used by reverse transcribing the RNA, followed by PCR of the RNA's complementary DNA as template. (Slatko et al., 2018).

The possibility of sequencing many samples consisting of a mix of different DNA-sequences arose with so-called next generation sequencing (NGS) or massive parallel sequencing (MPS). MPS offered the possibility of sequencing thousands of different sequences simultaneously. It also offered a "deeper" sequencing of environmental samples through a higher number of DNA reads, which made it

possible to identify more of the rare bacterial populations. The MPS technology developed fast in the two last decades and the dominating MPS-technique today is Illumina-sequencing (Slatko et al., 2018).

1.5 Study aims

This thesis is part of a larger research project called "RASbiome" funded by the ERA-NET Cofund BlueBio. Its overall aim is to investigate the potential use of alternative microbial processes to improve nitrogen-management in RAS. One of the investigated alternative microbial processes is PNA. The aim was to adapt PNA carriers from high TAN concentrations to low TAN concentrations and low temperatures, relevant for RAS conditions. Active PNA carriers from an AnitaMox process are supplied to the project by Nordre Follo wastewater facility.

PNA-based water treatment in RAS would have many benefits, most importantly the possibility of removing nitrogen from the rearing water and the effluent water. To our knowledge, treatment processes based on anammox or PNA have not previously been adapted to TAN concentrations as low as in RAS for smolt production. Compared to other wastewater systems, ammonium concentrations are radically lower in RAS (around 2 mg/L N). However, anammox bacteria have been found in several RAS biofilters (Lahav et al., 2009; Tal et al., 2006). Temperatures of RAS are similar to those of temperate mainstream municipal wastewater, around 11-13°C. Since RAS-water has a low C/N ratio and often use MBBR, it might be suitable for PNA-based treatment. Furthermore, RAS is fundamentally different from wastewater treatment since water is recycled, instead of passing through just once like it does in wastewater plants. So even if efficiency of anammox is lower at RAS conditions, the removal rate might be high enough to keep ammonium levels below the critical limit.

The aim of this master project was to adapt the carriers and to obtain anammox and PNA at RAS-relevant ammonium concentrations through different strategies.

The specific objectives were to:

1. Obtain PNA in an MBBR reactor which is started at low TAN concentrations directly and fed ammonium and acetate
2. Obtain anammox in an MBBR reactor fed ammonium and nitrite, where the ammonium and nitrite concentrations are decreased gradually over time
3. Characterize the active bacterial biofilm communities and identify potential bacterial populations responsible for nitrogen conversions by Illumina sequencing of 16S rRNA amplicons based on RNA extracts

2 Methods

Two MBBR reactors were operated during my lab work. The aim was to adapt the PNA carriers delivered from Nordre Follo to low TAN concentrations and still achieve anammox activity. The reactors had different strategies for operation and for lowering the TAN concentration. One reactor was operated anaerobically and fed ammonium and nitrite in a ratio of 1 to select purely for anammox. The nitrogen concentration was lowered gradually over a period of 10 months (Day 1-298). This reactor was called the AMX reactor (read as "The anammox reactor"; abbreviated "the AMX", read as "the anammox"). The second reactor was operated to select for PNA by feeding ammonium and aerating with intermittent aeration. It was also fed acetate as a DO-control strategy. This reactor was called the PNA reactor (or "the PNA") and was started directly at low TAN-N concentration. More details follow in section 2.1.

The work on these reactors was shared between myself and Zhitao Huang, a post-doc working in the project. I was involved in the operation and measurements of both reactors, but focused especially on the optimization of the PNA. I am using the data from the project until 14.02.22, but colleagues continued the work after my period was over.

2.1 Experimental design

Carriers with active PNA communities were delivered from the municipal wastewater treatment plant "Nordre Follo Renseanlegg" in two batches, on the 23.04.21 and 17.09.21, respectively. The carriers came from an environment with high COD and NH_4^+ concentrations since they had been used to treat high-ammonia sidestreams from an anaerobic digestion process in an Anita-MOX process. Conditions of the Anita-MOX process at "Nordre Follo Renseanlegg" are listed in Table 2-1. The carriers are K5 carriers with $800 m^2/m^3$ of surface area (Veolia, n.d.).

Table 2-1. AnitaMox average process values from Nordre Follo wastewater plant 2021 (Personal communication, Maria Dremé, Nordre Follo Renseanlegg).

Parameter	Values
Influent NH_4^+-N	800 mg/L
Influent pH	8
Influent temperature	26 °C
Influent COD	3000 mg/L
Effluent NH_4^+-N	130 mg/L
Effluent NO_2^--N	0.8 mg/L
Effluent NO_3^--N	15-20 mg/L
Effluent pH	6,9
Effluent temperature	32 °C
Effluent DO	0.4 mg/L
Reactor HRT	34 hours

The AMX reactor was set up first with carriers from the first batch of carriers from Nordre Follo. The PNA was set up some months later with the carriers from the second batch. The reactors were being fed with identical synthetic feeding water, except for the concentrations of ammonium, nitrite and acetate (Table 2-3). The synthetic feeding water was made in batches of 60 L. They were stored at 20 °C. Synthetic feeding water is sometimes referred to as "media". Growth media of the biofilm will be referred to as "biocarrier" or "growth media".

The PNA reactor was fed a synthetic feeding water of 10 mg/L NH_4^+-N, in addition to 8.82 mg/L of acetate-carbon. The acetate dosing was intended as a DO-control strategy by maintaining heterotroph growth and thereby heterotroph consumption of DO to keep the DO concentration low inside the reactor. The acetate-concentration corresponded to 9.3 mg/L COD or a C/N ratio = 0.35 (see appendix B), which is in the same order of magnitude as in a commercial RAS for smolt production in Norway (Personal communication, Ingrid Bakke). Intermittent aeration of air was used to aerate the system. The aeration was tuned to get higher removal of nitrogen in the reactor.

The AMX started with being fed 300 mg/L NH_4^+-N and 300 mg/L NO_2^--N and the concentration was lowered stepwise to 10 mg/L of both compounds. The reduction in concentration happened in three steps throughout the 10-month period. The nitrogen concentrations for different stages are shown in Table 2-2. Since anammox requires low DO, the reactor and its feeding water was continuously being stripped of oxygen by N_2-flushing to keep the DO concentration below 2mg/L in the incoming feeding water and below 0.5mg/L inside the reactor. The DO concentration was continuously monitored for the duration of the reactor operation and the reactor and its feeding water was flushed at regular intervals of 2min/72 min.

Table 2-2. NH_4^+-N and NO_2^--N concentrations of stages 1-4 in the AMX reactor.

Day	Stage	NH_4^+-N-concentration	NO_2^--N-concentration
11-70	1	300	300
71-108	2	150	150
109-202	3	50	50
203-298	4	10	10

The synthetic feeding water for the reactors also contained macronutrients and trace elements (stock solutions) and was made with tap water. The composition of micronutrients and trace elements was from Gonzalez-Silva (2017) and is listed in appendix C. Table 2-3 shows the complete composition of synthetic feeding water for both reactors, including concentrations of TAN-N, NO_2^--N and acetate-COD.

Table 2-3. Synthetic feeding water composition for PNA and AMX reactors.

Compound	Unit	PNA	15L AMX
NH_4HCO_3	mg/L NH_4^+-N	10	*
$NaNO_2$	mg/L NO_2^--N	-	*
$NaCH_3COO*3H_2O$	mg/L COD	9.4	-
KH_2PO_4	g/L	0.025	0.025
$CaCl_2 \cdot 2H_2O$	g/L	0.3	0.3
$MgSO_4 \cdot 7H_2O$	g/L	0.2	0.2
$NaHCO_3$	g/L	1	1
Trace element solution 1	mL/L	1	1
Trace element solution 2	mL/L	1	1

* given in table 2.2

Table 2-4 shows the reactor volume, filling degree, surface area and hydraulic retention time (HRT) of each reactor. Calculation of reactor-specific surface area can be viewed in appendix 6.1. The reactor setup is shown in Figure 2-1.

Table 2-4. Volume, filling degree and surface area for each reactor.		
Parameter	PNA	AMX
Volume (L)	15.2	15.2
Filling degree (%)	39	39
Surface area (m^2)	4.74	4.74
HRT* (hours)	42.7	42.7

* Theoretical HRT based on flowrate.

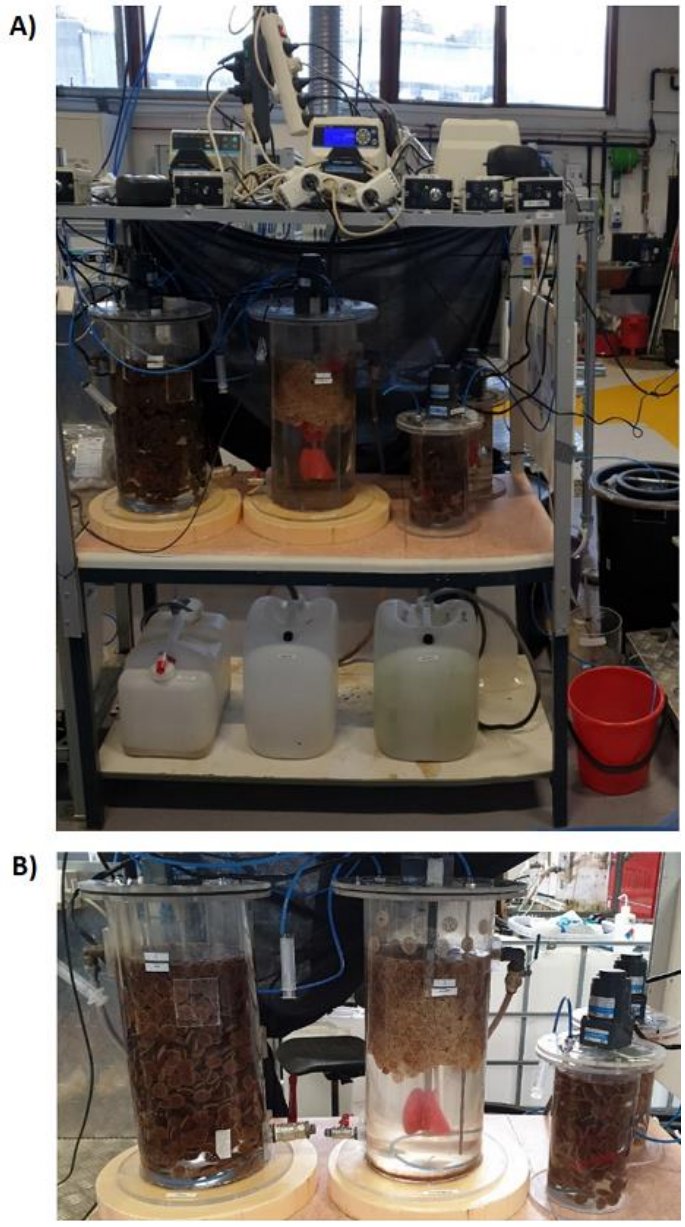

Figure 2-1. The MBBR reactors operated in this study. From left to right: PNA reactor, AMX reactor, and another small reactor operated as part of the RAS Biome project. A) The overall setup. On top of the reactors are the media pumps, rotor motors and aeration pumps. Below the reactors are the waste outlets. To the right is one of the tanks used to store synthetic feeding water. B) Close-up of the reactors.

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2.2 Monitoring of water quality parameters

The parameters to monitor water quality in this study included pH, DO, temperature, and the concentration of the three nitrogen compounds NH_4^+-N, NO_2^--N and NO_3^--N. These parameters were measured in the influent and effluent of both the reactors twice a week. In addition, chemical oxygen demand (COD) was measured twice a week in the influent and effluent of the PNA reactor, and once a week in the effluent of the AMX reactor. Depending on whether the sample was filtrated or not, the COD was called total COD (T-COD) or dissolved COD (D-COD), respectively. The unfiltrated water sample may include particles and cell mass, while the filtrated (0.45 µm filter) contained mostly dissolved, and not particulate compounds. Both T-COD and D-COD were measured in the AMX effluent, while in the PNA reactor, mostly D-COD was measured. No replicates were made for the concentrations of NH_4^+-N, NO_2^--N, NO_3^--N, and COD.

2.2.1 Sampling for water quality analyses

Samples for water quality analyses were taken from the influent and effluent of the reactors at the same time. Effluent samples were taken from the top of the reactor lids through designated sample points from within the reactors. Influent samples from the AMX were taken either directly from the feeding water batch tank or from the inlet tube in the reactor lid. For PNA, the influent samples were taken from the surface of the feeding water batch from Day 1-15, and from below the surface and after mixing the batch water by mechanical stirring from Day 15-138.

All water samples were taken with 20 mL syringes and the syringes were rinsed three times with water from the relevant reactor before taking the sample. Samples of both influent and effluent were filtered with 25mm Syringe Filter w/0.45µm one-time filters of polypropylene or polyethersulfone (VWR), except for the total COD samples.

2.2.2 Measurements of nitrogen compound and COD concentrations

The concentration of NH_4^+-N, NO_2^--N and NO_3^--N were measured by cuvette kits produced by Hach Lange. These kits contain ready-to-use test cuvettes with chemicals which form specific compounds in reaction with ammonium, nitrite and nitrate, respectively. These compounds have color, which makes it possible to quantify the amount of the compound in the cuvette by spectrophotometry. Spectrophotometry is a method which measures how much a chemical compound absorbs light by letting a beam of light pass through the sample and measure the light intensity (Vo, 2022). Since each compound absorbs light over a certain wavelength, different compounds can be separated and quantified by this method.

The Hach Lange kits have a certain range they can be relied upon to measure precisely within. In this project several different Hach Lange kits were used. Samples with higher concentrations than the range were diluted appropriately with MQ-water to fit the range of the kit.

The kit LCK303 (2-47 mg/L NH_4^+-N) was used to measure NH_4^+-N concentration for all reactors. LCK342 (0.6-6 mg/L NO_2^--N) and LCK341 (0.015-0.6 mg/L NO_2^--N) were used for NO_2^--N concentration and LCK340 (5-35 mg/L NO_3^--N) and LCK 339 (0.23-13.5 mg/L NO_3^--N) were used for NO_3^--N concentration. COD concentrations were determined with Hach Lange kits. The LCI400 kit (Hach) was used from Day 1-64 and LCK1414 was used from Day 64. The analyses were performed as described by the manufacturer.

2.2.3 Monitoring of feeding water for the PNA reactor

Since acetate is an easily degradable carbon source and the feeding water was not kept sterile, the feeding water was monitored to check whether the acetate-COD was being degraded in the tank. This was done by monitoring the concentration of D-COD and DOC for a possible drop during storage. In addition, water samples from one batch were spread on TSA agar plates to check for bacterial growth of heterotrophs living of the acetate in the feeding water. Three feeding water batches were monitored separately.

Water samples for measuring D-COD and DOC concentrations included a sample of the tap water used to make the batch, a sample of the newly made batch (Day 0) and samples of the batch on storage day 1, 2 and 3 (Day 1, 2 and 3). This was done for all three monitored batches.

Bacterial growth in the feeding water was monitored only for the first of the three batches. Water samples were taken from the newly made batch (Day 0) and from the batch after being stored at 20°C for 3 days (Day 3). 100µL of the water samples from Day 0 and Day 3, respectively, were spread on general TSA medium agar plates in a 1:10 dilution series from the undiluted sample to a 10^{-4} dilution. Three replicates were done for each sample and each dilution, to a total of 30 agar plates (2 samples x 5 dilutions x 3 replicates). The agar plates were incubated at 20°C for 7 days.

2.3 Ex-situ carrier activity tests

The capacity of ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and denitrifying bacteria (DNB) on the carriers was tested by exposing the carriers to solutions of NH_4^+, NO_2^- and NO_3^-, respectively. The concentrations of the nitrogen compounds were measured over time and associated conversion rates calculated. These tests are also referred to as potential activity tests of AOB, NOB and DNB on the carriers. Such tests were performed on the original carriers delivered by Nordre Follo the 17th of September and on the adapted carriers sampled from the PNA and AMX the 24th of January (corresponding to Experimental Day 113 for PNA and Experimental Day 277 for the AMX). The tests were performed in 0.5L glass bottles containing media and carriers, and the test was run over a period of 48 hours.

For the test of the original carriers from Nordre Follo, 40 carriers were used for each test (AOB, NOB and DNB). For the test of the adapted carriers, 35 carriers were used for each test for the PNA and AMX test bottles. The sampled carriers were dried by putting them on a paper towel to soak up water caught in the biofilm and then put into the test bottles containing media.

For the AOB, NOB and DNB tests the carriers were exposed to media containing 30 mg/L NH_4^+-N, 30 mg/L NO_2^--N and 30 mg/L NO_3^--N, respectively. The DNB test media also contained some acetate-carbon in a C/N-ratio of 5-6. In addition, all media contained the macronutrients and trace elements in the same concentration as given in table 2.3. AOB and NOB test bottles were aerated with aquarium pumps to a DO concentration of 8-9 mg/L, while the DNB test bottles were flushed with N_2-gas after each sample was taken and then shut firmly to keep the concentration of DO low. The DNB test bottles were stirred slowly at 100-150 rpm. The setup can be viewed in Figure 2-2.

DO concentration and pH were measured at regular time intervals during the 48-hour test period. In addition, water samples were saved for nitrogen concentration measurements for each sample point. The samples were taken with 20 mL syringes and filtered through 0.45µm filters.

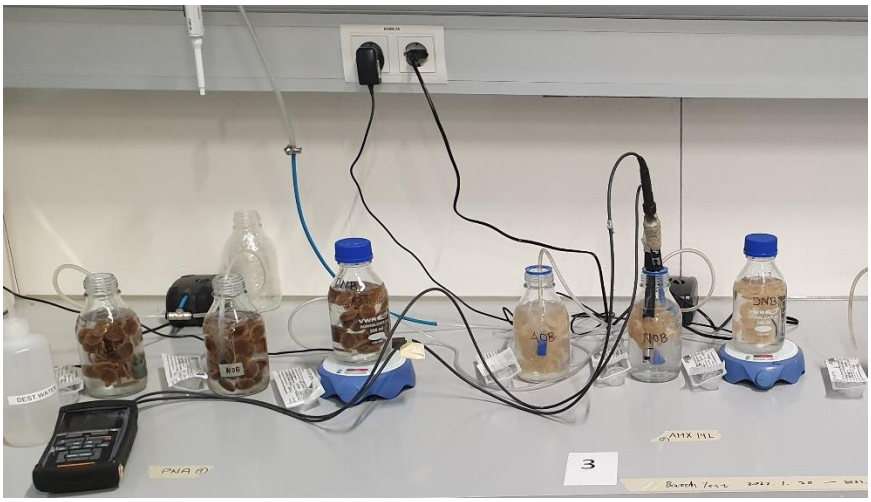

Figure 2-2. The ex-situ batch test performed on the adapted carriers from the PNA and AMX reactors. A total of six bottles were organized into groups of three; AOB, NOB and DNB test bottles for both the PNA and AMX. They were given each its respective synthetic media and carriers and DO, pH and nitrogen-concentrations were monitored for 48 hours.

2.4 Anammox activity tests

To get an estimate of the amount of anammox bacteria in the reactors, test kits from Hach Lange (LCK411) were used. These kits contain cuvettes which measure the concentration of heme c in a sample. Heme c is a very important co-factor in the main metabolic reactions of anammox bacteria (Ma et al., 2019) and is a good indicator of anammox presence and performance. Since the kit determines det concentration of heme, not the true activity rates, the results of these tests are viewed as an estimate of concentration of anammox present or as potential anammox activity, not a true activity estimate.

The kit is developed to analyze granular sludge, but with some adjustments we used the kit for biocarriers. Half a carrier was cut into pieces and put in a standard 10 mL tube (shown to the right in Figure 2-3). This tube corresponded to the LCW906 tube in step 3 of the kit protocol (appendix E; Hach, 2019) along with 5 mL of the reactor water. Step 4-12 of the procedure was followed as described by the manufacturer's protocol. These tests were performed by post-doc Zhitao Huang.

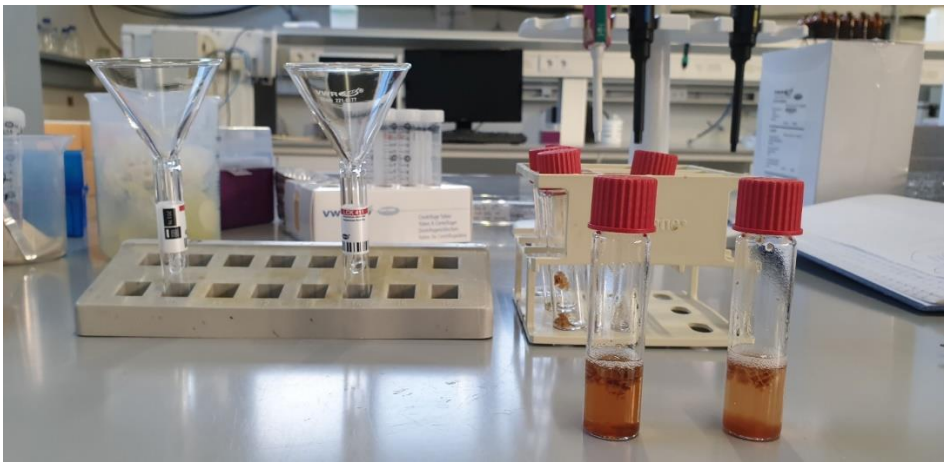

Figure 2-3. Anammox activity tests (Hach) performed on carriers from both reactors. Half a carrier was used for each test. These tests were performed according to the manufacturer's protocol except for using cut biocarriers with biofilm instead of granular sludge.

2.5 Weight of biofilm carriers

To estimate the amount of biomass on the carriers, dry weight of carriers with biofilm was measured for the original carriers delivered by Nordre Follo in the beginning of the experimental period and for carriers adapted to the PNA and AMX reactors at the end of the experimental period.

Sampled carriers were put on paper towels to soak up the water in the biofilm. Then, the carriers were put on individual aluminum plates and dried in an oven at 110 °C for around 45 minutes. The weight of the individual plates had been noted down previously. After the drying of the carriers, the weight of the plate with the dried carriers were weighed again. Five carriers were sampled of the original carriers, and three were sampled for each of the PNA and AMX reactors.

2.6 Biofilm microbiome analyses

2.6.1 Sampling

For the microbiome analyses, biocarriers with biofilm were sampled regularly over the experimental period of both reactors (see appendix E for sampling dates) to follow the development of the biofilm community over the experimental period. The samples dates for all three reactors are listed in appendix D. Three carriers were sampled from each reactor on each sample date. The water in the carriers was carefully soaked up by putting the carriers on paper towels before storing them at -20°C until further processing.

2.6.2 Microbial community analysis

The biofilm microbiomes of both reactors were characterized by Illumina sequencing of 16S rRNA amplicons. This work was performed in collaboration with post-doc Zhitao Huang.

To extract the total DNA/RNA from the samples Zymo Research's "Quick-DNA/RNA™ Magbead – CoPurification" kit was used. First, the bacterial cells in the biofilm on the carriers were lysed mechanically as follows: A quarter of each carrier was used for each DNA/RNA extraction and this quarter was cut into small pieces with scissors. RNaseZap® was used to clean the work surface, gloves and instruments used for cutting the carriers to minimize the carrier's exposure to RNase, a

common enzyme on skin which causes RNA breakdown. The pieces of the cut quarter carriers were put in standard 2 mL tubes with ~0.4 mL (equivalent to 0.55g) 0.1 mm Precellys 24 glass beads (Bertin Technologies) and DNA/RNA Shield™ solution. The solution has DNA/RNA conserving properties (ZYMO RESEARCH, n.d.) and was provided in the kit. The samples were mechanically lysed in a Precellys 24 Tissue Homogenizer (Bertin Technologies) at 5500 rpm for 2x30 seconds with a 30 second break.

The "Quick-DNA/RNA™ Magbead – CoPurification" kit used for DNA/RNA extraction is based on the use of magnetic beads and adapted to being used on a Kingfisher instrument. The "Thermo Scientific KingFisher Flex" is an automated benchtop extraction instrument which facilitates the simultaneous extraction for 24 or 96 samples depending on well plate used (ThermoFisher, n.d., a). The protocol for extraction provided in the kit was followed as it is described for swab samples.

To achieve RNA-based microbiome analyses reflecting the active part of the bacterial communities, around 50µL of the DNA/RNA extracts were treated with DNase to remove DNA and achieve a pure RNA sample. The reagents and protocol for DNase treatment were provided in the "Quick-DNA/RNA™ Magbead – CoPurification" kit (Zymo Research) and the protocol from the manufacturer was followed as it is provided. The DNase treatment was also performed on the Kingfisher. Elution volume for extractions and DNase treatments was 50 µL.

Then, complementary DNA was synthetized from the DNase-treated RNA using "iScript™ cDNA Synthesis Kit" (BIORAD) by PCR. The reaction mix and protocol was followed as it was given by the manufacturer, with the exception that the reagent volumes in the mix were halved. The RNA template volume was kept as suggested in the protocol (1 µL).

After the DNase treatment, the resulting RNA and cDNA were PCR-amplified and compared on gels to check the efficiency of the DNase-treatment. If the DNase treatment was efficient, no PCR amplification would be expected when RNA was used as template. This test indicated that DNA was still present in the DNase treated RNA extracts. The RNA extracts were therefore run a second time through the DNase treatment with some adjustments. Instead of 10 minutes incubation, 15 minutes were used, and sample size was reduced from 50 µL to 20 µL. cDNA was then synthesized again for the double DNase-treated RNA extracts as explained previously.

After the second DNase treatment, DNA and RNA concentrations in the DNase-treated extracts were measured on a Qubit Fluorometer. The Qubit quantifies nucleic acids by detecting fluorescent dyes that emit only when bound to the targets of interest (ThermoFisher, n.d., b), such as DNA and RNA. The Qubit™ dsDNA HS Assay Kit (Invitrogen) and Qubit™ RNA BR Assay Kit (Invitrogen) were used, and the protocol was followed as described by the manufacturer.

The cDNA was then used as a template for PCR amplification using the broad coverage primers Ill-341-KI and Ill-805-R (Table 2-5). The reaction mix consisted of 1x Phusion buffer HF (Thermo Scientific), 0.3 mM of each primer (Sigma-Aldrich), 200 µM each dNTP (VWR), 0.02U/µL Phusion Hot Start II DNA Polymerase (Thermo Scientific), and 1 µL undiluted cDNA as template. The total reaction volume was 25 µL. The cycle conditions involved an annealing temperature of 55°C and 34 thermal cycles (Table 2-6). The PCR for the amplification of the 16S rRNA gene was conducted on a T100™ Thermal Cycler (BioRad).

Table 2-5. Names and sequences of PCR primers used for the 16S rRNA amplification for Illumina sequencing. Illumina adapters are marked in bold.

Primer name	Sequence	Target region
Ill-341F-KI	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN CCT ACG GGN GGC WGC AG-3'	V3
Ill-805-R	5'GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN GAC TAC NVG GGT ATC TAA KCC-3'	V4

Table 2-6. Temperature cycling conditions in the 16s rRNA PCR amplification.

Step	Temperature [°C]	Time	
Denaturation	98	2 min	
Denaturation	98	15 sec	
Annealing	55	20 sec	x 34
Elongation	72	20 sec	
Final elongation	72	5 min	

The quality and quantity of the resulting PCR products, in addition to possible contamination of PCR products was examined using agarose gel electrophoresis. A 1% agarose solution was prepared by dissolving 1 g agarose in 100 mL 1x TEA-buffer by heating the solution (see appendix F for recipe for TEA-buffer). The 1% agarose solution was poured into gel chambers with gel combs and was allowed to settle (20-30 minutes). Samples (5 µL) mixed with 1 µL 6x DNA Loading Dye (Thermo Scientific) were applied to the gel wells along with Gene Ruler™ 1 kb Plus DNA Ladder (Thermo Scientific) as size marker. The gels were run at 110 V for 30-35 minutes. The DNA fragments were stained by adding GelRed® (Biotium) to the agarose solution before pouring it into the chamber and the DNA products were visualized in a UV-light chamber.

The further processing of the PCR products to generate the amplicon library that was sent to sequencing, was performed by technician Amalie Mathisen in the "Analysis and Control of Microbial Systems" research group at the Department of Food Science and Biotechnology. In brief, this included a first normalization and purification of the PCR products by using the Sequal Prep Normalization plate Kit (Invitrogen) as described by the manufacturer's protocol. Next, the normalized and purified PCR products were indexed in a second PCR, using the Nextera XT Index Kit Set D (Illumina) to provide each PCR product with a unique combination of eight forward and twelve reverse sequence tags. Each primer sequence index (2.5 µL each) were added to a PCR reaction mixture consisting of 1x Phusion buffer HF (Thermo Scientific), 0.25 mM dNTP (VWR), and 0.015 U/µL Phusion Hot Start DNA polymerase (Thermo Scientific), to a total reaction mix volume of 25 µL. The indexing PCR reactions were performed as described above (Table 2-6), except for a lower number of PCR cycles (12).

The indexed PCR products were examined using agarose gel electrophoresis as described above, and then normalized and purified using the Sequal Prep Normalization plate Kit (Invitrogen). Finally, the PCR products were pooled, and concentrated by using Amicon Ultra 0.5 Centrifugal Filter units (Merck Millipore, Ireland) following the manufacturer's protocol. The concentration and purity of the concentrated product was measured using NanoDrop™ One (Thermo Scientific) and the quality of the final sample was examined using agarose gel electrophoresis. The final amplicon library was sent to the Norwegian Sequencing Centre (NCS) for sequencing on one run on an Illumina MiSeq instrument (Illumina, San Diego, CA) with V3 reagents (Illumina).

Due to a delay in the delivery of the microbiome sequencing data set, the data processing and analysis was mainly performed by supervisor Ingrid Bakke and technician Amalie Mathisen, but was explained for the candidate. In brief, the sequencing data were processed using the USEARCH pipeline (version

11; https//www.drive5.com/usearch/). The command Fastq_mergepairs was used to merge forward and reverse reads, remove primer sequences and filter out reads shorter than 390 base pairs. A zero radius Operational Taxonomic Unit (zOTU; corresponding to the term Amplicon Sequencing Variants; ASVs) table was made by denoising using the Unoise3 command. As recommended in the Usearch documentation, zOTUs with abundances lower than eight reads in all samples were removed. Taxonomy assignment was performed applying the Sintax script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP (Ribosomal Database Projects) reference data set v16.

PAST v. 4.05 (Hammer et al., 2001) was used for statistical analyses. The Bray-Curtis similarity was used for comparison of community profiles within and between sample groups. Principal coordinate analysis (PCoA) was performed based on Bray-Curtis. One-way PERMANOVA (permutational multivariate analysis of variance) based on Bray-Curtis similarities was performed to test whether there were significant differences in the microbial community composition between sample groups.

2.7 Nitrogen mass balances and removal rates

All calculations of nitrogen removal are based on the mass balance below (2.1).

Nitrogen removal
$= \Delta N\ [mg\ N/L] = (C_{NH_4^+\text{-}N,in} + C_{NO_2^-\text{-}N,in} + C_{NO_3^-\text{-}N,in}) - (C_{NH_4^+\text{-}N,out} + C_{NO_2^-\text{-}N,out} + C_{NO_3^-\text{-}N,out})$ (2.1)

Where

$$C_{NH_4^+\text{-}N,in} + C_{NO_2^-\text{-}N,in} + C_{NO_3^-\text{-}N,in} = \text{total } N_{in} = \text{total influent nitrogen}$$

and

$$C_{NH_4^+\text{-}N,out} + C_{NO_2^-\text{-}N,out} + C_{NO_3^-\text{-}N,out} = \text{total } N_{out} = \text{total effluent nitrogen}$$

The concentration of nitrogen compounds is presented as mg/L of NH_4^+-N, NO_2^--N and NO_3^--N, while nitrogen removal is presented as nitrogen removal in mg/L or as nitrogen removal rate (NRR; eq. 2.2). Nitrate production is presented as nitrate production rate (NPR) (eq.2.3).

$$NRR\ [\%] = \frac{C_{\text{total N,in}} - C_{\text{total N,out}}\ [\text{mg N/L}]}{total\text{-}N,in\ [\frac{mg}{L}]} * 100 \quad (2.2)$$

$$NPR\ [\%] = \frac{C_{NO3\text{-}N,out} - C_{NO3\text{-}N,in}\ [\text{mg N/L}]}{C_{total\text{-}N,in}\ [\frac{mg}{L}]} * 100 \quad (2.3)$$

Anammox activity is associated with a small nitrate production of 0.26 moles per mol NH_4^+ (Eq.1.8). If it is assumed that all nitrogen removal is due to anammox then the associated NO_3^- production can be calculated thus:

$$x + 1.32x = \text{total N rem.} + 0.26x$$
$$x = (\text{total N removed} / 2.06) * 0.26 \quad (2.4)$$

Denitrification activity is associated with nitrate/nitrite and organic carbon consumption. For denitrification, 3.5 g acetate or 3.74 g COD is needed for every 1 g nitrate-N (Chiu & Chung, 2003).

3 Results

3.1 Performance of the PNA reactor

The strategy for the PNA reactor was to lower the influent TAN concentration directly and to feed some acetate-carbon as a DO control strategy. The reactor was fed 10 mg/L NH_4^+-N as the sole nitrogen source and around 10 mg/L acetate-COD across the whole experimental period (Table 2-3). From Day 1-34 the reactor operated with the feeding water as the sole source of DO. Oxygenation was increased three times between Day 35 – 58 to get higher NH_4^+-N removal (see appendix D for intermittent aeration rates). The performance of the PNA reactor is reported for Day 1- 134.

The nitrogen removal rate (NRR; see section 2.7) gradually increased and reached around 74% towards the end of the experiment (Figure 3-1). Between Day 1-25 both influent and effluent NH_4^+-N oscillated, but from Day 25 influent NH_4^+-N was stable around 10 mg/L N and effluent NH_4^+-N began to sink. Sudden peaks in NRR, or the corresponding drops in effluent NH_4^+-N, were observed several times and are believed to be part of the adaptation process or due to an increase in the aeration. The last increase to aeration was done on Day 58, and the NRR continued to increase after that. The drop in effluent NH_4^+-N observed on Day 71 (marked * in Figure 3-1) was caused by the inlet media pump being left off for two days, thus leaving the reactor in batch mode. This drop was followed by the further lowering in effluent NH_4^+-N concentrations. The NRR increased over the whole experimental period and peaked at 91% on day 120. The average NRR of the last three days of operation was around 74%. There was no traceable production of NO_2^- or NO_3^- during the experimental period, and NO_2^- was always below the detection limit of the kit.

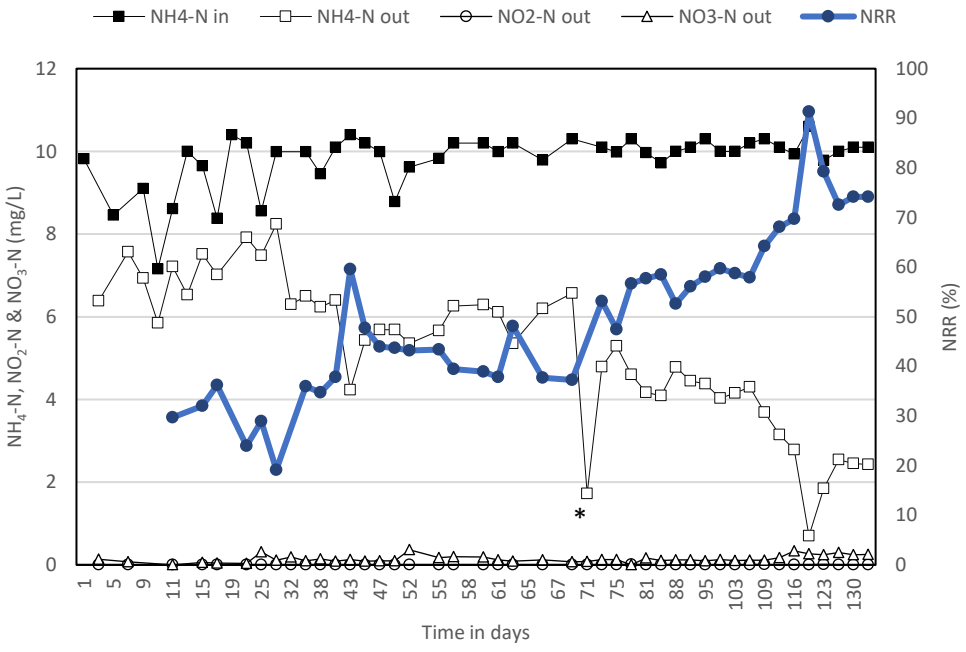

Figure 3-1. Concentrations (mg/L) of influent and effluent nitrogen compounds in the PNA reactor throughout the experimental period of 134 days. During this period the reactor was fed 10 mg/L NH_4^+-N and 9.3 mg/L COD. The aeration rate was increased on Day 35, 39, 52 and 58 (see appendix D). Influent samples were taken from the feeding water tank, while effluent samples were taken from inside the reactor. NH_4^+-N in: influent ammonium-N; NH_4^+-N out: effluent ammonium-N; NO_2^--N out: effluent nitrite-N; NO_3^--N out: effluent nitrate-N; NRR: nitrogen removal rate (%). *: inlet media pump had stopped, leaving the reactor in batch mode over the weekend.

The influent and effluent DO concentration were relatively stable during the experimental period (Figure 3-2), with an average of 6 and 0.26 mg/L, respectively (Table 3-1). Although the aeration was increased, the effluent DO concentration did not increase. This indicates that the added oxygen was being consumed immediately in the reactor.

Table 3-1. Average values and standard deviation (SD) of operation parameters between Experimental Day 1-134 for PNA.

Parameter	Average value	SD
DO in [mg/L]	6.06	1.06
DO out [mg/L]	0.26	0.05
pH in	7.95	0.30
pH out	7.72	0.27
D-COD in [mg/L]	15.67*	2.96*
D-COD out [mg/L]	15.99	4.13

*Excluding the point marked with a * in Figure 3-1

The dissolved-COD (D-COD) concentrations varied over time and the variation was larger than expected (Figure 3-2). Influent D-COD concentrations varied between 12-22 mg/L and were often higher than expected based on the amount that was theoretically added through acetate (9.3 mg/L D-COD) and tap water (DOC concentration in tap water is estimated to be around 3 mg C/L (Personal communication w/ Stein Østerhus Wold)). Effluent D-COD concentrations were sometimes higher and sometimes lower than the influent D-COD concentrations (Figure 3-2). The average D-COD concentrations in the influent and effluent were almost equal (Table 3-1).

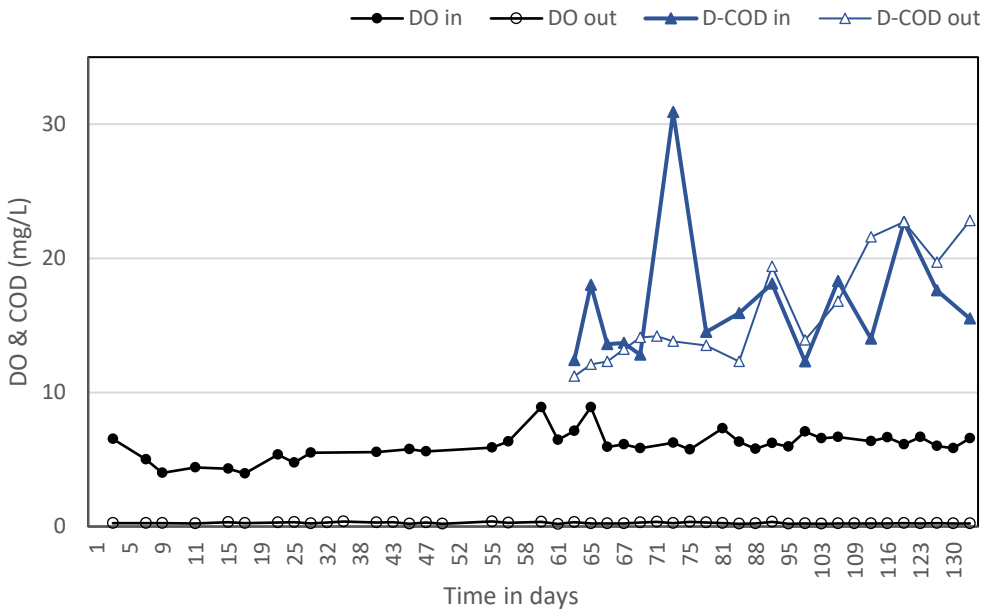

Figure 3-2. Influent and effluent D-COD and DO concentrations for PNA from Day 1-134. DO in: influent DO (mg/L); DO out: effluent DO (mg/L); D-COD in: influent D-COD (mg/L); D-COD out: effluent D-COD (mg/L). The D-COD concentration is only reported for Day 64-134. The concentrations from before Day 64 were measured with a kit which was meant for higher concentrations and these measurements were therefore disregarded.[1]

[1] The used kit from Day 1-64 (LCI400) had a range of 0-1000 mg/L COD and since the concentration in our system was closer to 10-20 mg/L, the measurements were significantly inaccurate.

3.1.1 Monitoring of COD concentration and bacterial growth in feeding water

Feeding water was made in batches of 60L and fed over 4-5 days. Since the COD concentrations varied more than expected, the COD concentrations in the feeding water were monitored to get an indication of whether acetate-carbon was being consumed and whether there was heterotrophic growth in the batch during storage.

Three batches of synthetic feeding water were monitored: Batch 1, 2 and 3. The D-COD and DOC concentrations were measured in a sample of the tap water used to make the batch, in the newly made batch (Day 0), and in the batch after storage for one, two, and three days.

The DOC concentrations were relatively stable over the three days of storage (Figure 3-3). The concentrations differed somewhat from each other between batches, but were stable within the batch. The D-COD concentrations varied more and generally gave a higher estimate of the organic carbon than the DOC concentrations. However, the D-COD concentrations generally showed the same trends as the DOC concentrations. The same was observed in the measurements of tap water. The DOC concentrations were relatively stable, between 2.8-3.8 mg/L (Table 3-2).

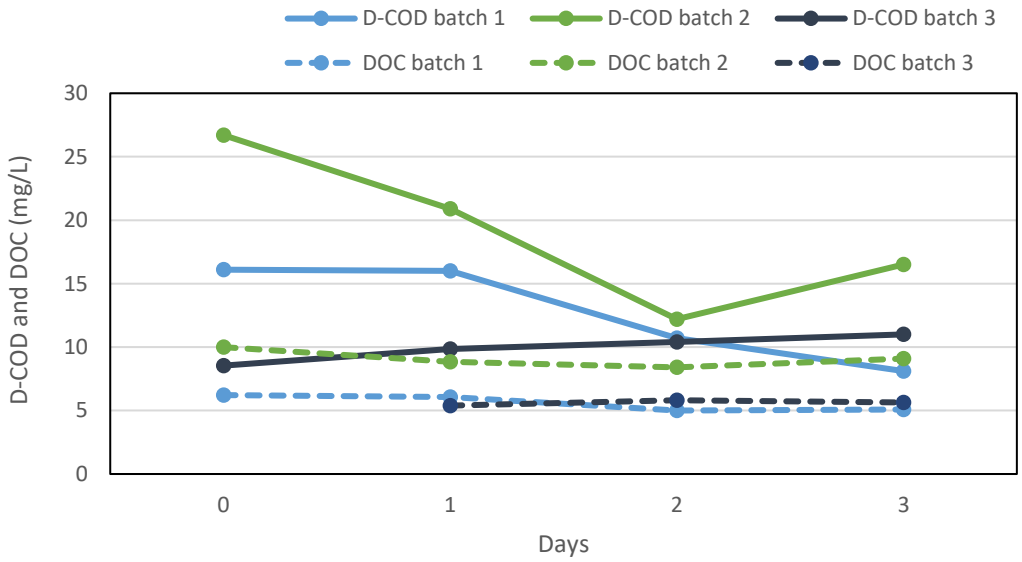

Figure 3-3. D-COD and DOC concentrations for three batches of synthetic feeding water containing acetate for the PNA reactor. Batch 1: Feeding water batch nr. 1 (fed Day 78-81); batch 2: feeding water batch nr. 2 (fed Day 92-95); batch 3: feeding water batch (fed Day 106-109). On the x-axis: Tap water: the sample of tap water used to make the respective batch; Day 0: sample of newly made batch (within 3 minutes); Day 1-3 are storing days nr. 1, 2 and 3. The DOC concentrations are averages of three replicates. Standard deviations for these DOC concentrations are too small to be visible in the figure.

Table 3-2. D-COD and DOC concentrations (mg/L) in tap water for the three batches monitored.

Day	D-COD	DOC
73	-	3.799
78	7.64	3.2490
92	4.27	2.8257
106	6.82	2.8958

The colonies on the TSA agar plates with water samples from a newly made batch and from storage day number 3 were counted after the 7-day incubation period. 1-2 colonies were observed on 7 plates out of 30 plates in total (Table 3-3). 5 of the plates with colonies were on plates with no dilution. These results do not seem to imply that acetate is being consumed in the batch tank due to heterotroph growth during storage of the batch.

Table 3-3. Number of bacterial colonies on TSA agar plates incubated with water samples from Day 0 (newly made synthetic water) and Day 3 (third day of batch storage) of a synthetic feeding water batch. 100 µL of the water sample was spread on each plate. Blank spaces indicate no colonies on the agar plate.

Dilution	Replicate	Day 0	Day 3
1	1		1
	2	1	1
	3	2	1
10^{-1}	1	1	
	2		
	3		
10^{-2}	1		
	2		
	3		
10^{-3}	1	1	
	2		
	3		
10^{-4}	1		
	2		
	3		

3.1.2 Mass balance calculations

Microbial nitrogen removal can be caused by either anammox or denitrification. To investigate whether the concentrations of nitrogen compounds in the PNA reactor fitted with a situation in which anammox were responsible for the N-removal, the expected nitrate concentration for that situation was calculated based on the stoichiometric equation of anammox (eq. 2.4) and the observed total nitrogen removed.

The observed nitrate-N concentration in the effluent was considerably lower than expected if anammox were responsible for all the observed nitrogen removal (Figure 3-4). This suggests that denitrification happened in the reactor.

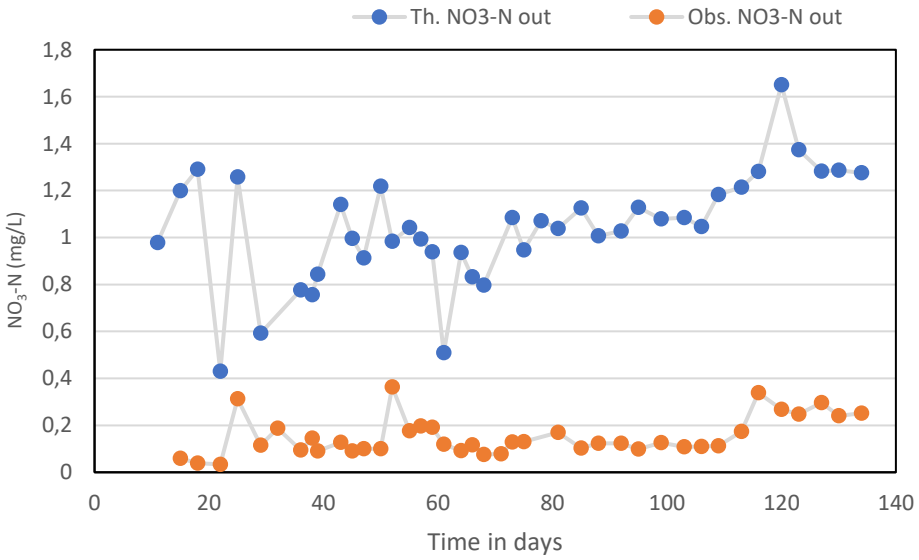

Figure 3-4. Theoretical effluent NO_3^--N concentration based on anammox activity and observed effluent NO_3^--N concentration. Th.NO_3^--N out: influent NO_3^-N (mg/L) + expected NO_3^--N production (mg/L) associated with anammox activity (eq.2.4); Obs. NO_3^--N out: observed NO_3^--N concentration in the effluent.

To get an indication of whether the nitrogen removal could be due to nitrification and denitrification instead of partial nitrification anammox, the theoretical consumption of DO and organic carbon was calculated for a situation in which all the observed nitrogen removal was due to nitrification and denitrification.

The theoretical consumption of DO (in mg/L) was calculated by using the observed concentration of consumed NH_4^+-N and the molar ratios of NH_4^+ and O_2 for AOB (eq.1.1) and the molar ratio for NO_2^- and O_2 for NOB (eq.1.2). The consumed DO in the system was lower than what was theoretically needed for the nitrification of the consumed NH_4^+-N to NO_3^--N (Figure 3-5). The theoretical DO needed for nitrification increased over the experimental period since the consumption of NH_4^+-N increased as well. However, the observed DO consumption in the reactor stayed around 5-7 mg/L. Therefore, nitrification can probably not explain the removed NH_4^+-N in the reactor.

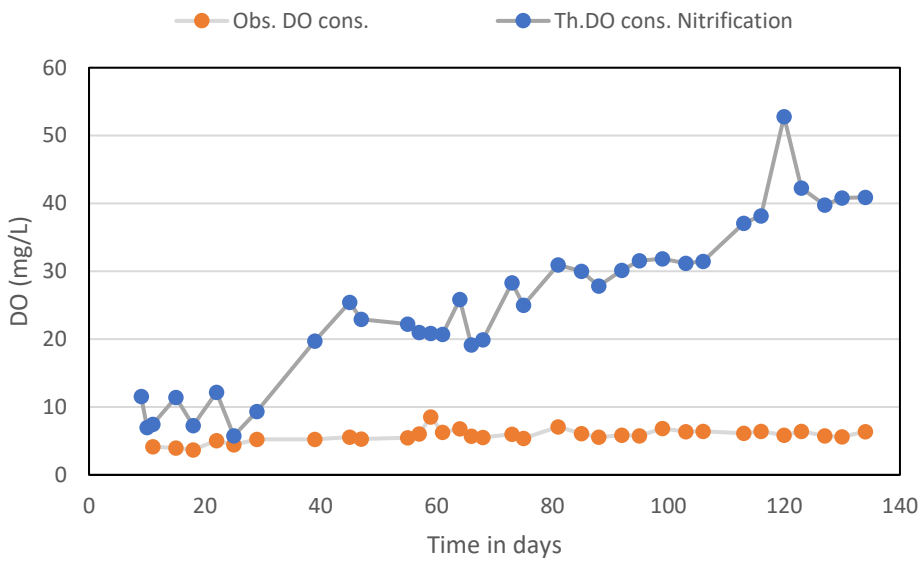

Figure 3-5. Theoretical DO consumption for nitrification of the consumed NH_4^+-N and observed DO consumption in the reactor. DO consumption is given as mg/L. Th.DO cons. Nitrification: the theoretical DO consumption for nitrification of the consumed NH_4^+-N to NO_3^--N in mg/L; Obs. DO cons.: the observed DO consumption in the reactor in mg/L (determined as the difference between influent and effluent DO concentration). The theoretical DO consumption is based on the molar ratios between NH_4^+ and O_2 (eq.1.1) and NO_2^- and O_2 (eq.1.2) and the concentration of consumed NH_4^+-N.

Then, the theoretical acetate consumption for denitrification was calculated based on the concentration of consumed NH_4^+-N (corresponding to production of NO_3^--N) and the NO_3^--N/acetate ratio (see section 2.7). For the observed amount of organic carbon, the added amount of acetate (8.82 mg/L; see appendix B) was used instead of measured COD concentration since these varied (section 3.1.1). The available acetate concentration was too low to explain the nitrogen removal by denitrification (Figure 3-6). Therefore, partial nitrification and denitrification cannot explain the removed nitrogen.

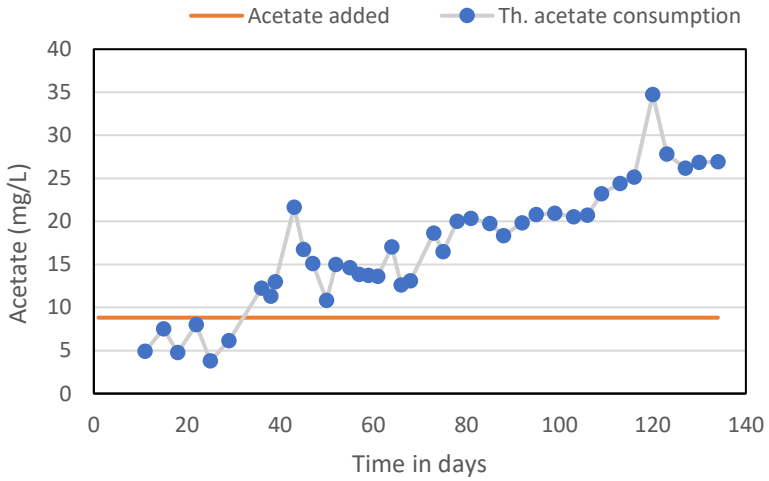

Figure 3-6. The added amount of acetate and the theoretical acetate consumption needed for denitrification. The acetate needed for a theoretical denitrification of NO_2^--N from the consumed NH_4^+-N increases over the experimental period because the NH_4^+-N consumption increased over the experimental period (Figure 3-1). The added acetate to the synthetic feeding water was consistently 8.82 mg/L (section 2.1).

To sum up, the theoretical calculations indicated that there was a mix of anammox and denitrifying bacteria in the PNA reactor and that they both contributed to the nitrogen removal and that nitrification and denitrification could not explain the nitrogen removal.

3.1.3 Ex-situ tests for nitrification and denitrification activity on carriers from the PNA reactor

Ex-situ tests for nitrification and denitrification were performed on the original carriers from Nordre Follo (Original Carriers) and on the carriers which had been adapted to the conditions in the PNA and AMX reactors. This was done by exposing the carriers to solutions of NH_4^+, NO_2^- and NO_3^- in batch reactors for 48 hours, measuring the concentrations of mentioned nitrogen compounds and calculating the conversion rates to estimate the potential of AOB, NOB and DNB activity (see section 2.3). Comparing the slopes of these removal rates sheds light on how the carrier's capacity to perform ammonium- and nitrite-oxidation and denitrification changed during adaptation to the low TAN-concentrations.

The conversion rates of AOB, NOB and DNB decreased from the Original Carriers to the PNA-Adapted Carriers (Figure 3-7). The DNB activity dropped the most, followed by NOB and then AOB activity. The percent decline in the conversion rates from the Original Carriers to the PNA-Adapted Carriers for DNB, NOB and AOB were 67%, 40% and 23%, respectively. The NOB conversion rate was the highest for both Original and PNA-Adapted Carriers and suggests strong NOB capacity.

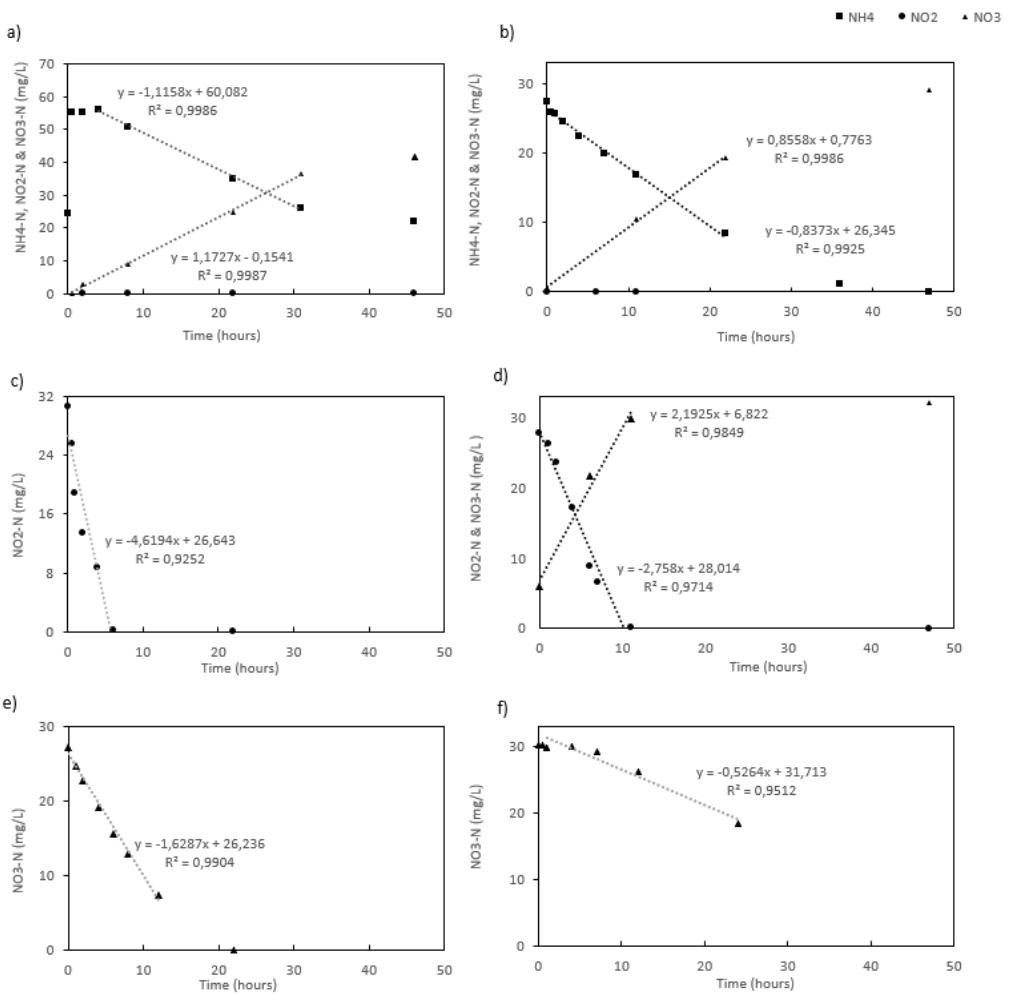

Figure 3-7. Ex-situ tests of AOB, NOB and DNB activity of Original Carriers (Day 1; a, c, e) and PNA-Adapted Carriers (Day 113; b, d, f). The AOB, NOB and DNB batches were fed ammonium, nitrite, and nitrate, respectively, and the concentrations of the compounds were determined over 48 hours of batch experiments. The AOB and NOB batches were aerated, while the DNB batches were flushed with N_2 gas. AOB-, NOB- and DNB-activity are shown by the conversion rates of NH_4^+-N, NO_2^--N and NO_3^--N over time in concentrations, respectively. The slope of NH_4^+-N conversion by AOB dropped from -1.12 (a) to -0.84 (b); the slope of NO_2^--N conversion by NOB dropped from -4.62 (c) to -2.76 (d); and the slope of NO_3^--N conversion by DNB dropped from -1.63 (e) to -0.53 (f).[2]

[2] The start concentration of NH_4^+-N in the Original Carrier AOB-test (a) was higher than in the other tests. This is probably due to the water on the carriers not having been soaked up before they were put in the test bottle, thus releasing ammonium in the biofilm to the water in the test bottle.

3.2 Performance of the AMX reactor

The strategy for the AMX was to adapt the carriers to a low TAN concentration through a gradual decrease over time. Anammox was selected for by feeding ammonium and nitrite. The reactor was not fed any organic carbon, but the feeding water tank and the reactor was flushed with N_2 gas at regular intervals to keep the DO concentration low (see section 2.1). The influent concentration of both NH_4^+-N and NO_2^--N started at 300 mg/L (Stage 1; Day 11-70), and was lowered to 150 mg/L, 50 mg/L, and 10 mg/L during Stage 2 (Day 71-108), 3 (Day 109-202) and 4 (Day 203-298), respectively (Table 2-2). The performance of the AMX is reported from Day 1-298.

The nitrogen removal rate (NRR) was high in the beginning of operation, but it decreased steadily over the experimental period (Figure 3-8, A). During Stage 1 and 2 the NRR was between 75-90%. In Stage 3-4, however, the NRR dropped and the effluent nitrate-N increased. In Stage 3, all the nitrite-N and most of the ammonium-N was converted to nitrate and in Stage 4 all the influent nitrogen was being converted to nitrate (Figure 3-8, B). The decreasing NRR was reflected in the increasing nitrate production rate (NPR; Figure 3-9), which oscillated between 12-58 % in Stage 3 and increased to 75% in Stage 4.

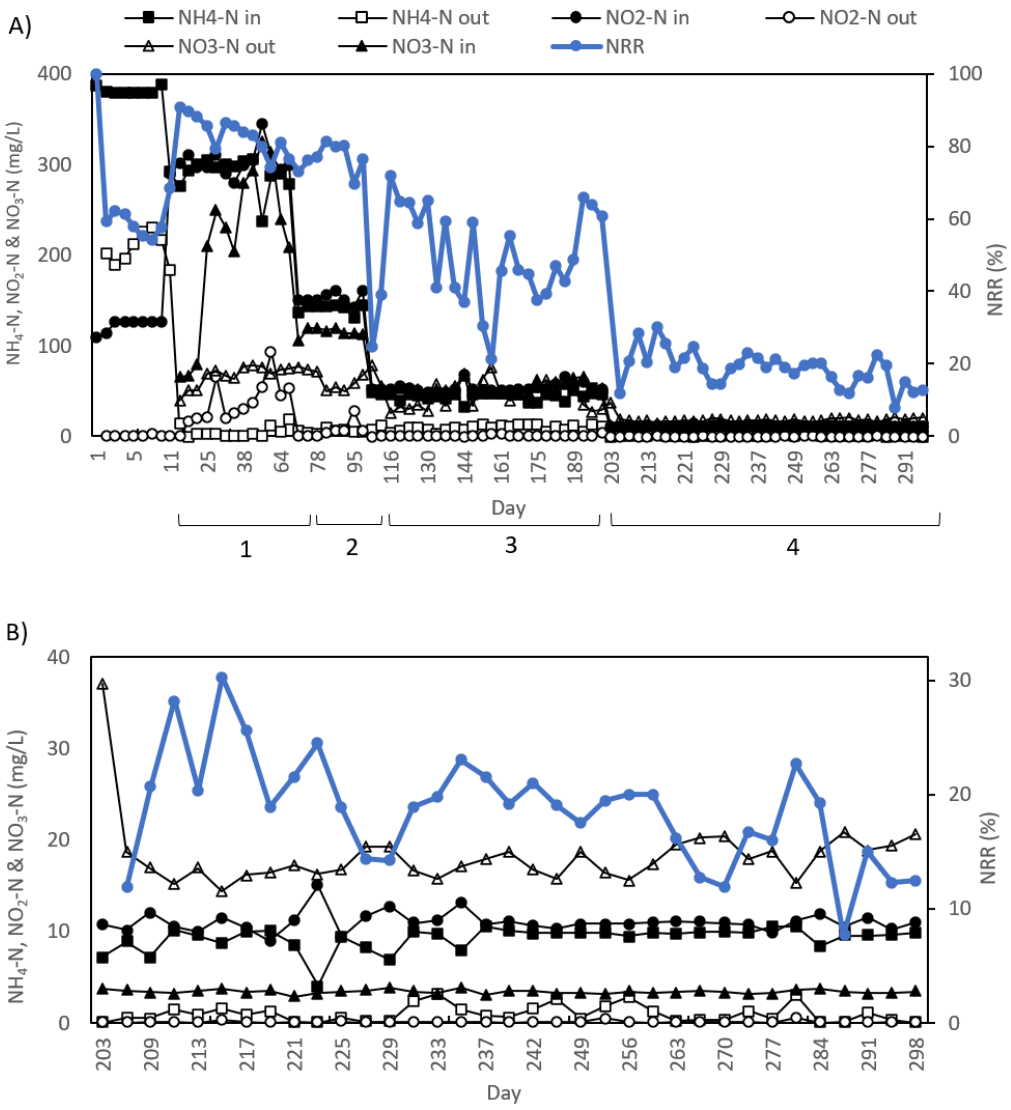

Figure 3-8. Concentrations (mg/L) of influent and effluent nitrogen compounds in the AMX reactor throughout the experimental period of 298 days. The reactor was fed NH_4^+-N and NO_2^--N in a ratio of 1 and the concentrations were lowered gradually over 4 stages (section 2.1). The stages of operation are shown at the bottom of the figure A. Influent samples were taken from the inlet tube on the top of the reactor, while effluent samples were taken from inside the reactor (see section 2.2.1). NH_4^+-N in: influent ammonium-N; NH_4^+-N out: effluent ammonium-N; NO_2^--N out: effluent nitrite-N; NO_3^--N out: effluent nitrate-N; NRR: nitrogen removal rate (%; see section 2.7). NRR started at 90% in the beginning of Stage 1, then decreased to around 75% during Stage 2, further decreased to 50% removal during Stage 3 and after Stage 4 the NRR had dropped to 12%. In Stage 4 (figure B), the effluent ammonium and nitrite are low, while the effluent nitrate is around 20 mg/L. It is apparent that the ammonium and nitrite were being converted to nitrate.

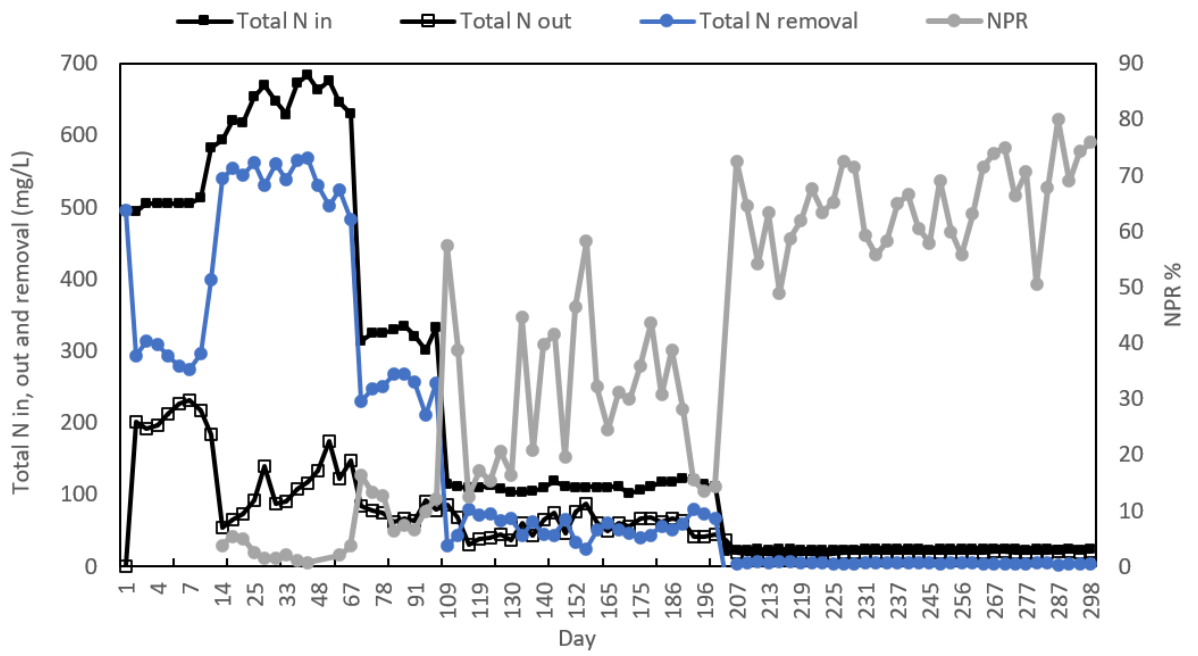

Figure 3-9. Influent and effluent total nitrogen, total nitrogen removal and nitrate production rate (NPR) in the AMX reactor throughout the experimental period of 298 days. Total N in: total influent nitrogen (mg/L); total N out: total effluent nitrogen (mg/L); total N removal: total nitrogen removed (total influent nitrogen–total effluent nitrogen; mg/L); NPR: nitrate production rate (%; see section 2.7).

The average influent concentration of DO for the whole experimental period of the AMX was 1.60 mg/L ± 0.94. The influent and effluent DO concentrations (Table 3-4) corresponded to an average DO consumption of 1.77, 2.02, 0.77 and 1.48 mg/L for Stage 1, 2, 3 and 4, respectively. Both dissolved and total COD concentrations were measured in the AMX reactor, but only in the effluent. Both effluent T-COD and D-COD concentrations showed a decline over the four stages (Table 3-4). Since the T-COD is the total COD in unfiltered samples, a high T-COD concentration indicates high amounts of biomass in the sample, and a decreasing T-COD concentration over time could indicate a loss of biomass on the carriers.

Table 3-4. Average values of operation parameters and total nitrogen-removal for stages 1-4 for the AMX reactor. DO in: influent DO concentration; DO out: effluent DO concentration; DO consumed: DO in – DO out; pH in: influent pH; pH out: effluent pH, T-COD out: effluent total COD concentration; D-COD out: effluent dissolved COD concentration.

Parameter	Stage 1	Stage 2	Stage 3	Stage 4	All stages*
DO in (mg/L)	2.01	2.55	1.31	1.76	1.60
DO out (mg/L)	0.23	0.53	0.55	0.28	0.46
DO consumed (mg/L)	1.77	2.02	0.77	1.48	1.13
pH in	8.35	8.31	8.30	8.67	8.56
pH out	7.80	7.35	7.59	8.19	7.95
T-COD out (mg/L)	234.50	199.00	49.26	21.30	-
D-COD out (mg/L)	73.24	65.50	28.46	13.53	-
Total N-removal (mg/L N)	528.4	247.8	54.3	3.70	-

* The values given for the "All stages" column are averages across the whole experimental period, not of the averages given for Stage 1, 2, 3 and 4 in this table.

3.2.1 Mass balance calculations

To investigate whether the nitrogen concentrations in the reactor fitted with a situation where anammox was responsible for the nitrogen removal, theoretical nitrate production associated with anammox activity and the observed nitrate production were compared. The theoretical nitrate production was calculated based on the stoichiometric equation of anammox (eq. 2.4) and the observed total nitrogen removed.

In the beginning of the experimental period (Stage 1), the observed NO_3^--N concentration in the effluent was lower than the theoretical NO_3^--N concentration associated with anammox activity (Figure 3-10), indicating that in the beginning of operation (Stage 1) denitrification contributed to the nitrogen removal. In Stage 2, the theoretical and observed effluent NO_3^--N concentrations were similar thereby compatible with a situation in which anammox removed the nitrogen, while in stages 3 and 4, the observed NO_3^--N concentration was higher than the theoretical. The nitrate production therefore had to be due to other microbial processes than anammox. This high NPR observed for Stage 4 (Figure 3-9) corresponded to the low NRR (Figure 3-8) and the low total nitrogen removal (Table 3-4). These findings were surprising since there seemed to be an efficient nitrification in Stages 3 and 4, despite the low DO concentration (Table 3-4).

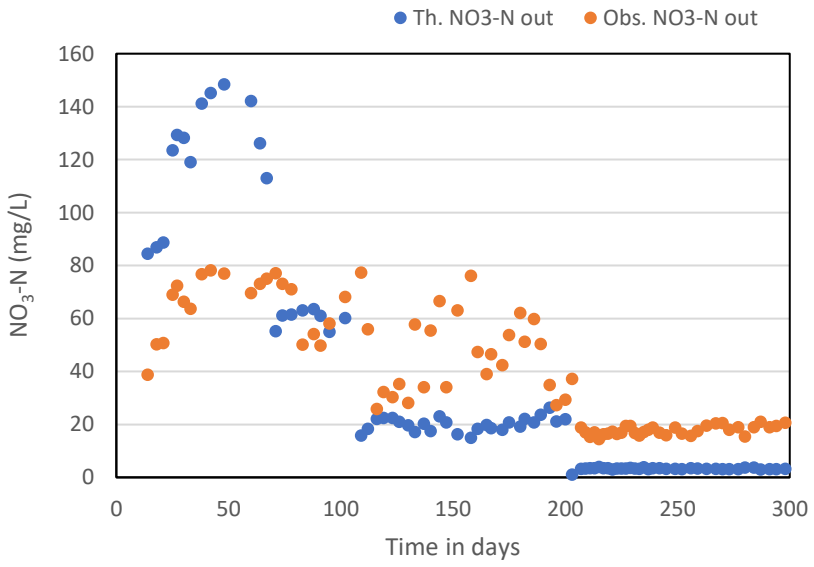

Figure 3-10. Theoretical effluent NO_3^--N concentration based on anammox activity and observed effluent NO_3^-N concentration. Th.NO_3^--N out: influent NO_3^--N + expected NO_3^--N production associated with anammox activity (eq.2.4); Obs. NO_3^--N out: observed NO_3^--N in the effluent.

To get an indication of whether there was enough DO in the system for the nitrate production in Stage 3 and 4, the DO needed for the nitrification of ammonium-N and nitrite-N to nitrate-N was calculated and compared to the consumed DO in the system. This calculation was based on the removed NH_4^+-N and NO_2^--N concentrations and the molar ratios between O_2 and NH_4, and O_2 and NO_2 (eq. 1.1 and 1.2). There was not enough DO in the system for the complete nitrification to nitrate observed in Stage 3 and 4 (Figure 3-11).

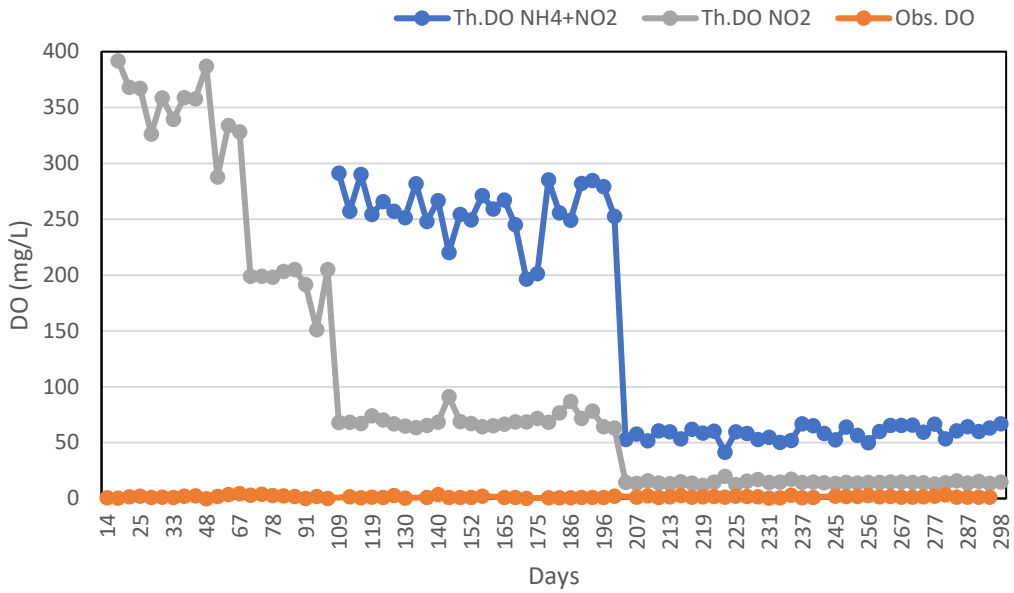

Figure 3-11. The theoretical DO consumption for nitrification and the observed DO consumption in the AMX over the experimental period. DO consumption is given in mg/L. Th.DO NH4+NO2: Theoretical DO consumption for nitrification of both NH_4^+-N and NO_2^--N to NO_3^--N; Th. DO NO2: Theoretical DO consumption for nitrification of NO_2^--N to NO_3^--N; Obs. DO: the observed DO consumption in the reactor (influent – effluent DO concentration). The theoretical DO consumptions are based on the concentration of removed NH_4^+-N and NO_2^--N and the molar ratios between these and O_2 (eq. 1.1 and 1.2). Average DO-concentrations for Th.DO NH4+NO2, Th. DO NO2 and Obs. DO in Stage 4 were 58.6, 14.6, and 1.7 mg/L, respectively.

In sum, the theoretical calculations show that anammox and denitrifiers probably removed nitrogen in combination in Stage 1 and 2, but in Stage 3 and 4 almost all the nitrogen was being converted to nitrate. This nitrate production could not be explained by anammox activity, nor was the DO consumption high enough to explain the nitrate production by nitrification by AOB and NOB.

3.2.2 Ex-situ tests for nitrification and denitrification activity

As for the PNA (see section 3.1.3), the activity rates of AOB, NOB and DNB were tested for the carriers which had been in the AMX reactor for 277 days. This was done by exposing the carriers to solutions of NH_4^+, NO_2^- and NO_3^- in batch reactors for 48 hours, measuring the concentrations of mentioned nitrogen compounds and calculating the conversion rates to estimate the potential of AOB, NOB and DNB activity (see section 2.3). By comparing the slopes of the removal rates of the Original Carriers from Nordre Follo and the carriers adapted in the AMX reactor (AMX Adapted Carriers), the carrier's capacity to perform ammonium- and nitrite-oxidation and denitrification before and after the adaptation period in the AMX (Day 277) can be compared.

All the conversion rates of AOB, NOB and DNB decreased from the Original Carriers to the AMX Adapted Carriers (Figure 3-12). Similarly to the PNA, the DNB activity dropped the most, followed by NOB and then AOB activity. The percent decline in the conversion rates from the Original Carriers to the carriers adapted to the AMX for DNB, NOB and AOB were 99%, 47% and 13%, respectively. It follows that AOB activity stayed relatively stable, while DNB activity was almost non-existent after the adaptation period in the AMX. That is interesting since the AMX was supposedly operated anaerobically. As for the PNA, the NOB activity rate was the highest rate in the AMX reactor as well.

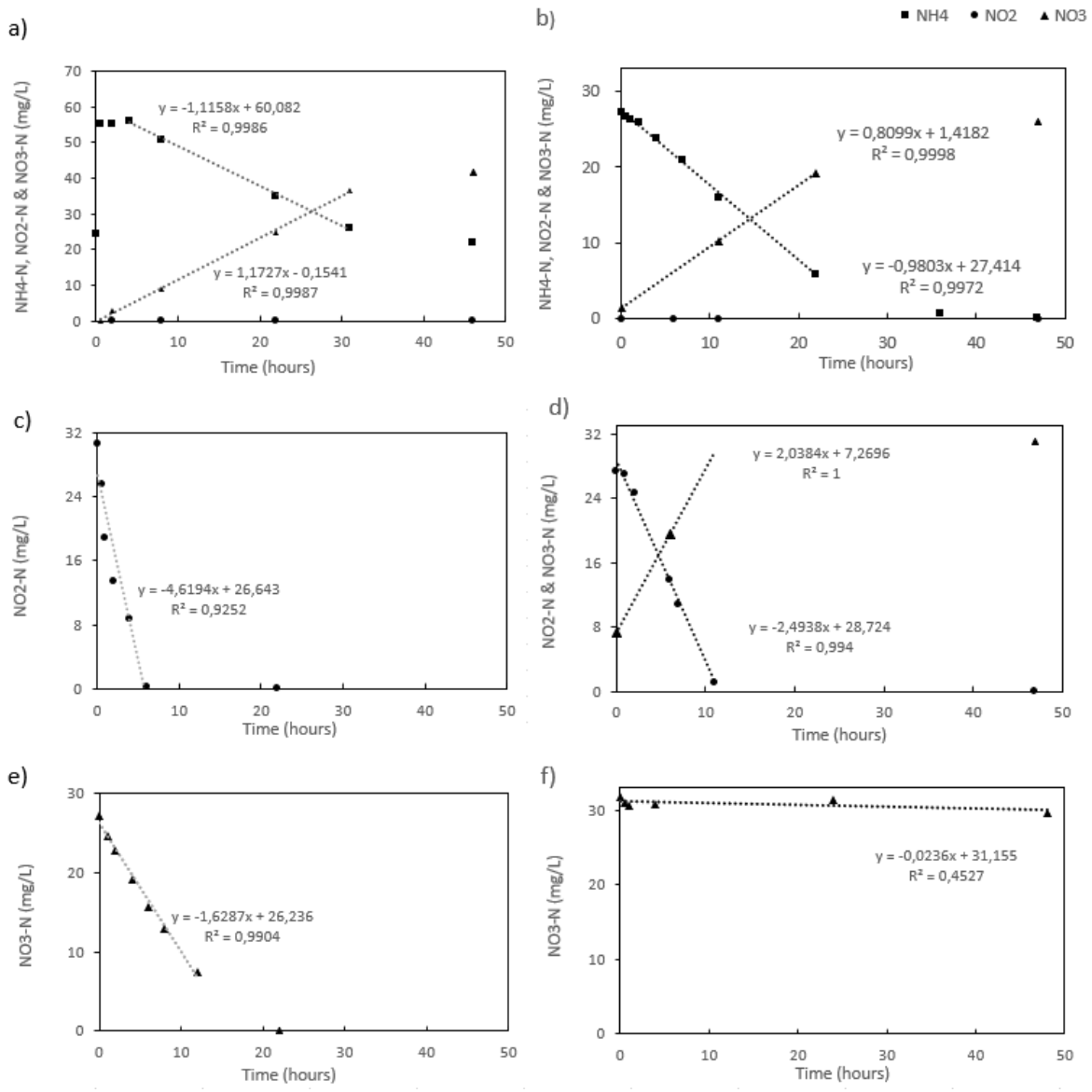

Figure 3-12. Ex-situ tests of AOB, NOB and DNB activity of Original Carriers (Day 1; a, c, e) and AMX Adapted Carriers (Day 277; b, d, f). The AOB, NOB and DNB batches were fed ammonium, nitrite, and nitrate, respectively, and the concentrations of the compounds were determined over 48 hours of batch experiments. The AOB and NOB batches were aerated, while the DNB batches were flushed with N_2 gas. AOB-, NOB- and DNB-activity are shown by the conversion rates of NH_4^+-N, NO_2^--N and NO_3^--N over time in concentrations, respectively. The slope of NH_4^+-N conversion by AOB dropped from -1.12 (a) to -0.98 (b); the slope of NO_2^--N conversion by NOB dropped from -4.62 (c) to -2.49 (d); and the slope of NO_3^--N conversion by DNB dropped from -1.63 (e) to -0.02 (f).

3.3 Anammox activity test

The anammox activity tests were performed on the original carriers and on carriers from both reactors during their experimental periods. The carriers' anammox activity is represented by the concentration of heme in the carrier's biofilm. Heme is a good indicator for anammox performance, and the amount of heme in biofilm from the carriers was found by cuvette kits for anammox activity (LCK411). The same amount of carrier material was used for each sample, meaning that a general reduction in biofilm on the carriers will contribute to lower anammox ability. We used the results to compare anammox presence and performance in the reactors, not to quantify the anammox biomass.

The heme-concentration in the carrier's biofilm decreased over time for both reactors (Figure 3-13), but dropped the most in the AMX reactor. The initial heme-concentration in the carrier biofilm was 30 µmol/L and in the AMX, the heme-concentration dropped to around 8 µmol/L within Day 91 (Stage 2), further to 1.6 µmol/L on Day 202 (the end of Stage 3) and heme was undetectable after this. For PNA, the heme concentration dropped to 13 µmol/L after 37 days (Day 202 in the figure) and stayed stable there until Day 277. The final heme concentration measured in the PNA was at 6.9 µmol/L. The decrease in heme concentration could be due to a general decrease in biomass on the carriers (see section 3.4), however the trend does show that the heme concentration on the carriers from the AMX decreased more than carriers from the PNA reactor.

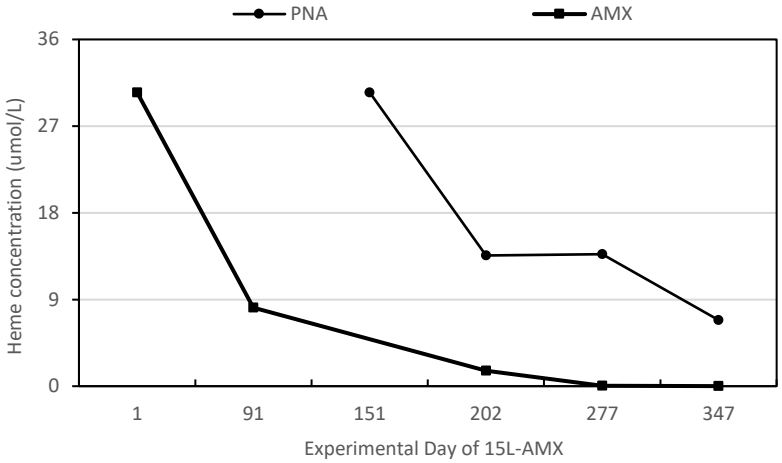

Figure 3-13. Anammox activity tests for the PNA and AMX reactors. The experimental days of the AMX are showed on the x-axis, since the AMX was started first. Day 151 marks the start of the PNA reactor. The initial heme concentrations of the carriers are given on Day 1 (AMX) and Day 151 (PNA), while the other days show the activity of the carriers over the experimental period. The Day in the figure corresponds to the following days of operation for the PNA reactor: Day 151: Day 1; Day 202: Day 38; Day 277: Day 113; Day 347: Day 183. The heme concentrations were determined by Hach kits for anammox activity (LCK411; section 2.4). The concentrations of heme are averages of two replicates for each sample.

3.4 Weight of biofilm carriers

To estimate the amount of biomass on the carriers, the carrier dry weight was measured for the original carriers and the carriers from the PNA and AMX reactors at the end of the experimental periods. Biomass loss occurred on the carriers of both PNA and AMX (Table 3-5). This was expected due to the lowering of influent nitrogen concentration. The carriers from the PNA reactor showed a smaller biomass loss than the carriers from the AMX reactor. This biomass loss in the reactors was visibly apparent (Figure 3-).

Table 3-5. Mean dry weight of carrier with biofilm of the Original Carriers from Nordre Follo, PNA-Adapted Carriers and AMX-Adapted Carriers.

Carriers	Experimental Day	Mean weight of dried carrier with biofilm
Original carriers	0	0.415
PNA-Adapted Carriers	113	0.411
AMX-Adapted Carriers	277	0.361

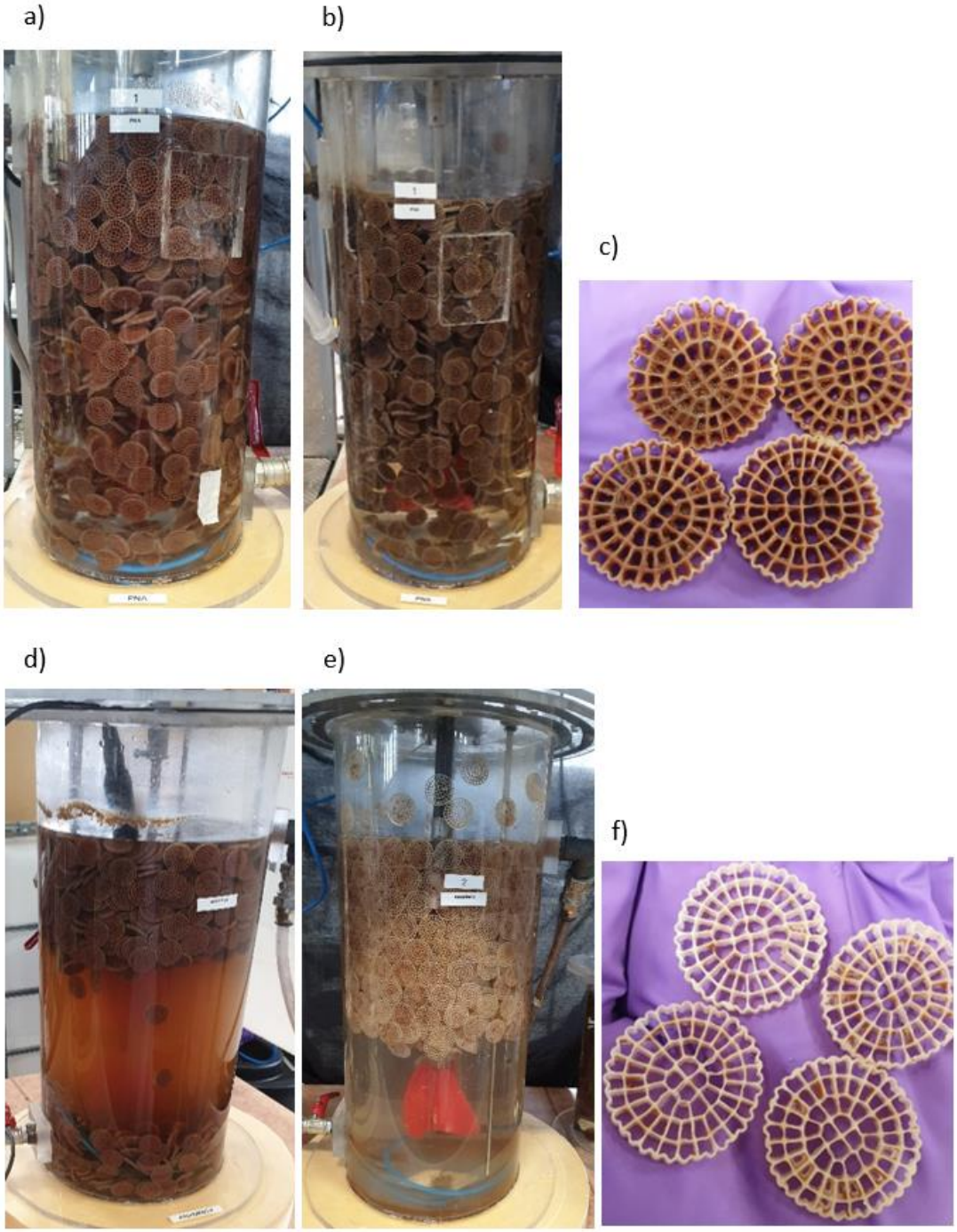

Figure 3-14. Carriers in the PNA (a-c) and AMX reactor (d-f) over their respective experimental periods. a) & c) Day 10 of PNA operation, b) Day 129 of PNA operation, d) Day 1 of AMX operation, e) & f) Day 174 of AMX operation. There is visibly less biomass on the carriers of the AMX reactor than in the PNA.

3.5 Microbial community analysis

3.5.1 PCR amplification of 16S rRNA from the RNA extracts

The microbiome characterization by Illumina sequencing of 16S rRNA amplicons was based on RNA extracts from the biofilm samples. To ensure that the PCR products represented the RNA, and not DNA from the DNA/RNA extracts, they were treated with DNase, and subsequently the RNA was reverse transcribed to cDNA which was used as templates in the PCR reactions.

The efficiency of the DNase-treatment was checked by running PCR of the DNase-treated extracts and the cDNA. If the DNase treatment had been efficient, no products were expected for the PCR of the RNA templates. However, PCR products were observed both for RNA and cDNA templates, suggesting presence of DNA in samples that had been DNase-treated. DNase-treatment was therefore run again with some modifications to the protocol (see section 2.6.2). After the second DNase-treatment of the RNA extracts, the DNA and RNA concentrations were measured using a Qubit-instrument for a representative selection of samples. The measurements show that there was a 1000-order magnitude difference between the amount of DNA and RNA (Table 3.3) in the samples after having run the samples twice by DNase-treatment, but the RNA extracts were still not completely free from DNA. Since there was such a large predominance of RNA, we consider it probable that most of the cDNA represents the RNA and not the DNA from the original DNA/RNA extracts.

Table 3.3. DNA and RNA concentration in RNA extracts treated twice with DNase as measured by a Qubit meter.

Sample*	RNA (µg/µL)	DNA (ng/µL)
4B	13,7	34
6B	16,8	49,8
7B	28,9	41,6
10B	16,4	31
14B	13,5	10,4
15A	9,4	1.61
18B	13,4	10,4
20B	74,9	35,2
23B	32,3	34,8
25C	17,9	24,6
27C	16,7	33,4
31A	41,1	31,8
32C	131	54,2

*13 random samples were chosen for DNA and RNA concentration measurement after the second DNase treatment

Then, cDNA was synthesized from the double DNase treated RNA extracts and used as template for the PCR amplification. PCR products of the expected length were successfully obtained for all samples (Figure 3-14). The sequence diversity of the PCR products, reflecting the diversity of the biofilm microbiomes, were further characterized by Illumina sequencing.

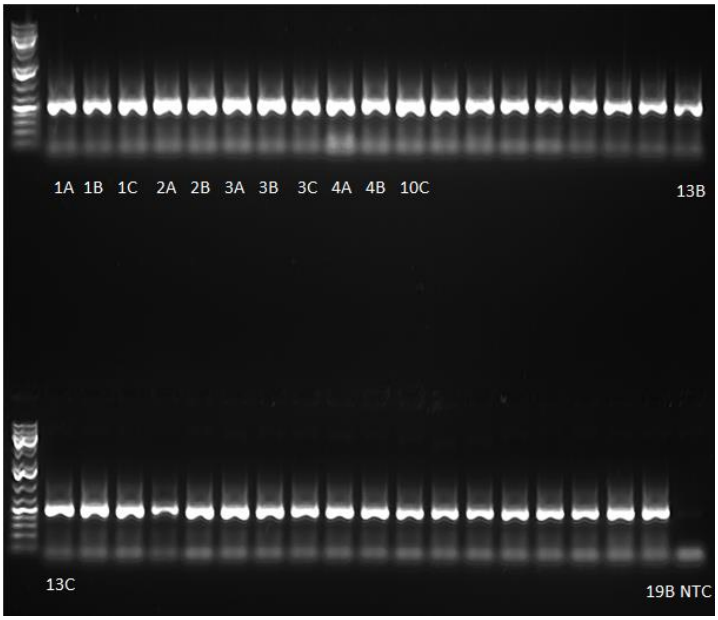

Figure 3-14. Agarose gel (1%) showing the v3+v4 16S rRNA amplicons obtained with the primers Ill-341F-KI and Ill-805-R. Samples represent a selection of the PCR-products of cDNA-templates from double DNase-treated RNA extracts. PCR reactions were run with an annealing temperature of 55 °C and 34 temperature cycles. Numbered and lettered samples indicate carrier samples while NTC represents a PCR non-template control. A negative DNA extraction control ("kit blank") was also among the samples. The ladder Gene Ruler™ 1 kb Plus DNA Ladder (Thermo Scientific) was used as a size marker and a volume of 3µL was used.

3.5.2 Characterization of biofilm-microbiomes in the PNA and AMX reactors

The bacterial biofilm communities in the PNA and AMX reactors were characterized by Illumina sequencing of the 16S rRNA amplicons. The PCR was based on RNA extracts, and thus, the dataset represents the active part of the microbial communities in the reactors (see section 1.4 and 2.6.2). The resulting ASV table was normalized to 45000 reads per sample and included samples of the original carriers from Nordre Follo and the biofilm samples from biocarriers from the PNA and AMX reactors. We observed a total of 9594 unique ASVs in the dataset; 9063 and 9371 ASVs for the samples from the PNA and AMX reactor, respectively. The data processing, analyses and production of the following figures in this section was performed by supervisor Ingrid Bakke and technician Amalie Mathisen.

A principal coordinate analysis (PCoA) ordering diagram based on Bray-Curtis-similarities indicated that the biofilm communities in both the reactors changed over time and that these communities differed from each other (Figure 3-15). A PERMANOVA test based on Bray-Curtis similarities confirmed that the communities were significantly different from each other (p=0.0001). Furthermore, it seems like the bacterial community in the PNA reactor resembled the community on the original carriers more than the community in the AMX reactor did.

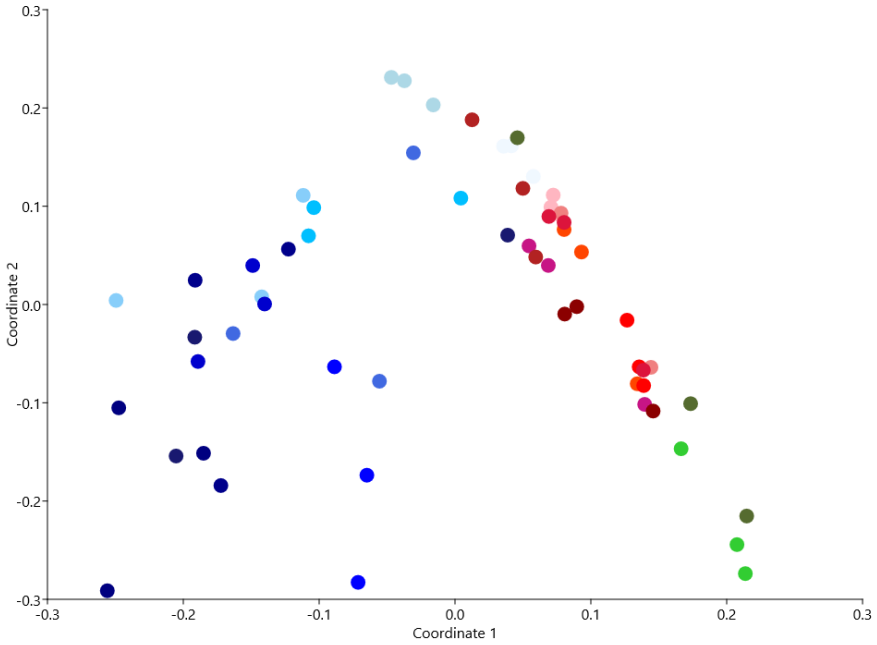

Figure 3-15. Principal coordinate ordination based on Bray-Curtis similarities for comparison of the bacterial biofilm communities on the biocarrier samples from the PNA and AMX reactors. Blue and red dots represent samples from the AMX reactor and the PNA reactor, respectively. The shades of the color indicate sampling time, where a darker color indicates a later experimental sampling day. Green dots represent carriers provided by Nordre Follo wastewater facility: the light green dots represent samples of carriers used for startup of the AMX reactor, while dark green represent samples of carriers used for startup of the PNA reactor. For each sampling time, there are three replicate samples from three different biofilm carriers.

The community composition at the phylum level revealed Planctomycetes as the most abundant phylum in almost all the samples (Figure 3-16). The Nitrospira phylum was considerably more abundant in the AMX samples than the PNA samples and its abundance seemed to develop and increase over time. Many of the ASVs were classified only on the "Domain"-level (the "Unassigned" in Figure 3-16) and therefore, we decided to investigate the community composition of the biofilm on ASV level. Two ASVs were clearly the most abundant in all samples from both the reactors; ASV_1 (Brocadiaceae) and ASV_2 (Chloroflexi; Figure 3-17). The two ASVs constituted in average 40% of all the reads in the samples from both reactors. A striking difference between the AMX and PNA reactors was the much higher abundance of ASVs related to different *Nitrospira* genera in the AMX reactor (Figure 3-17). Interestingly, the relative abundance of Nitrospira increased over the experimental period since it corresponded with the increasing nitrate production. In the two last biofilm samples Nitrospira constituted in average 17% of the relative abundance. A remarkable high number of ASVs were classified as Planctomycetes or as Bacteria, but most probably Planctomycetes: 3105 ASVs (of 9594 in total) of the whole data set (Figure 3-18). The majority of these ASVs were suggested to be part of the *Kuenenia* genus, but with low confidence. The number of these Planctomycetes ASVs was higher in the PNA samples than in the AMX samples (in average 1402 ± 261 and 789 ± 338, respectively; Figure 3-18). The Planctomycetes ASVs also constituted a higher relative abundance of the total reads in the PNA samples compared to the AMX samples (46% ±7 and 27% ±10, respectively; Figure 3-18).

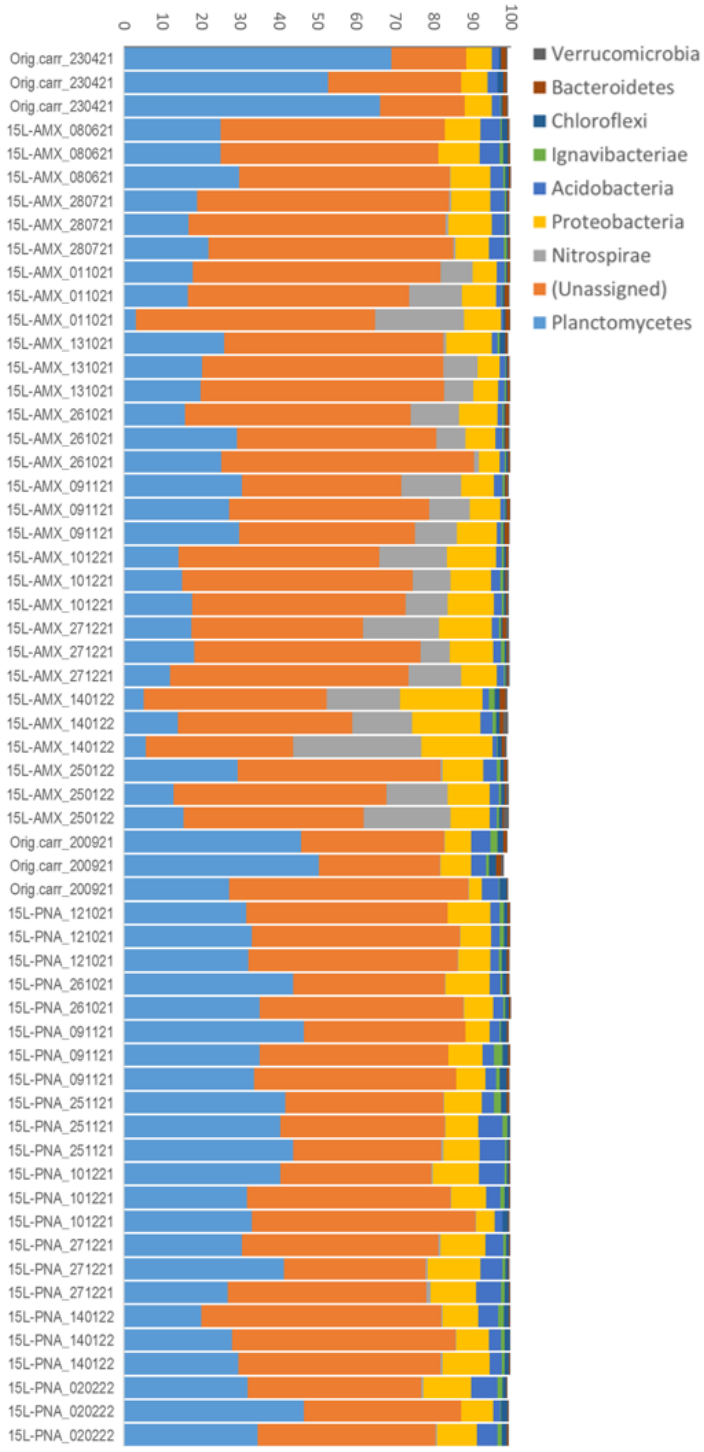

Figure 3-16. Community composition at the phylum level for the bacterial biofilm microbiomes. The abundance of phyla are given in %. Only phyla with relative abundances higher than 1 % of the total in at least one samples are included. The names of samples show the reactor name with "15L-" in front and the sample date at the end in the format of DDMMYY. Orig.carr: original carriers. At the top of the figure are the samples of original carriers from the 23.04.21 which were incorporated in the AMX reactor, then the biocarrier samples from the AMX reactor, then the original carrier sample for the PNA and the following biocarrier samples from the PNA reactor.

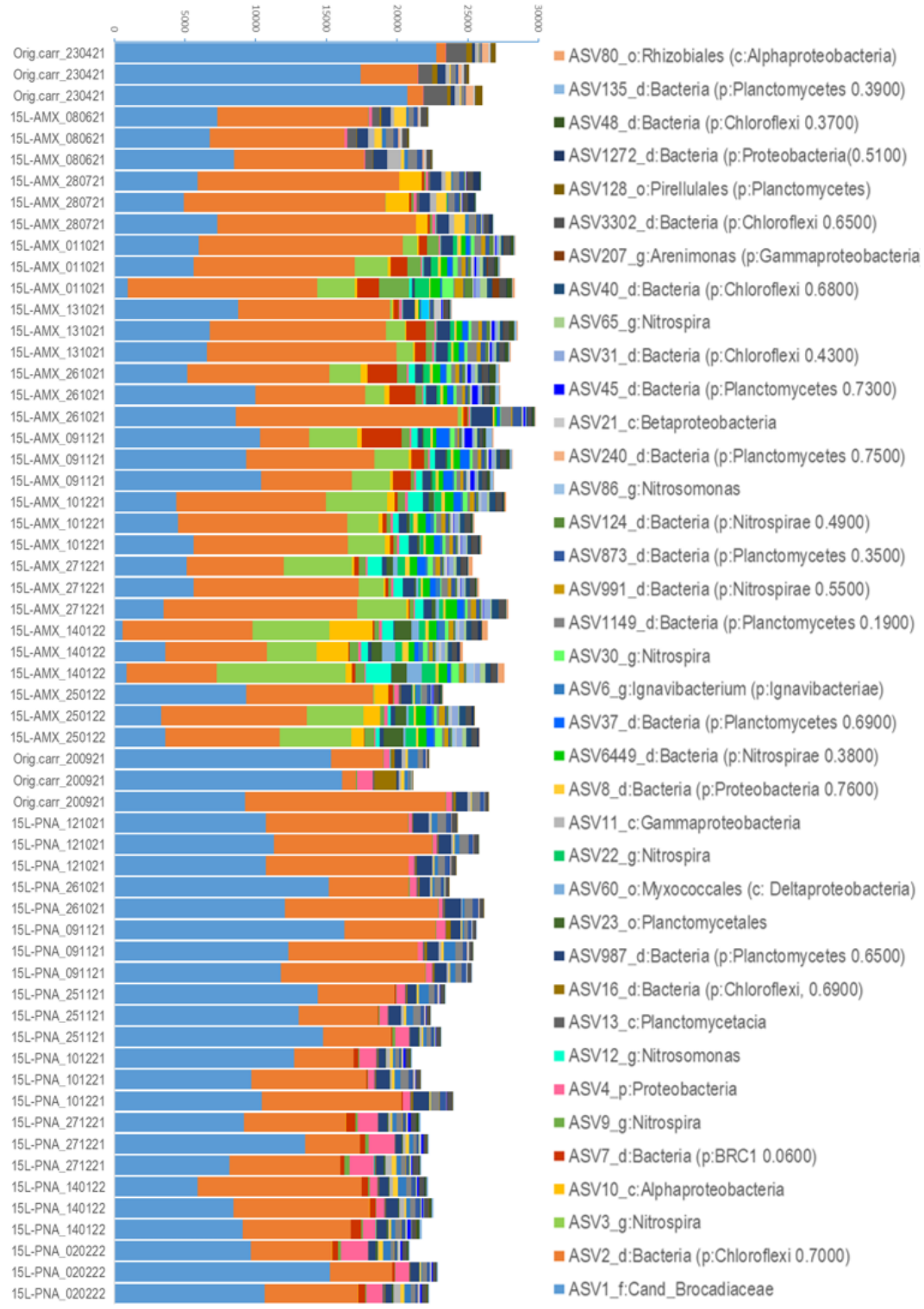

Figure 3-17. Relative abundance of ASVs in the bacterial biofilm microbiomes. The abundance is given as the number of reads, and the total number of reads for each sample was 45000. Only the ASVs with abundances higher than 1% of the total reads (i.e. more than 450 reads) in at least one sample were included. The names of samples show the reactor name with "15L-" in front and the sample date at the end in the format of DDMMYY. The taxonomy given for each ASV is the lowest level for which taxonomic assignment was obtained with the Sintax Usearch command. For ASVs that could only be classified at the domain level (as Bacteria), the most probable phylum as suggested by the taxonomic assignment is provided together with the confidence threshold.

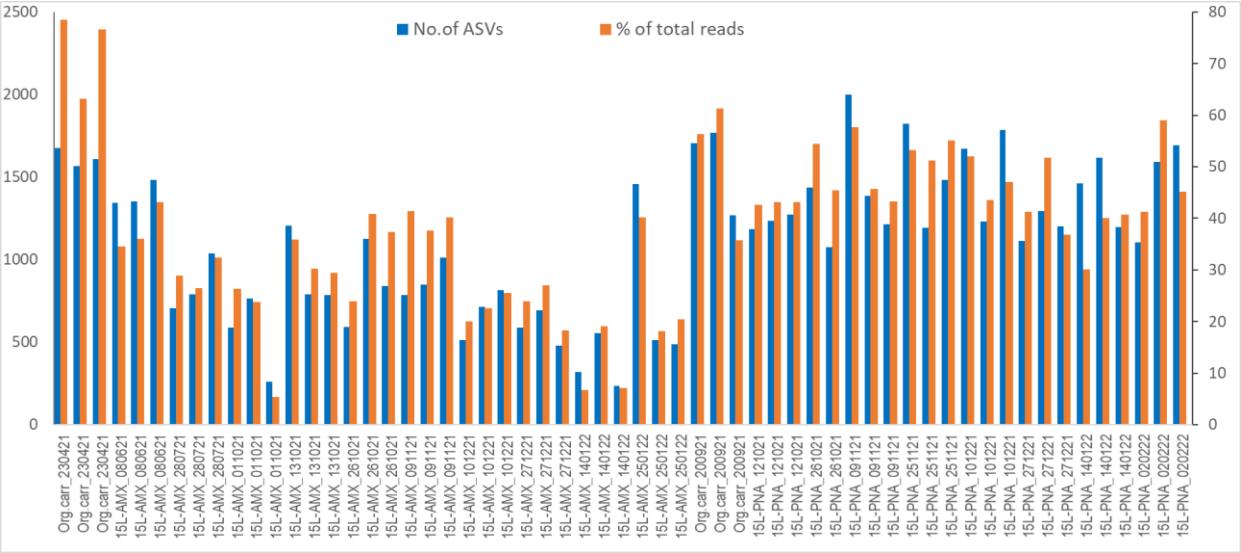

Figure 3-18. Number of ASVs suggested to belong to the Planctomycetes phylum and their relative abundances (%) in the biofilm samples. The names of samples show the reactor name with "15L-" in front and the sample date at the end in the format of DDMMYY. The ASVs were either classified to the phylum Planctomycetes (confidence threshold for classification >0.8) or to the domain Bacteria, but with Planctomycetes specified as the most probable phylum (confidence threshold <0.8).

4 Discussion

4.1 Evaluation of methods

This study was done as a study of two continuous MBBR lab scale reactors of 15 L with synthetic feeding water made batchwise. The MBBR setup with carriers was chosen as our partner in the project, Nordre Follo, used this setup for their partial nitrification and anammox process. Nitrogen and COD concentrations were measured with Hach Lange kits, while DO concentrations and pH were measured manually by sensor. Ideally, there should have been run replicates of these reactors, but since running such reactors is quite labor intensive, having replicates is not usually done.

To analyze the microbial community, it was chosen to sequence the actively growing population, the RNA, of the biofilm. This was to get an indication of which microbes were active in the reactor under the conditions we had set. The RNA sequencing does not show dormant, suppressed, or rare microbial taxa which are also present in the biofilm, such as DNA-based sequencing does.

4.2 Anammox-denitrification activity in the PNA reactor

The startup of the experimental period of the PNA reactor was characterized by oscillating influent TAN concentrations[3] and low NRR. Over time, however, the nitrogen removal rate (%) in the PNA reactor increased. The calculations of the available and theoretical DO and organic carbon (acetate) concentrations in the reactor showed that there was not enough DO or organic carbon in the system for the nitrogen removal to have been caused by nitrification and denitrification. However, it was difficult to estimate the amount of consumed oxygen in the reactor. In the calculations, only the oxygen supplied through the feeding water was considered, because the DO concentration in the feeding water was high (around 6 mg/L), and therefore expected to contribute more to the available DO in the MBBR than the relatively low levels of intermittent aeration.

The supplied oxygen through feeding water and intermittent aeration was rapidly consumed in the reactor, and the average DO concentration in the PNA reactor was 0.26 mg/L throughout the experimental period even thought the intermittent aeration was increased. This oxygen was probably consumed by heterotrophic bacteria and ammonium nitrifiers. In competition for DO it is believed that heterotrophic bacteria outcompete nitrifiers and AOB outcompete NOB (Li et al., 2011; Qiu et al., 2019). The feeding of some organic carbon was therefore thought to be beneficial for PNA operation since it promotes the exploitation of heterotrophic bacteria in removing DO from the system, thus keeping the DO concentration low (Li et al., 2018). Intermittent aeration is thought to be a robust strategy for partial nitrification and anammox since it introduces small amounts of oxygen (Li et al., 2011; Xu et al., 2015), thus potentially may suppress NOB and prevent anammox from oxygen inhibition (Qiu et al., 2019).

The anammox activity tests showed declining anammox activity. However, these tests measured the heme concentration of the biomass on half a carrier each time and thus, the total amount of biomass of the carrier will influence the result, not just the relative abundance of anammox. Since the carriers were originally used in a process of much higher TAN-N and COD concentrations, a reduction of biomass on the carriers is expected and may explain the decreasing anammox activity. This does not exclude the possibility of anammox having become more abundant in the biofilm relatively to other microorganisms.

Some denitrification activity was indicated by the mass balance of expected and observed nitrate production, which showed that less nitrate was observed in the effluent than was expected based on

[3] Suspected reasons included stratification of the batch, imprecise pipetting, and kit sensitivity. From Day 18 the batch was mixed before sampling, resulting in more consistent influent NH_4^+-N concentrations.

anammox activity (Figure 3-4). This suggests some activity of denitrifying bacteria. The ex-situ batch tests showed that there was DNB activity on the carriers which had been in the PNA reactor for 113 days (Figure 3-7f), but the activity rate had dropped with 67% and was the DNB activity was the lower than the AOB and NOB activities. It therefore seems likely that denitrifiers were active and contributed to nitrogen removal, but that their activity was limited due to low dosing of carbon to the system. Low C/N ratios are reported to be important to select for anammox over denitrification in anaerobic systems (section 1.3.2.2; Jenni et al., 2014; Trinh et al., 2021), and this might have played a role in the successful operation of this reactor.

The ex-situ batch tests also suggested a high abundance of nitrite oxidizers in the carrier biomass and high activity rates if given the right conditions (figure 3-7, c & d). Similar findings of high NOB activity from batch tests are reported in a similar study of the microbial community in a PNA process at low nitrogen concentrations (Persson et al., 2017). Strong NOB presence in PNA biofilms presses the urgency of controlling the DO concentration to suppress NOB activity in a PNA reactor (Li et al., 2018; Ma et al., 2020).

The acetate-carbon fed to the PNA reactor corresponded to a theoretical D-COD concentration of 9.3 mg/L. In addition, some COD was added through the tap water, but this is believed to consist of mainly non-degradable compounds such as humus and does therefore not contribute to the organic carbon accessible to microbes in the reactor. However, variable D-COD concentrations of 12-25 mg/L were measured in the PNA reactor. To investigate whether heterotrophic consumption of the easily degradable acetate in the feeding water batch tank was the reason of the varying COD concentrations, the bacterial density in water samples taken from a batch of deeding water during a storing period of 4 days was estimated by CFU analysis. This showed that there was very low bacterial numbers in the feeding water tanks and it was therefore concluded that the acetate was probably not consumed during storage, but entered the reactor and was probably consumed in the biofilm community. A Hach Lange kit for determination of the concentration of organic acids might have confirmed that this was the case, but there was not time to order the kit during this study. Other reasons for the varying COD concentrations may be that in systems with low DO, fermentation may also generate easily biodegradable organics (Ji et al., 2020) which might contribute to effluent COD concentrations.

Inoculation of a reactor with carriers with active PNA biofilm as inoculum was probably beneficial to get the desired PNA activity (Qiu et al., 2019). However, it was surprising and exciting that carriers from a high-strength sidestream could be implemented in a process of low nitrogen and COD concentrations directly and still show high activity of anammox and high nitrogen removal. Although anammox happens at low concentrations in nature (Dalsgaard & Thamdrup, 2002), to our knowledge, this is the first time anammox has been adapted to these low nitrogen concentrations (10 mg/L NH_4^+-N) in a controlled reactor. Previous research has adapted PNA to 20 mg/L NH_4^+-N (Laureni et al., 2016).

4.3 Decreasing nitrogen removal and increasing nitrification in the AMX reactor

In the AMX reactor a very different trend in the nitrogen removal rate (NRR) was observed compared to the PNA reactor. The NRR for the AMX was high in the beginning of the experimental period, but declined over the experimental period as the influent nitrogen was lowered. In the beginning of the experimental period, it is likely that anammox and/or denitrification removed nitrogen in combination, while later in the experimental period nitrogen removal decreased and nitrification increased. In Stage 4, almost all the fed ammonium-N and nitrite-N was being converted to nitrate-N. Furthermore, the calculations of available DO from the feeding water showed that there was not theoretically enough DO for nitrification. There is some uncertainty connected with these DO calculations, however, there does seem to be a large amount of DO lacking for the nitrification observed in the AMX reactor.

The declining nitrogen removal and the increasing nitrate production in the reactor was surprising since the DO in the feeding water was not theoretically enough for the nitrification. Since the nitrification increased gradually over the experimental period, it is likely that this development was due to the activity and development of slow-growing bacterial populations. Nitrification has been reported at low DO concentrations (0.5 mg/L; Bellucci et al., 2011; How et al., 2018; Liu, 2012, p. 88–95), but they did not explain how nitrification could take place at the low DO concentration.

However, there might be other sources of oxygen in the reactor than the O_2 introduced through the feeding water. Autotrophic bacteria produce oxygen in the CO_2 fixation process and since the feeding water consists of high concentrations of carbonate, CO_2 fixation might contribute to the DO in the reactor. Another possibility is the involvement of ammonium oxidizing archaea (AOA). AOA are highly abundant in marine environments and play a key role in the marine nitrogen cycle (Martens-Habbena & Qin, 2022). These archaea have long been known to oxidize ammonia to nitrite using oxygen (Könneke et al., 2005). However, they have also been discovered in strictly anoxic marine environments (Sun et al., 2021). Recently, a study demonstrated that AOA produced N_2 and O_2 under anoxic conditions, and nitrite and nitrous oxide are believed to be the key intermediates of this process (Kraft et al., 2022). It has been suggested that this O_2 production by the AOA provides excess oxygen which can be utilized by NOB (Martens-Habbena & Qin, 2022). Previous research has suggested that AOA growth is facilitated by low DO concentrations and long retention time (Park et al., 2006), which is consistent with the conditions in our reactor. Of course, there is no way of estimating how much these factors might have influenced the DO concentration of the system, but they may have contributed to the nitrification.

The decreasing NRR was reflected in the anammox activity tests which showed a declining heme-concentration in the carriers' biofilm over the experimental period. These tests are influenced by the total biomass loss on the carriers since the test was performed on half a carrier each time. Furthermore, the biomass loss was substantial in the AMX reactor (Table 3-5) so a declining heme concentration is expected. But the heme concentration declined much further and faster in the AMX reactor than in the PNA reactor (Figure 3-13).

In the AMX, both dissolved and total COD were measured in the effluent. Both the T-COD and D-COD concentrations in the AMX reactor effluent decreased over the experimental period, indicating a considerable loss of biomass in the AMX reactor. This was supported by the dry weight of carriers and visual observations. A much lower biomass loss was observed for the PNA reactor (Table 3-5, Figure 3-). The biomass loss in the AMX reactor could be due to the lowering of nitrogen concentrations in the feeding water and the lack of any organic carbon. However, this did not seem to be the case in similar studies where PNA was gradually adapted to lower nitrogen concentrations (630 to 70 mg N/L and 500-45 mg N/L in Hu et al., 2013 and Persson et al., 2017, respectively). These studies reported successful PNA activity. It might be possible that the provided acetate in the PNA reactor played a role in sustaining the biofilm. A denser biofilm on the carriers would, in theory, provide more protection for the anammox bacteria against oxygen inhibition since the oxygen concentration decreases deeper inside the biofilm (see Figure 1-3).

4.4 The microbial communities in the PNA and AMX reactors

There was a higher number of and relative abundance of the ASVs classified as Planctomycetes and potentially representing anammox bacteria in the PNA reactor than the AMX reactor. This supports the other findings which indicate anammox activity in the PNA reactor. In the biofilm communities in the PNA reactor, the second most abundant ASV (ASV_2) probably represented a bacterium belonging to the Chloroflexi phylum (Figure 3-17). This phylum is often found together with anammox bacteria, but its role is still uncertain and being researched (Pereira et al., 2017). It has been suggested

that these microbes use organic compounds from lysed cells and thus prevent the accumulation of organic waste products in biofilm (Kindaichi et al., 2012). A bacterium from the Chloroflexi phylum has also been shown to perform nitrite oxidation (Sorokin et al., 2012). Since the Chloroflexi in our bacterial community could not be classified at a lower level with high confidence, it is possible that there were members of the Chloroflexi phylum which contributed to the nitrate production in the AMX reactor.

The anammox activity in the PNA reactor depends on ammonium oxidation, since the reactor was not fed nitrite. However, there were no apparent candidates for ammonium oxidation in the bacterial community (Figure 3-17). In a similar study, AOB were not identified due to a much lower relative abundance compared to other bacteria (Bae et al., 2010). The candidate for ammonium oxidation may also be concealed within the phylum of Proteobacteria (Figure 3-16), as AOB are found within this phylum (Pereira et al., 2017). Another possibility is that ammonium oxidation is being performed by ammonium oxidizing archaea, but unfortunately, Archaea were not targeted by the primers used in this study.

In the AMX reactor, the relative abundance of *Nitrospira* increased over time. Although the nitrate production observed in the reactor indicated presence of nitrite oxidizers, it was still surprising that the *Nitrospira* developed toward higher abundance in the reactor over time since there was not theoretically enough oxygen present. In PNA systems, a DO of 0.2 mg/L is recommended to suppress NOB, such as Nitrospira (Li et al., 2011; Qiu et al., 2019), but the feeding water of the AMX reactor contained around 2 mg/L DO and the DO concentration inside the reactor was often above 0.2 mg/L (Table 3-4). Therefore, the development of Nitrospira under those conditions could be expected, but it still does not explain how nitrification could have happened with the DO consumption observed and based on the molar ratio of NOB (eq.1.2).

The nitrifying microbial community of low DO concentrations is believed to differ from the nitrifying community at high DO concentrations (How et al., 2018). Persson et al. (2017) reported that despite careful DO control, NOB activity could not be repressed in their MBBR biofilm and this resulted in a low nitrogen removal efficiency (11%) in the final period of their operation. This resembles our findings of decreasing nitrogen removal due to higher nitrate production and Nitrospira presence. Nitrospira activity has also been observed in other systems with low DO concentration (Gilbert et al., 2014; How et al., 2018; Keene et al., 2017). In addition, other sources of oxygen might have contributed to nitrification, as mentioned in section 4.3.

4.5 Future work and perspectives on the integration of anammox in RAS

The anammox process was adapted to low TAN concentrations of 10 mg/L N. The PNA reactor is still in operation in the RAbiome project. The influent TAN-N concentration has been lowered to 5 mg/L and is operational and removes most of the incoming TAN. The next step is to lower the temperature to RAS conditions as well. If this proves successful, then the PNA reactor will be implemented in a small experimental RAS without fish.

Many challenges remain before this process can be implemented in RAS. First, the DO concentration needs to be kept low for anammox to work. However, the DO concentration in RAS water is high due to oxygenation for the fish. Another challenge is the low HRT used in RAS biofilters. A possible way to integrate the PNA process in the RAS loop which might work with the mentioned challenges of high DO concentration and high HRT, could be to integrate the PNA process in a sidestream. This would allow for longer HRT and therefore also lower DO concentrations. Including an aerobic nitrification step after the anammox process should be considered as a preemptive measure against any accumulation of NH_4^+ or NO_2^-. Much remains to be understood about the roles of the different

bacterial populations in the biofilm communities. More studies are needed to chart the contribution to nitrogen removal of the different populations.

5 Conclusion

Research on applying anammox and PNA to low-strength wastewater is on-going. Previous studies have lowered the influent TAN-N to 20-30 mg/L and achieved PNA activity (Laureni et al., 2016; Persson et al., 2017). In this study, two MBBR reactors were operated to obtain anammox and PNA activity at low TAN concentrations relevant to RAS conditions. To our knowledge, this is the lowest nitrogen concentration used for PNA and anammox.

Biofilm carriers from Nordre Follo wastewater facility were used to inoculate the reactors. These carriers came from a process of 800 mg/L TAN-N and 3000 mg/L COD and were operated directly in a reactor with an influent ammonium-N concentration of 10 mg/L (the PNA reactor). This reactor was fed ammonium as the only nitrogen source and some acetate-carbon as a DO control strategy, in addition to being intermittently aerated. Partial nitrification and anammox activity was successfully obtained in the reactor. The nitrogen removal in the PNA reactor increased over the experimental period and the final NRR was 74%.

In the reactor which was lowered stepwise, fed ammonium and nitrite and where oxygen was removed from the feeding water and reactor by N_2 flushing, the nitrogen removal decreased over time while the nitrate production increased (to around 75%). Thus, this strategy with gradual decrease of the nitrogen concentration did not prove successful for obtaining anammox activity at low nitrogen concentrations.

The biofilm communities in both reactors had high relative abundances of the Planctomycetes phylum in all samples, and especially the *Brocadia* genus. The two ASVs of Brocadiaceae and probably Chloroflexi constituted a big part of the biofilm communities, and PNA contained the highest abundance of these. The nitrification in the AMX reactor was probably caused by the increasing Nitrospira population, which constituted in average 17% of the relative abundance in the last two biofilm samples.

6 Reference list

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7 Appendices

Appendix A - Technical specifications of reactors

Working volume, filling degree and HRT of the PNA and AMX reactors

d = 22 cm
h = 40 cm
Working volume, V = 3.14 * 11^2 * 40 ≈ 15.2 L

Carriers: 6 L
Filling degree: 6 L / 15.2 L = 39 %
Measured water volume of working volume = 12.9 L

Theoretical flowrate: 5 mL/min = 0.3 L/h = 7.2 L/d
Theoretical HRT = V/Q = 12.9 L / 0.005 L*min^{-1} = 43 hours

Surface area

The surface area in the reactors was calculated using the equation below from Veolia (2010). The protected surface area of the K5 biocarrier is 800m^2/m^3 (Veolia, n.d.).

$$SA\ (m^2/reactor) = \left[\left(\frac{800m^2}{m^3}\right) * \left(reactor\ volume\ (L) * \left(\frac{1m^3}{1000L}\right) * filling\ degree\ \left(\frac{m^3}{m^3}\right)\right)\right]$$

$$SA\ (PNA\ \&\ AMX) = \left(\frac{800m^2}{m^3}\right) * 15.2L * 0.39 * \left(\frac{1m^3}{1000L}\right) \approx\ 4.74\ m^2$$

Appendix B – Calculation of C/N ratio and COD concentration in the PNA reactor

3.39 g NH_4HCO_3 and 1.2 g $NaCH_3COO*3H_2O$ were added to a 60 L feeding water batch.

The NH_4^+-N concentration was:
3.39 g NH_4HCO_3 / 60 L = 0.0565 g/L NH_4HCO_3 = 56.5 mg/L NH_4HCO_3
56.5 mg/L * (14 $gmol^{-1}$/79 $gmol^{-1}$) = 10.01 mg/L NH_4^+-N

The amount of N added to the batch = 10.01 mg/L N
The amount of C added to the batch = 1.2 g $CH_3COONa*3H_2O$ / 60 L = 0.02 g/L = 20 mg/L
20 mg/L * (2*12 $gmol^{-1}$/ 136.1 $gmol^{-1}$) = 3.5 mg/L C
The C:N ratio = 3.5/10 = 0.35

To find the COD-concentration, the acetate/COD-ratio was found first.
$CH_3COOH + 2O_2 \rightarrow 2\ CO_2 + 2H_2O$
For every mole of acetate, 2 moles of O_2 are necessary.
$2O_2$ = 32 g/mol
Molecular weight (Acetic acid, CH_3COOH) = 60 g/mol
Molecular weight (Sodium Acetate Trihydrate, $CH_3COONa*3H_2O$) = 136.1 g/mol
1 mg acetic acid = 2 * 32 $gmol^{-1}$/60 $gmol^{-1}$ = 1.07 mg COD
It is necessary with 1.07 mg COD to convert 1 mg of acetate.

The COD concentration from 1.2 g of added $CH_3COONa*3H_2O$ was then found to be
1.2 g $CH_3COONa*3H_2O$ / 60 L = 0.02 g/L $CH_3COONa*3H_2O$
0.02 g/L * (60 $gmol^{-1}$/136.1 $gmol^{-1}$) = 0·00882 g/L CH_3COOH = 8.82 mg/L CH_3COOH
8.82 mg/L CH_3COOH * 1.07 = 9.3 mg/L COD

Appendix C – Composition of trace element solutions for feeding water

The composition of trace elements in the trace element solution used in the synthetic feeding water is shown in table C-1. The recipe is from Gonzalez-Silva et al. (2017).

Table C-1. Composition of trace element solution 1 and 2 (g/L).	
Solution 1:	
$FeSO_4 \cdot 7H_2O$	5
EDTA	5
Solution 2:	
EDTA	15
$ZnSO_4 \cdot 7H_2O$	0.43
$CoCl_2 \cdot 6H_2O$	0.24
$MnCl_2 \cdot 4H_2O$	0.629
$CuSO_4 \cdot 5H_2O$	0.25
$Na_2MoO_4 \cdot 2H_2O$	0.25
$NiCl_2 \cdot 6H_2O$	0.19
$Na_2SeO_4 \cdot 10H_2O$	0.21
H_3BO_3	0.014
$NaWO_4 \cdot 2H_2O$	0.05

Appendix D – Aeration in the PNA reactor

Table D.1. Intermittent aeration rates in PNA.

Day	IMA rate
0-34	No aeration
35-38	1min/3 hour
39-51	2 min/3 hour
52-57	2 min/2 hours
58-134	2 min/72 min

Appendix E – Protocol for anammox activity tests (Hach, 2019)

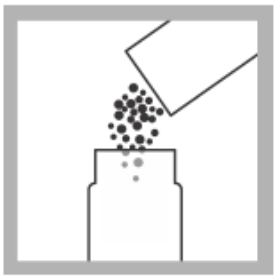
Procedure

1. Pre-heat the thermostat **(LT200)** to 70 °C/158 °F.

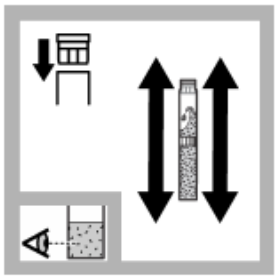
2. Stir the sample in a **baffled** flask while pipetting. Ensure **turbulent** mixing.

3. Pipet **5.0 mL sample** into a cuvette LCW906.

4. Add **0.5 mL** of **reagent A**.

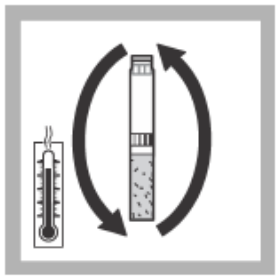


5. Add **1 Permachem** 2187569.

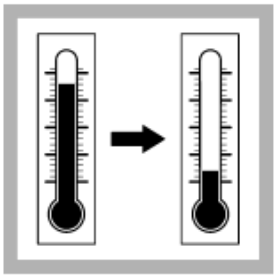


6. Close the cuvette and shake it until the **reagent is dissolved**.

7. Thoroughly clean the outside of the cuvette. Place the cuvette into the thermostat:
LT200 (pre-heated to 70 °C/158 °F): for **10 minutes** at 70 °C.

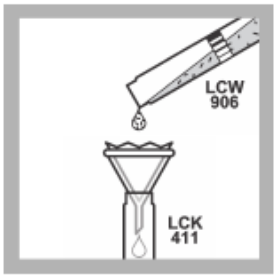


8. Invert the cuvette.
Caution: the cuvette is **still hot**.



9. Allow to **cool** to room temperature.

10. Open the cuvette. Insert a filtration paper in to the funnel and place it on a cuvette **LCK411**.



11. Filtrate digested sample (LCW906) into **LCK411**.
Note: the sample may be turbid. This does not disturb the measurement.
It is sufficient, if the bottom 25 mm of the cuvette is filled with filtrate.

12. Close the cuvette with a stopper.
Thoroughly clean the outside of the cuvette.

Appendix F – Carrier samples for microbiome analysis

Table F.1. Sampling dates for biocarrier samples from the PNA and AMX reactors. Original carriers were sampled directly from the delivery batch from Nordre Follo before putting them in the reactors.

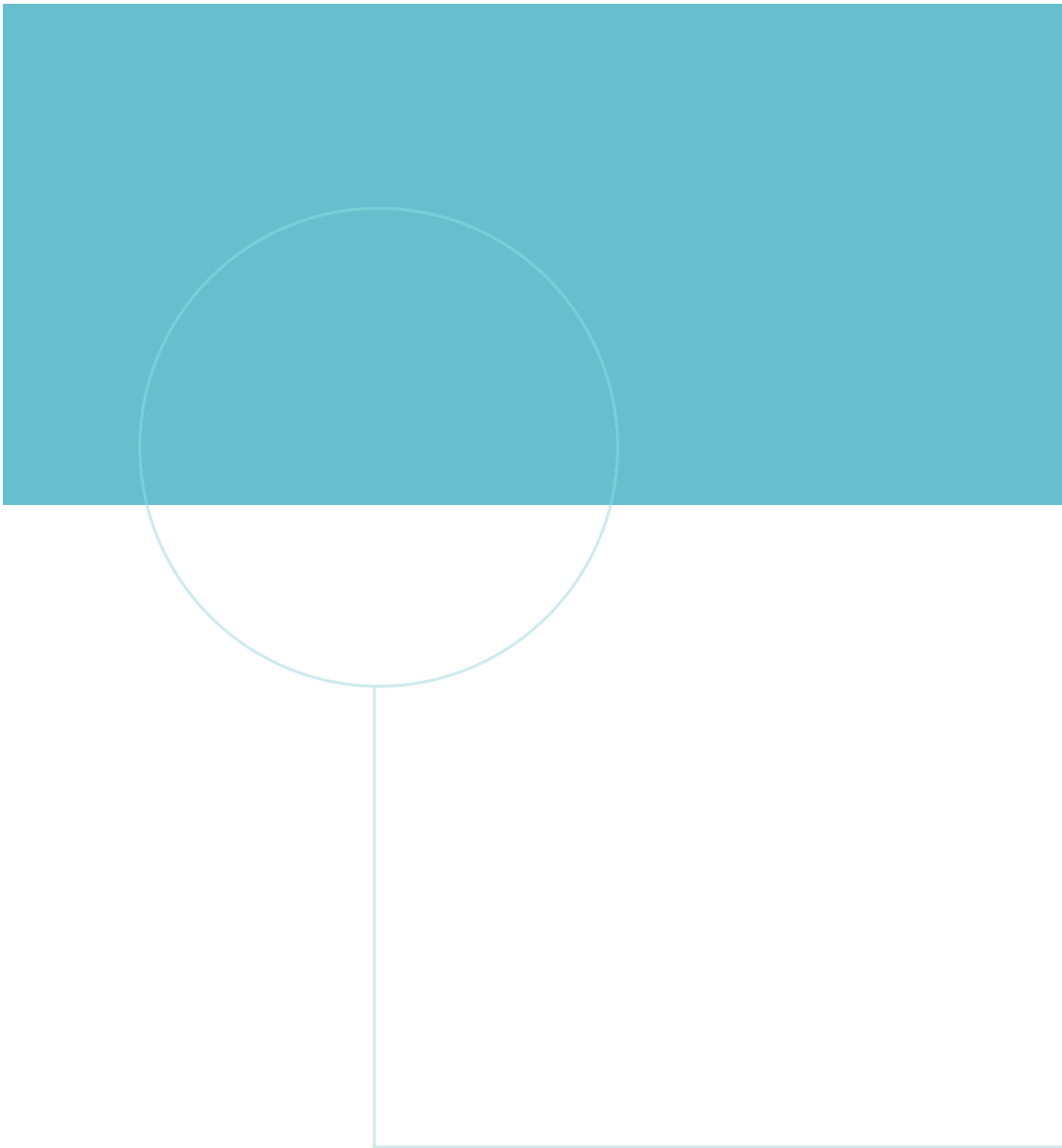
	PNA	AMX
Original carriers	20.09.21	23.04.21
	13.10.21	08.06.21
	26.10.21	28.07.21
	09.11.21	01.10.21
	25.11.21	13.10.21
	10.12.21	26.10.21
	27.12.21	09.11.21
	14.01.22	10.12.21
	25.01.22	27.12.21
	02.02.22	14.01.22
		25.01.22
In total	10	11

Appendix G – TEA buffer

Recipe for 50x TAE-buffer is shown in Table E-1. 1x TAE-buffer was prepared by diluting 40 mL 50x TAE-buffer in 1960 mL MQ-water.

Table G-1. Recipe for 50x TAE-buffer.

Component	Amount
Tris base	242 g
Glacial acetic acid	57.1 mL
0.5M EDTA pH 8.0	100 mL
dH_2O	To a total volume of 1 L



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