Bachelor Thesis

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Start-up and operation of a continuous stirred tank reactor performing stable anammox process with *Candidatus Brocadia fulgida*

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Declaration

I hereby declare that I have written the bachelor thesis “Start-up and operation of a continuous stirred tank reactor performing stable anammox process with *Candidatus Brocadia fulgida”* independently and without the use of further aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works. I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice. This paper has neither been submitted for evaluation to another examination authority nor has been published in any other form.

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Abstract

Nitrogen removal from wastewater is an energy and resource intense operation. In response, anaerobic ammonium oxidising (anammox) bacteria are being increasingly applied as a cost effective and sustainable wastewater treatment. Despite their utilisation worldwide, very little is understood about kinetic parameters and factors impacting their niche differentiation. The lack of enriched planktonic cultures is limiting further investigations into anammox bacteria and the optimisation of wastewater treatment. In this study, a Continuously Stirred Tank Reactor (CSTR) inoculated with *Candidatus Brocadia fulgida* to obtain an enriched planktonic culture. *Ca. Brocadia fulgida* was selected due to *Ca. Brocadias* widespread application in Wastewater Treatment Plants (WWTPs), and the lack of planktonic cultures of this anammox species. The cultivation of anammox bacteria is a time-consuming operation, due to the slow growth rate. This operation was conducted for 84 days, and variety of analytical and microbial techniques were used to monitor bioreactor performance and anammox bacteria enrichment. An analysis of consumption and production patterns was performed to investigate anammox metabolism. Changes in the microbial community were also assessed, which indicated a culture enriched in anammox bacteria. The establishment of a planktonic culture enriched in *Ca. Brocadia fulgida* in a CSTR was not achieved, and further research is needed in order to successfully apply this innovative approach to anammox bacteria cultivation.

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Abbreviations

Anammox	Anaerobic ammonium oxidising
AOB	Ammonia Oxidising Bacteria
BLAST	Basic Local Alignment Search Tool
BNF	Biological Nitrogen Fixation
BNR	Biological Nutrient Removal
BSA	Bovine Serum Albumin
CAS	Conventional Activated Sludge
CSTR	Continuously Stirred Tank Reactor
COD	Chemical Oxygen Demand
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dissolved Oxygen
FBR	Fluidised Bed Reactor
HRT	Hydraulic Retention Time
K_s	Saturation Constant
MBR	Membrane Bioreactor
NCBI	National Centre for Biotechnology Information
NLR	Nitrogen Loading Rate
NOB	Nitrite Oxidising Bacteria
N_r	Reactive Nitrogen
NRR	Nitrogen Removal Rate
PCR	Polymerase Chain Reaction
PN/A	Partial Nitrification/Anammox
rpm	Rotations per minute
SBR	Sequencing Batch Reactor
SRT	Solid Retention Time
t_d	Doubling Time
TN	Total Nitrogen
UAB	Upflow Anaerobic Bioreactor
WWTP	Wastewater Treatment Plant

1 Introduction

1.1 Nitrogen in Environment and Engineering systems

1.1.1 Nitrogen Cycle in Nature

Nitrogen (N) comprises 78 % of the Earth's atmosphere and is the fourth most abundant element in biomass (Stein and Klotz, 2016). Nitrogen gas (N_2) is a very stable compound due to its triple bond, requiring large amounts of energy to break (Bernhard, 2010). The nitrogen cycle is the interchange between inert nitrogen abundant in the atmosphere, and reactive nitrogen (N_r), which are nitrogen compounds that support or are products of cellular metabolism and growth (Stein and Klotz, 2016). The nitrogen cycle is almost entirely a microbe mediated process and a key process for life on Earth. Nitrogen flows through terrestrial and marine ecosystems, and is transformed into variety of compounds, before returning to the atmosphere. In a natural cycle free from human influence, the only sources of N_r would be the result of biological nitrogen fixation (BNF) and the production of nitrous oxides (NO_x) by lightning. These are primarily nitric oxide (NO) and nitrous oxide (N_2O) (Fowler et al., 2013) .

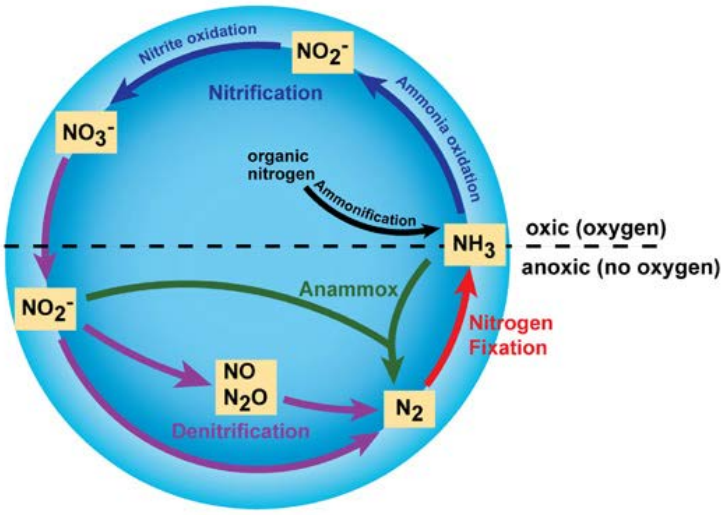

Figure 1: Major transformations in the nitrogen cycle (Bernhard, 2010)

Understanding of the nitrogen cycle has developed. It was traditionally divided into three processes; nitrogen fixation, nitrification and denitrification, with microbes labelled with their respective role. Nowadays, there are five accepted nitrogen-transformation flows: ammonification, nitrogen fixation, nitrification, denitrification and anammox (Stein and Klotz, 2016). Figure 1 displays these processes within the nitrogen cycle, including whether the process occurs under anoxic or oxic conditions.

Ammonification is the mineralisation of organic nitrogen compounds (i.e. compounds with ammino groups containing NH_2) such as those found in the tissues of plants and animals into ammonia (NH_3) and ammonium (NH_4^+) (Strock, 2008). The bacterial decomposition of tissue releases inorganic nitrogen into the ecosystem, where it is once more available for uptake by other organisms (Bernhard, 2010). This process occurs under oxic conditions.

Nitrogen fixation comprises the breakage of the nitrogen triple bond, producing biologically usable ammonium for uptake by plants and animals (Cabello et al., 2009, Stein and Klotz, 2016). Nitrogen is the limiting nutrient in primary production, despite the abundance of nitrogen gas in the atmosphere (Bernhard, 2010, Galloway, 2005). The majority of this process, when performed in nature and not synthetically, is executed by a select group of prokaryotes. A limited amount of nitrogen is also fixed abiotically by lightning (Bernhard, 2010). Nitrogen fixation occurs under anoxic conditions and is an extremely oxygen sensitive process (Stein and Klotz, 2016).

Nitrification is the aerobic conversion of ammonia to nitrite then to nitrate. Complete nitrification is a two-step process. The initial stage involves the oxidation of ammonia to nitrite by ammonia oxidising bacteria (AOB). This nitrite is then further oxidised to nitrate by nitrite oxidizing bacteria (NOB). Nitrifying bacteria are autotrophs, requiring no organic carbon for their metabolism. This is in comparison to heterotrophs, which utilise organic compounds as an energy source. AOB and NOB are utilised in nitrogen removal in Wastewater Treatment Plants (WWTP), transforming nitrogenous compounds and preventing water pollution (Bernhard, 2010).

Denitrification is the reduction of nitrate and to nitrogen gas, with intermediates such as nitrite, nitric oxide and nitrous oxide. The latter two are potent greenhouse gases.

Denitrification is an anaerobic process performed by chemoorganotrophs, which must have an organic carbon source. Denitrification is an important process in WWTPs, removing unwanted nitrates from water effluent (Bernhard, 2010).

Anammox, or anaerobic ammonium oxidation, is the oxidation of ammonium coupled to the reduction of nitrite which produces dinitrogen gas (Stein and Klotz, 2016). Anammox bacteria are responsible for the removal of fixed nitrogen from the environment in anoxic environments (Kartal et al., 2007). This process will be further discussed in greater detail in section 1.2.

1.1.2 Nitrogen Cycle Imbalance and Associated Problems

Under natural conditions, the nitrogen cycle is well balanced and nitrogenous compounds are recycled through the cycle with no accumulation. However, nitrogen is a limiting factor for plant growth, and this has resulted in human interference in the nitrogen cycle. Since the 19th century, legume cultivation and the application of animal manure has provided a nitrogen rich substrate for crop production, with minimal negative consequences (Galloway, 2005). The development of nitrogen-based fertilisers, industrially produced by the Haber-Bosch process, has facilitated an enormous increase in food production and a global population increase (Gruber and Galloway, 2008). The industrial production of fertilisers is therefore essential to support humanity, with almost half the world population dependent on fertiliser for their food supply (Fowler et al., 2013, Galloway, 2005).

In 2010, the extent of nitrogen fixation by the Haber-Bosch process (120 Tg-N yr^{-1}) process is almost double that of the natural cycle (63 Tg-N yr^{-1}) (Fowler et al., 2013). One Tg is equal to 10^6 tonnes. However, less than 20 % of industrial fertiliser is taken up by crops, making it a very inefficient process. The rest is lost to the environment; either to the atmosphere, hydrosphere or stored in soils (Galloway, 2005). All of the nitrogen resulting from agricultural activities, in addition to nitrogenous gases generated through the burning of fossil fuels, are dispersed into the environment (Gruber and Galloway, 2008). The excess nitrogen cascades through the Earth's ecosystems, disrupting the natural cycle and processes, adversely impacting human health, ecosystem services, biodiversity and contributing to climate change.

(Erisman et al., 2013). Figure 2 shows the significant anthropogenic influences on the nitrogen cycle. A selection of the associated environmental issues are discussed below:

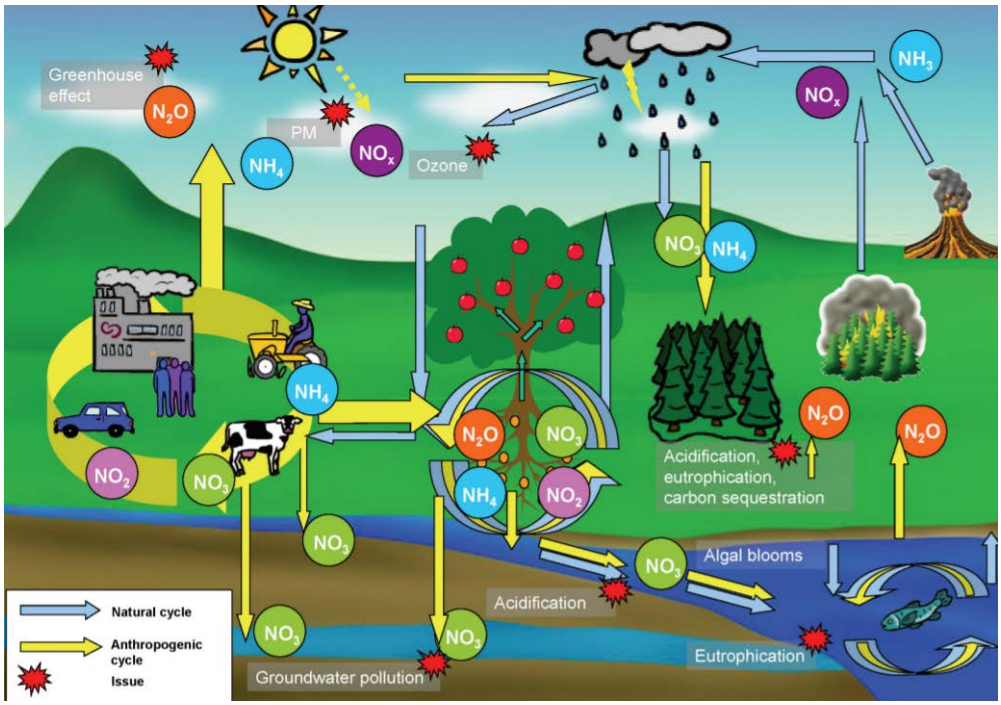

Figure 2: Overview of anthropogenic impacts on the nitrogen cycle (Erisman et al., 2011)

Fertiliser run off or sewerage discharge can enrich the nitrogen content of fresh and coastal water ecosystems. Biomass and biodiversity may temporarily increase with the increased nutrient load, but eventually phytoplankton are favoured. These algal or cyanobacterial blooms are referred to as eutrophication. They choke waterways and can lead to the release of toxic compounds. Decomposition of phytoplankton biomass decreases oxygen content in the water, leading to hypoxia, or 'dead zones'. This results in reduced species diversity, as only tolerant species can survive. Disruption to lower trophic levels can affect species up the food chain, including mammals and birds (Erisman et al., 2013).

Imbalances in the nitrogen cycle can also negatively affect human health. NO_x can result in increased tropospheric ozone (O_3) formation, as well as toxic smog, particulate matter (PM) and aerosols (Erisman et al., 2013). These have been linked to respiratory illnesses, cancer and cardiac disease in humans (Galloway, 2005). The pollution of groundwater from nitrate

is recognised as a human risk factor, with chronic effects being experienced at just 3 mg NO_3^- L^{-1}. Agricultural activities are responsible for groundwater and surface water pollution, threatening human drinking water (Erisman et al., 2013).

Industrial nitrogen fixation has allowed for increased food production yet has come with undesired consequences on human and environmental health, including biodiversity loss and climate impacts (Erisman et al., 2013, Fowler et al., 2013). With the global population predicted to continue its current growth rate, so too will demand for food and energy. It is therefore probable that anthropogenic nitrogen fluxes, and associated negative consequences, are destined to increase (Gruber and Galloway, 2008).

1.1.3 Need for Wastewater Treatment

Urbanisation and population growth worldwide has resulted in increased wastewater generation. With increasing awareness of sustainability, WWTPs are evolving from primarily focused on water recovery, to resource recovery, while minimising chemical and energy inputs (Guven et al., 2019). As discussed in section 1.1.2, nitrogen is a key concern for water quality. To prevent ecological and human health impacts, wastewater must be treated before release into the environment. In many countries this is enforced by legislation (Farazaki and Gikas, 2019). A variety of methods exist; chemical, physicochemical and biological, for the removal of wastewater charged with nitrogen (Ni et al., 2016).

1.1.4 Nitrogen Removal in Conventional Wastewater Treatment Plants

WWTPs are designed to achieve the removal of pollutants before release into the environment, mainly organic matter (C), nitrogen (N) and phosphorus (P) (Ni et al., 2016). Conventional activated sludge (CAS) is the most applied treatment of municipal wastewater in developed countries (Guven et al., 2019). Activated sludge is a mixture of the microbial community, responsible for the biodegradation of sewerage substrates, combined with inert solids (van Loosdrecht and Brdjanovic, 2014). This process traditionally referred exclusively to the biological treatment or oxidation of carbonaceous organic matter, although significant

improvements have been developed to include the removal of nitrogenous compounds and phosphorous. However, these CAS systems are very energy intensive, and high carbon removal efficiencies limit the effectiveness of later denitrification steps (Guven et al., 2019). In CAS operations, organic matter is initially converted into carbon dioxide (CO_2) and the assimilation of new biomass in the activated sludge. Ammonia is then nitrified to nitrite then nitrate, before reduction to nitrogen gas (Guven et al., 2019).

Not all WWTPs are equipped or specialised for nitrogen or phosphorous removal, but those that do are called biological nutrient removal (BNR) plants (Guven et al., 2019). BNR is the most implemented approach to nitrogen removal of wastewater (Ni et al., 2016). With this method, microbial communities exist in activated sludge under different conditions to achieve the removal of both organics and nutrients. Both the nitrification and denitrification steps must be completed for complete nitrogen removal (Farazaki and Gikas, 2019, Ni et al., 2016).

While aerobic conditions are required for the nitrification process, which is performed by autotrophs requiring only inorganic carbon for growth, the denitrification process requires a significant organic carbon load and anoxic conditions (Farazaki and Gikas, 2019). To achieve these opposing environments, biological reactors with different conditions are serially linked. A mix of wastewater and activated sludge flows through the reactors, before a clarification step where the sludge settles and is retained, while the treated water is discharged. The entire process can be seen outlined in Figure 3.

The first tank is where denitrification occurs under anoxic conditions. Nitrates and organic carbon from raw wastewater are consumed. The effluent then flows to subsequent aeration tank where nitrification and thus the production of nitrate occurs. Recirculation between the anoxic and aerated tanks allows for nitrates to be consumed (Farazaki and Gikas, 2019). This process can transform 75 % of nitrogen from the influent into nitrogen gas, and an additional 20 % is incorporated into sludge, requiring further treatment. Approximately 5 % of the nitrogen remains in the effluent (Ni et al., 2016). The process is effective with regards to nitrogen removal; however, the aeration requirement of nitrification makes it a very energy intensive process (Guven et al., 2019, Ni et al., 2016).

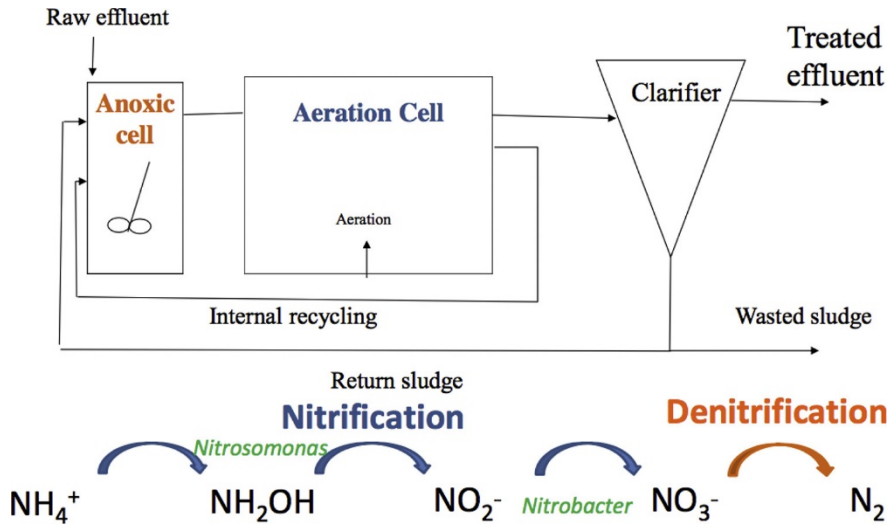

Figure 3: BNR in a WWTP (Raper et al., 2019)

1.1.5 Problems with Conventional Treatment

The most significant drawback of BNR is the high requirement for aeration, and its associated energy costs. Aeration has the highest energy demand of WWTPs, comprising up to 60 % of total energy consumption. Sludge treatment accounts for a further 35 % and pumping an additional 15 % (Guven et al., 2019). BNR process have additional oxygen demand to CAS, in order to supply oxygen for the nitrification stage, and the additional pumping required for recirculation and sludge treatment (Guven et al., 2019).

Heterotrophic denitrification requires an organic carbon electron donor. The organic carbon demand for denitrification is significant, while the organic matter present in most wastewater streams is comparatively low. This necessitates the addition of large quantities of organic matter. Methanol is the most widely utilised carbon source, as it is cheapest in terms of cost per mass of nitrogen removed, but a variety of other carbon substrates can be used (Ni et al., 2016). Denitrification is coupled both to energy production and bacterial growth. This results in a high production of biomass resulting from heterotrophic denitrification, the treatment and removal of which adds considerable cost to the operation of the WWTP (Ni et al., 2016).

Of increasing concern in escaping intermediates of the denitrification process, particularly nitrous oxide, a greenhouse gas 300 times more potent than carbon dioxide. There are reports that up to 1.8 % of the nitrogen loading rate (NLR) is lost as nitrous oxide, contributing to climate damage (Ni et al., 2016). Different denitrification enzymes have varying

environmental sensitivities which can lead to the accumulation of intermediates. This results in incomplete denitrification and the presence of undesirable nitrogen compounds in the emissions and effluent (Ni et al., 2016). Modern technologies and processes can be introduced for more efficient and less energy intensive nitrogen removal. These innovations, when coupled with anaerobic sludge digestion for biogas production, can result in an energy neutral WWTP (Guven et al., 2019, Wen et al., 2020). One promising alternative to conventional treatments is the anammox process.

1.2 Anammox Process

1.2.1 Anammox Bacteria

The existence of the anammox metabolism was proposed in the 1970's, but it was not until the 1990s that the presence of anammox bacteria was confirmed (Wu et al., 2020). Anammox bacteria belong to the Planctomycetes phylum. They are chemolithoautotrophic, obtaining their energy from chemical reactions without the use of organic compounds, and are thought to be responsible for 50 % of nitrogen gas that is released into the atmosphere (Kartal et al., 2013). Consequently, anammox bacteria are widely considered to play a key role in the global nitrogen cycle (Kartal et al., 2013, Oshiki et al., 2013, Zhang and Okabe, 2020). Since their initial discovery they have been identified in a variety of ecosystems with low oxygen availability. Six genera have been described so far, including *Candidatus Brocadia, Ca. Kuenenia, Ca. Scalindua, Ca. Anammoxoglobus, Ca. Jettenia* and *Ca. Anammoximicrobium.* All are found in freshwater systems, with the exception of *Ca. Scalindua* which is exclusively found in saline environments (Wu et al., 2020).

1.2.2 Anammox Metabolism

Anammox bacteria are capable of the oxidation of ammonium in anoxic environments, utilising nitrite as an electron acceptor. Nitrate and dinitrogen gas are produced (Zhang and Okabe, 2020). The proposed stoichiometry can be seen in Equation 1 below (Strous et al., 1998). Anammox bacteria are chemolithoautotrophic, utilising only inorganic compounds to derive their energy. Carbonate serves as the sole carbon source for biomass synthesis (Jetten

et al., 2009). The reaction occurs in a highly specialised, membrane bound organelle called the anammoxosome. The proposed equation for the anammox process is as follows:

$$1\ NH_4 + 1.32\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^-$$

Equation 1: Anammox nitrogen stoichiometry (Strous et al., 1998).

Anammox bacteria are found in natural and engineered environments, where substrates are scarce and nitrite is typically limiting (Kartal et al., 2007, Zhang and Okabe, 2020). They are characterised by high substrate affinity, utilising substrates at very low concentrations (< 5 µM) (Jetten et al., 2009).

Anammox bacteria grow slowly, with a doubling time (t_d) reported from one to several weeks and variations between the species studied and reactor type (Wen et al., 2020, Wu et al., 2020). This contributes to the difficulty in enriching and isolating anammox (Jetten et al., 2009). No anammox species has been successful isolated, though several have been highly enriched (Wu et al., 2020). Enrichment cultivations of free living anammox cells require large amounts substantial amounts of time, in the order of 100 to 200 days (Zhang and Okabe, 2020). This concept has been recently challenged by the cultivation of *Ca. Kuenenia stuttgartiensis* which was successfully enriched to 87 %, with a doubling time of 3 days after just 30 days of reactor operation (Ding et al., 2018).

In addition to ammonium, organic acids have been shown to serve as the electron donor (Kartal et al., 2007, Wu et al., 2020). While the presence of organic carbon can inhibit growth, anammox bacteria have been shown to co-metabolise fatty acids (Wu et al., 2020). Anammox bacteria can use fatty acids as electron donors to reduce nitrite and nitrate in order to outcompete heterotrophic denitrifiers in the presence of ammonium (Jetten et al., 2009, Kartal et al., 2007). *Ca. Brocadia fulgida* in particular has been identified as having a superior ability to oxidise acetate compared to other species, leading to the enrichment of *Ca. Brocadia fulgida* within a mixed culture (Wu et al., 2020).

1.2.3 Microbial Interactions

The lack of pure cultures of anammox bacteria means that other microbes are always present in anammox-based systems. Interactions between other bacteria can be both cooperative and competitive (Zhang and Okabe, 2020). WWTPs streams typically have a high concentration of ammonium with nitrite limiting. However, anammox bacteria require both nitrite and ammonium. Thus, partial nitration, which involves the partial oxidation of ammonium to nitrite by AOB, is required before subsequent anammox treatment. AOB activity simultaneously ensures an anoxic environment, through the consumption of oxygen, in addition to providing substrate for the anammox bacteria (Zhang and Okabe, 2020). On the other hand, anammox bacteria must compete against AOB for ammonium (Wu et al., 2020). While AOB are present in anammox reactors, they grow extremely slowly (Wu et al., 2020). Anammox bacteria must also compete against NOB and denitrifiers for nitrite. Denitrifiers can effectively compete with anammox bacteria for nitrite in the presence of suitable organic electron donors (Wu et al., 2020). Anammox bacteria with high oxygen tolerance and high substrate affinity are most suited to compete with other nitrogen cycle microbes (Zhang and Okabe, 2020). While microbes also compete with anammox bacteria, cooperation within microbial communities have significant potential for enhancing the nitrogen removal in WWTPs (Wu et al., 2020).

In addition to competition between different nitrogen cycle bacteria, there also exists competition between different anammox species. In this interspecific competition, different anammox species compete for resources or living space. This results in the decline or exclusion of the less adapted species, resulting in niche differentiation (Zhang and Okabe, 2020). Key factors in shaping community include the environmental conditions, growth kinetics and metabolic flexibility (Zhang and Okabe, 2020). There is a lack of understanding on the mechanisms affecting the niche differentiation of anammox bacteria (Zhang and Okabe, 2020).

Anammox bacteria have a predisposition to form biofilms or aggregations (Zhang and Okabe, 2020). With many interactions occurring at the oxic/anoxic boundary, the formation of granules is beneficial for all nitrogen cycle bacteria. In single-stage wastewater treatment systems, AOB can dominate the outer region, proving nitrite for NOB. Nearer the centre of

the granule, the anoxic conditions are well suited to denitrifiers and anammox bacteria, which then transform nitrogenous substrates to nitrogen gas (Figure 4). The bacteria on the outer layer are more active, as the diffusion of substrate is limited by the granule (Ali et al., 2016). The interactions between anammox species, and between other nitrogen cycle microorganisms are both fragile and complicated, and require further investigation to understand niche differentiation mechanisms (Zhang and Okabe, 2020).

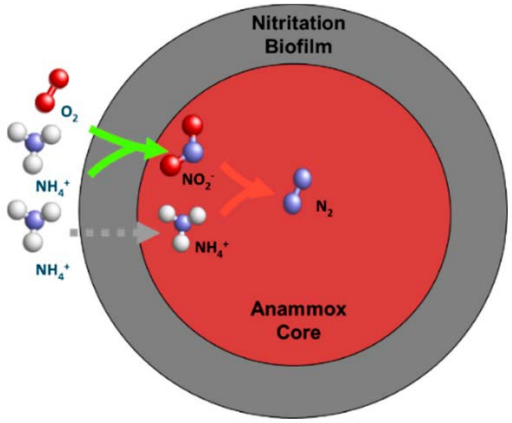

Figure 4: Simplified cooperation between diverse nitrogen cycle microbes in a single-system granule (Driessen and Hendrickx, 2021)

1.2.4 Niche Differentiation of Anammox Bacteria

The factors affecting how different anammox species find their own ecological space, i.e., anammox niche differentiation, remain to be answered (Zhang and Okabe, 2020). Substrate availability, and the relevant substrate affinity are believed to be key parameters. *Ca. Brocadia sinica* was able to proliferate at higher NLR, whereas *Ca. Jettenia caeni* was only able to grow at low nitrite concentrations (Zhang et al., 2017a). The saturation constant (K_s) is considered a key parameter for understanding microbial growth and niche differentiation. However, the K_s for different anammox species have been reported in a wide range, with ammonium (K_{NH4}+: 3.0–640 μM) and nitrite (K_{NO2}-: 0.2–370 μM) (Zhang and Okabe, 2020). Many of these values have been obtained from granules or sludge. Therefore, substrate transport limitations and mixed cultures contributed to the lack of a clearly defined K_s value and require reassessment (Zhang et al., 2017b, Zhang and Okabe, 2020).

The presence of carbon compounds can also influence the ability of a species to dominate in a culture. In one study, where anammox bacteria was enriched from WWTP sludge under identical operation conditions except for the addition of a carbon source, the addition of acetate lead to the dominance of *Ca. Brocadia fulgida,* while *Ca. Anammoxoglobus propionicus* proliferated with the availability of propionate (Jetten et al., 2009). Trace elements, including those found in the groundwater, can also result in a population shift. A mature culture enriched in *Ca. Brocadia sinica* from Sapporo, Japa, shifted to *Ca. Kuenenia stuttgartiensis* when cultivated in Niigata, Japan. The only difference was the use of the local groundwater. Interestingly, when the *Ca. Kuenenia* dominant culture was returned to Sapporo, the reactor reverted to a culture enriched with *Ca. Brocadia sinica* (Zhang and Okabe, 2020). This indicates that minor changes in the environment can provide species with a competitive advantage (Jetten et al., 2009). The importance of understanding the factors influencing niche differentiation allows for the selection of the most suitable anammox species for wastewater applications and preventing unwanted population shifts (Zhang and Okabe, 2020). So far, clear guidelines to cultivate one anammox species over another has not been established and further research is required in this area.

1.2.5 Planktonic Cells and Aggregated Sludge

Anammox bacteria tend to form aggregations including other microbes. This results in oxygen concentration gradients within the granules which allows competing species to coexist in their preferred environment within the aggregation (Zhang and Okabe, 2020). It has been found that, due to substrate diffusion limitations, populations nearer the surface of the granule are more active than those in the centre (Oshiki et al., 2020). The ability of anammox bacteria to aggregate varies among species. Increased hydrophobic cell surfaces, such as those detected on *Ca. Brocadia sinica,* relates to an increased tendency to aggregate (Zhang and Okabe, 2020).

These granules provide a protective environment against environmental inhibitors, while the formation of biofilms prevents washout. While aggregate and biofilm formation is a useful survival tactic, it makes understanding the factors and mechanisms of niche differentiation and kinetic studies difficult to investigate (Zhang and Okabe, 2020). In addition, the

biochemistry and kinetic parameters remain uncertain, mainly due to the lack of planktonic free-living cultures. Planktonic cells are essential for further investigation of the anammox process. An enriched, planktonic culture is suitable for obtaining purified proteins, required for further research into the biochemistry of anammox pathways. They are also crucial to deepen our understanding of anammox physiological characteristics (Oshiki et al., 2013).

1.2.6 Parameters and Operational Conditions

Parameters such as the pH, temperature, dissolved oxygen (DO) concentration and substrate concentrations must be monitored to provide suitable conditions for anammox activity and prevent inhibition. Anammox bacteria have been identified in many natural and engineered anoxic and suboxic environments, which is the interface between anoxic and oxic zones characterised by low oxygen concentrations (Kartal et al., 2007). These vary from soil and ocean environments to WWTPs (Wu et al., 2020, Zhang and Okabe, 2020). Anammox bacteria are obligate anaerobes whose metabolism are reversibly inhibited at increased oxygen levels (Jetten et al., 2009). Low oxygen conditions, with DO concentrations below 0.1 mg L^{-1} are favourable growth conditions for *Ca. Brocadia sinica* (Oshiki et al., 2013). DO concentration must therefore be controlled during the process as excessive oxygen will enable the growth nitrifiers and inhibit anammox activity. This competition can lead to an accumulation of nitrogen within the system (Wen et al., 2020).

Oxygen tolerance is dependent on the aggregation state of the biomass, whether it be aggregated or planktonic cells. The presence of oxygen appears to only inhibit the anammox process, and can be reversed (Zhang and Okabe, 2020). Planktonic anammox cells are more sensitive to oxygen, especially at low cell density (Oshiki et al., 2020).

During the anammox process, hydrogen ions are consumed resulting in an increasing pH within the reactor (Zhang and Okabe, 2020). Many anammox species to date have been enriched at pH ranging between 6.8 and 8.5 (Zhang and Okabe, 2020). The pH could play an significant role in regulating AOB, which in turn influences the system dynamics and impacts anammox growth (Zhang and Okabe, 2020).

Anammox bacteria have a wide temperature tolerance which varies dependent on the species. In order to adapt to temperature changes, anammox species have been observed to alter their lipid composition (Wu et al., 2020). Maximum anammox activity is reported between 35 °C and 40 °C, however, above 45 °C biomass lysis occurs and activity is irreversibly lost. Lower temperatures (15 °C) result in lower activity and the accumulation of nitrite (Wu et al., 2020).

Freshwater anammox species have been enriched at temperatures between 27 °C and 37 °C, and marine species between 15 °C and 25 °C. Maximum activity was observed at 37 °C for freshwater species, including *Ca*. *Brocadia sinica* (Zhang et al., 2017a, Zhang and Okabe, 2020). Marine species favour cooler environments to those found in freshwater systems (Wu et al., 2020).

Anammox bacteria are autotrophic, and the presence organic carbon matter can inhibit their growth. This has been shown at concentrations (measured in chemical oxygen demand (COD)) of over 237 mg COD L^{-1} (Wu et al., 2020, Zhang and Okabe, 2020). Methanol in particular has been identified as a strong inhibitor of most anammox bacteria (Narita et al., 2017). Nitrite too, although also a substrate, can inhibit anammox activity at high concentrations and is important to control during the operation (Zhang et al., 2017b). Partial inhibition occurs when concentrations exceeded 3.6 mM nitrite and total inhibition was observed at levels over 7.2 mM nitrite (Wu et al., 2020, Zhang and Okabe, 2020). Other studies report 50 % inhibition of a culture containing both *Ca*. *Brocadia* and *Ca. Jettenia* at 11.6 mM nitrite, allowing for a much wider range of nitrite tolerances (Narita et al., 2017). The importance of maintaining the nitrite below toxic levels, especially during the initial phase of operation has been strongly emphasised (Wu et al., 2020). Nitrite sensitivity also varies between species and aggregation states, with *Ca*. *Brocadia sinica* tolerating over 16 mM nitrite concentrations. In comparison, *Ca*. *Jettenia caeni*, was only determined to tolerate up to 11 mM (Zhang et al., 2017a). This might account for the ability of *Ca*. *Brocadia* to recover better and have a higher abundance than *Ca*. *Jettenia* after a high dose of substrate (Wu et al., 2020). High concentrations of ammonium and nitrate (between 25 and 50 mM) have been linked to anammox activity inhibition (Wu et al., 2020). Other inhibitors observed include sulfide (>5 mM), phosphate (>50 mM) (Zhang and Okabe, 2020), and high concentrations of calcium and magnesium (Wu et al., 2020).

1.2.7 The Anammox Process in Wastewater Treatment

For the treatment of wastewater containing high concentrations of ammonia, the anammox process is an innovative discovery (Wen et al., 2020). Over 200 anammox facilities are in operation worldwide, with wastewater from monosodium glutamate, pharmaceutical and agricultural industries, as well as landfill leachate have successfully been treated with the anammox process (Wen et al., 2020, Wu et al., 2020). The anammox process has been implemented in conjunction conventional nitrification and denitrification processes. One important set up involves the partial nitrification (or nitritation) followed by the anammox process (PN/A). As seen in Figure 5, this can occur in a single tank or multiple reactor systems, both reporting similar total nitrogen (TN) removal rates over 90 % (Jetten et al., 2009, You et al., 2020). In this way, AOB produces nitrite from ammonium in the influent. NOB activity is supressed. The anammox process then combines nitrite and ammonium to produce nitrogen gas, effectively removing nitrogen from the wastewater (Wu et al., 2020). A significant challenge posed by single stage nitrogen removal is the suppression of NOB, to prevent the full nitrification of ammonium to nitrate. Although inhibition and wash out strategies have been developed, none have proved reliable (Ni et al., 2016).

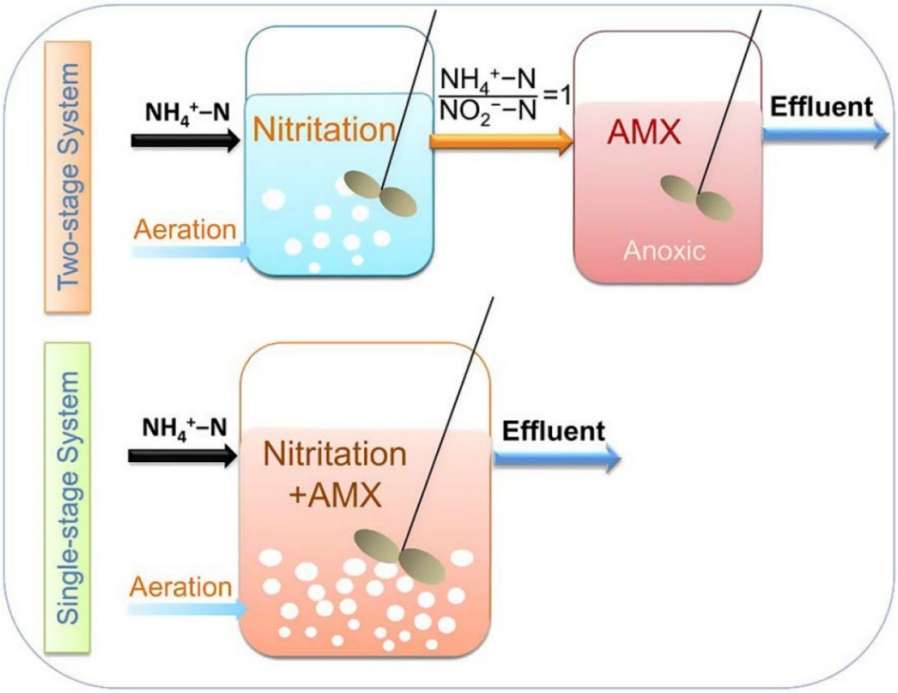

Figure 5: PN/A process in single and two staged systems (Li et al., 2018)

Nitrite is the limiting substrate in both natural and fabricated environments, therefore it is important to increase the concentration of nitrite of wastewater prior to feeding to the anammox tank (Zhang and Okabe, 2020). In the start-up procedure of full-scale plants, granular biomass is obtained from the cultivation of mature lab and pilot scale reactors is used to inoculate the full-scale plant, reducing the time until operation commences (Wu et al., 2020, Zhang and Okabe, 2020). Anammox plants also use anammox granular sludge or biomass retained in plastic carriers. The success of the world's first industrial scale anammox reactor is owed to the quantity of high-density granules with high settling velocities (Jetten et al., 2009). Settling velocities of granules have been reported higher than 100m s^{-1}. This reduces the operation time for clarification steps, allowing for increased treatment efficiency (Wu et al., 2020).

1.2.8 Advantages and Disadvantages of Wastewater Treatment via Anammox Process

Disadvantages of anammox systems include the large quantities of biomass required for seeding, hindering its implementation (Wu et al., 2020). The world's first anammox reactor in Rotterdam, The Netherlands, required over 3 years until it was operational (Ni et al., 2016). Anammox processes will continue to compliment traditional denitrification and nitrification treatments as a side stream process, as further investigation is required before it can be applied as a stand-alone wastewater treatment process (Wen et al., 2020).

However, the anammox process is a cost effective and ecofriendly approach to nitrogen removal from wastewater (Narita et al., 2017, Zhang and Okabe, 2020). Because of the anammox metabolic pathway, oxygen demand is reduced by 60 %, organic carbon is no longer required, and sludge production is reduced by 90 %. This results in substantial energy savings (Wen et al., 2020). This reduced energy demand, combined with the anaerobic digestion of organic carbon makes energy or energy positive treatment plants a possibility (Wen et al., 2020, Wu et al., 2020).

1.3 Anammox Cultivation in Bioreactors

1.3.1 Relationship between Reactor Operational Parameters and Growth

Traditional microbial techniques are not optimal for the isolation of slow growing organisms (Jetten et al., 2009). One of the features of a successful enrichment is the wash out of undesired bacteria, while retaining the desired species. This can be achieved by the application of a high flow rate, resulting in a short hydraulic retention time (HRT) removing cells suspended in the supernatant (Zhang and Okabe, 2020). The solid retention time (SRT) is how long biomass is retained in the reactor and is linked to the growth rate (μ). If the SRT is lower than a bacteria's growth rate, it will be unable to replicate before being washed out of the reactor. This applies selection pressure to those bacteria that cannot grow faster than the SRT, resulting in the growth and enrichment of the desired bacteria well suited to the reactor environment. CSTRs with single cells are useful tools to control the bacterial growth since the dilution rate of the reactor (inverse of HRT) is equal to the growth rate of the culture.

The SRT is a crucial parameter to control in bioprocesses, particularly for those involving slow growing bacteria (Reino Sanchez, 2016). A minimum value of SRT must be applied to increase the biomass concentration in the reactor. When SRT is too low, washout of desired species can occur (Jubany et al., 2009). Membrane bioreactors (MBR) are presented as an effective tool for slow-growing bacteria. An MBR allows for total retention of planktonic cells and are therefore an ideal tool for enrichment (Oshiki et al., 2013, Zhang and Okabe, 2020). In a an MBR it is possible to control the HRT and SRT independently, making it a popular configuration for enrichment (Zhang and Okabe, 2020). Another cultivation alternative is the use of a well-mixed CSTR. In these systems the HRT is equal to the SRT (Ding et al., 2018).

1.3.2 Current Cultivation Strategies

Typically, different reactors have been used for enrichment of anammox bacteria with different states of aggregation. While a sequencing batch reactor (SBR) is useful for anammox granular biomass, upflow anaerobic bioreactors (UAB) are implemented for growth of annamox biofilms and MBRs are the preferred tool for cultivating anammox planktonic cells (Zhang and Okabe, 2020). The use of membranes retains the biomass in the reactor, resulting

in longs SRTs. This is useful in counteracting the slow growth of anaerobic organisms such as anammox bacteria. This results in the decoupling of SRT and HRT, allowing low HRTs with high SRTs (Dong, 2015). No relationship between the reactor type and enrichment of particular anammox species has been observed (Zhang and Okabe, 2020). Various reactor configurations and operational conditions have resulted in different anammox enrichments. This ranges from 74 % in an SBR to 97.7 % purity in an MBR (Wu et al., 2020). *Ca. Jettenia*, *Ca*. *Brocadia* and *Ca*. *Scalindua* have successfully been enriched in MBRs from granular biomass. These operations require a considerable time investment typically between 100 and 200 days (Zhang et al., 2017b). MBRs are also prone to fouling, and accumulated biomass must be periodically removed (Dong, 2015).

Despite the high enrichment achieved in MBRs, the mechanisms are not well understood (Zhang and Okabe, 2020). It was believed that anammox cultures could only be planktonically enriched in MBRs, but recently this has also been demonstrated in a CSTR (Ding et al., 2018).

1.3.3 Cultivation in a CSTR

A CSTR has been proposed as a promising tool for anammox bacteria cultivation, after the successful enrichment *of Ca. Kuenenia stuttgartiensis* in UFZ, Leipzig, Germany (Ding et al., 2018). This study showed a rapid cultivation of at culture to an enrichment of 87%, with of a doubling time of just 3 days within 16 days of operation (Ding et al., 2018). This is significantly faster than the previously recorded of doubling times of 1-2 weeks in MBRs (Ding et al., 2018, Oshiki et al., 2013). A homogenous reactor effluent was attained, with a cell density of 8.5×10^{-7} cells mL^{-1} by day 30 (Ding et al., 2018).

A CSTR is positioned as a superior tool for planktonic anammox enrichment. The straightforward design does away with a sludge pump or membrane system, allowing for a less complex system. The proliferation of planktonic cells in a CSTR is desirable to study the microbiology, physiology and biochemistry of anammox bacteria (Ding et al., 2018). A disadvantage of CSTR operation is the inability to decouple HRT and SRT. The HRT therefore cannot be set above the growth rate at the risk of washing out desired microorganisms (Ding et al., 2018). It is very exceedingly difficult to operate a CSTR with biomass at steady state, as

the biomass produced must be equal to that washed out from the reactor. Care must also be taken not to operate too close to wash out conditions (Wittrup, 2007).

1.4 Planktonic *Ca. Brocadia* cultivation in a CSTR

1.4.1 Strain Selection

While *Ca. Scalindua* are the most abundant anammox species in natural environments, *Ca. Brocadia* species have been found to dominate in both municipal and industrial WWTPs (Chi et al., 2018, Kuenen, 2008, Oshiki et al., 2016). However, there is limited information regarding niche differentiation, kinetic parameters, and the physiology of anammox species, particularly regarding *Ca. Brocadia fulgida*. Further research is a requirement to better understand and optimise the process for application in WWTPS. An additional reason for the selection of *Ca. Brocadia fulgida* in this enrichment project was the ready availability of granules from a steady and active SBR. No planktonic cultures enriched in *Ca. Brocadia fulgida* have previously been reported.

1.4.2 Rational for the Planktonic Enrichment of *Ca. Brocadia* in a CSTR

With a clearly defined need for planktonic anammox cultures, new enrichment strategies are required. This approach is to investigate if the rapid enrichment and cultivation in a CSTR can be replicated with a granular sludge inoculum of *Ca. Brocadia fulgida* (Ding et al., 2018). This approach will also implement strategies reported from the successful start-up of planktonic *Ca*. *Brocadia* from a granular inoculum in an MBR (Oshiki et al., 2013). A literature review unearthed no studies relating to the planktonic cultivation of *Ca. Brocadia* in a CSTR, therefore this is an innovative approach. This culture could then be used for further biochemical and physiological studies optimise the application of the anammox process, specifically *Ca. Brocadia,* in WWTPs.

1.5 Objectives

The importance of enriched planktonic cell cultures of anammox bacteria has been outlined above. The main aim of this project was to achieve the stable operation of a CSTR inoculated with *Ca. Brocadia fulgida* performing the anammox process. The development of a planktonic cell culture, free from aggregations, was also pursued. Characterisation of the culture was undertaken to investigate changes in microbial population and niche differentiation.

2 Materials and Methods

2.1 Reactor

Choosing the appropriate reactor is important consideration in any operation. A CSTR was selected as an appropriate tool for cultivation as it provided a homogenous outflow, could be easily controlled and was affordable lab-scale configuration.

2.1.1 Setup

A lab scale CSTR was operated in semicontinuous mode pursuing the anammox process. The vessel had a working volume of 1 L, a diameter of 10 cm and a height of 23.5 cm. The reactor had ports for medium exchange, sampling, pressure, and temperature monitoring. All sensors were attached to a LabJack converter for data reading and storage. The monitoring and control were via a Raspberry Pi microprocessor. The reactor set up can be seen in Figure 6 and 7. A top-down view can be found in Appendix 6.1. Pressure due to gas accumulation in the reactor headspace was constantly monitored. To prevent pressure build-up and potential breakage, an automatic valve was installed in the gas outlet. When pressure was recorded above 400 mV, this triggered the release valve to open for 5 sec releasing gas and reducing pressure in the vessel. Temperature was controlled through a heating blanket and thermometer to 32 °C ± 0.6 °C. The pH was not controlled, but monitored using a FiveEasy pH meter (Mettler Toledo, Germany), and ranged between a pH of 7.0 and 8.4. No pH adjustments were needed. The DO concentration was monitored using adhesive sensors and Fitbox 4 Trace DO meter (PreSens, Germany). DO concentration was lowered, when required, through the intermittent addition of 100 mM Na_2SO_3, which is an oxygen scavenger.

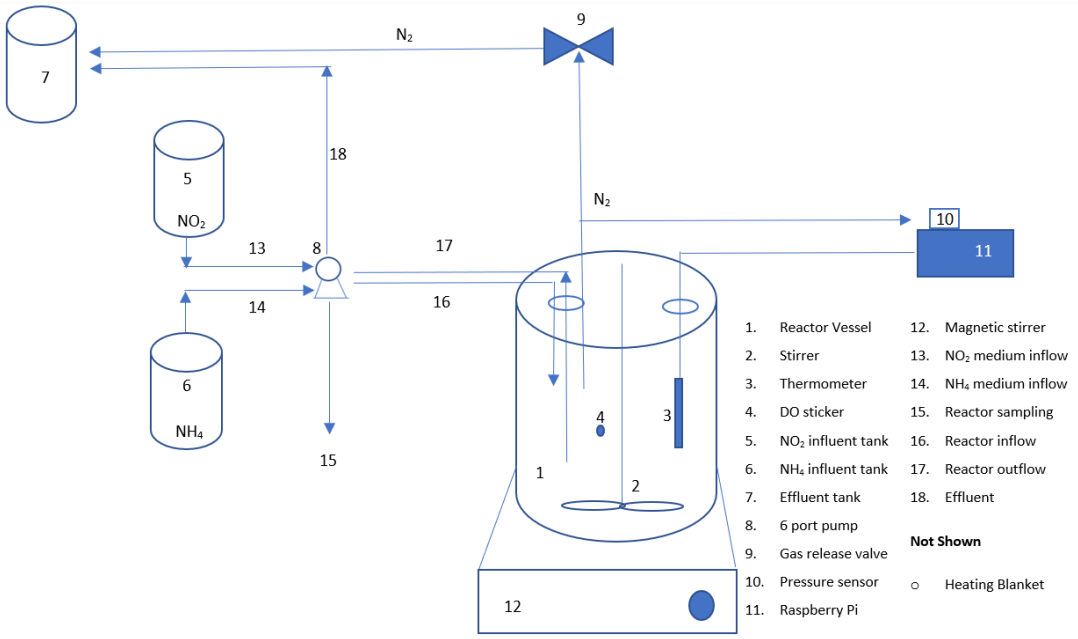

Figure 6: Schematic of CSTR start up

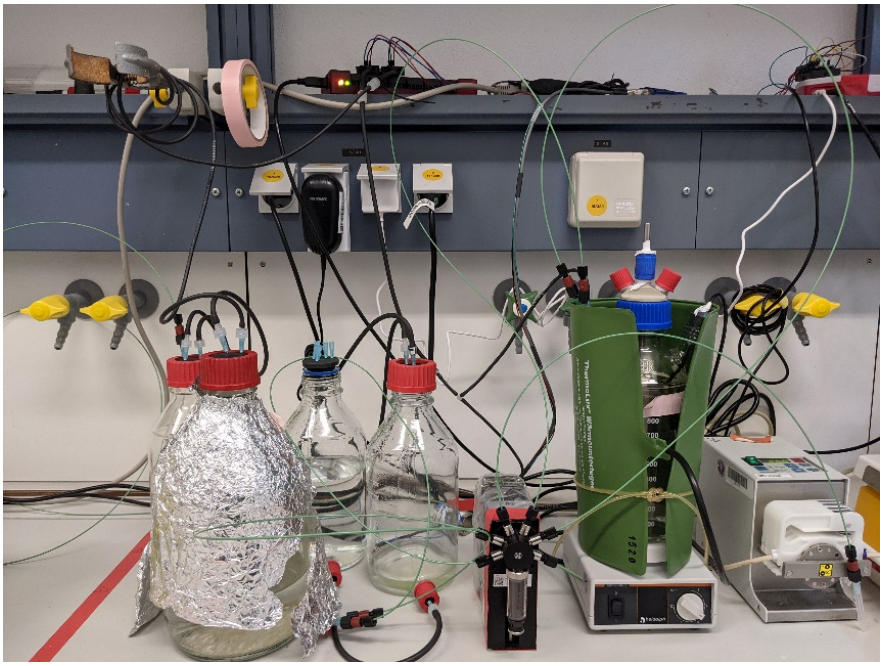

Figure 7: Operation Set Up in UFZ Laboratory, Leipzig, Germany

Supernatant was removed from the reactor (on days 41 and 77) and replaced with fresh medium. The influent mediums were stored in a bottle flushed with nitrogen gas, however, DO was present in the medium and was reduced through the periodic addition of Na_2SO_3 to maintain concentrations under 0.1 mg L^{-1}.

Ammonium was in excess to prevent a low redox potential and prevent accumulation of, and inhibition by nitrite. The reactor was fed a synthetic wastewater medium, described in Appendix 6.2. This medium composition was selected as it had been proven to be a suitable medium for the cultivation of anammox bacteria in a CSTR.

2.1.2 Inoculation

The CSTR was inoculated with granules from an active SBR anammox reactor operating in UFZ laboratories. The inoculum contained a mixed culture, enriched in *Ca. Brocadia fulgida.* 950 mL of anoxic medium was prepared, as described in Appendix 6.3. The medium was flushed for 45 minutes with nitrogen gas, without ammonium chloride, nitrite and sodium bicarbonate, which was later added. Inoculation occurred inside an anaerobic tent to prevent oxygen inhibition. Granules were taken from the SBR, and supernatant was removed for a total volume of 50 mL. The remaining biomass was 7.5 mL, or approximately 5 g wet biomass. The biomass was then vortexed for 1 minute to facilitate cell detachment, before being inoculated into the medium. This can be seen in Appendix 6.4. The reactor had initial concentrations of 5 mM ammonium, 3 mM nitrite with a pH of 7.81 and a DO concentration of 6 mg L^{-1}. The bioreactor was continuously mixed by a magnetic stirrer at 200 rpm.

2.1.3 Operation Conditions

After 3 days, feeding commenced as nitrite levels had depleted. The feeding was controlled by a Python script via a Raspberry Pi microprocessor. This then operated the pumps, with the signal transferred via cables. Each cycle began with the removal of 4.17 mL of reactor contents. This was then replaced with 1.67 mL ammonium and 2.5 mL nitrite, so the reactor volume was maintained. Initially the medium was fed every 2 hours. This resulted in a HRT of 20 days, and an NLR of 6.35 mmol day^{-1}. Because the ammonium medium was more

concentrated than nitrite, (160 mM and 105 mM respectively) the flow into the system was adjusted to maintain a molar ratio of approximately 1:1. This ratio was selected to prevent nitrite accumulation in the reactor. Operation conditions varied throughout the operation in response to reactor performance and were adjusted to promote the growth of anammox bacteria. Cycle times were increased with feeding occurring every 8 hours on day 16. This resulted in a HRT of 80 days. At times, feeding was stopped due to perceived accumulation in the reactor. On day 41, it was decided the mediums should be diluted. This was to enable a lower NLR while maintaining a shorter HRT. At this point, the cycle was also amended to feeding every 4 hours.

2.2 Microbiological Techniques

2.2.1 DNA Isolation

DNA extraction was achieved using the commercial PowerSoil DNA kit (Qiagen, Germany) following the manufacturer protocol. Samples of biomass were taken from the inoculum and the final day of operation. The quality and quantity of DNA extracted was measured by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). More details on DNA extraction results can be found in Appendix 6.5.

2.2.2 Polymerase Chain Reaction

DNA samples were amplified using Polymerase Chain Reaction (PCR). Anammox specific bacterial genes were amplified using primer set AMX649PF/AMX960RGC. The general bacterial primer pair 341FGC/518R12 was selected for 16s RNA genes. The amplifications were performed with a 25 µL GoTaq Green Master Mix (Promega, Germany), comprising 21.5 µL sterile water, 0.5 µL of both forward and reverse primers with a concentration of 10 µM, and 2.5 µL of DNA sample, for a total volume of 50 µL. Reactions were performed in the peqSTAR 2x Gradient Thermocycler (PreqLab, Germany) under the following thermal profile: 95 °C for 2 min, then 38 cycles of 95 °C for 40s, 62 °C for 45s, 72 °C for 30 s, followed by one cycle of 72 °C for 5 min and 8 °C hold. Amplification of the DNA was then confirmed using Gel Electrophoresis. Supporting information can be found in Appendix 6.6. A gel was

prepared comprising 1 % agarose in a 100 mL 1x TAE Buffer and was then run at 100 V for 1 hour. The gel was incubated and stained for 15 minutes in a bath of Ethidium bromide, then rinsed in water. The stained gel was then observed in the Molecular Imager Gel Doc XR (Bio-Rad, Germany).

2.2.3 Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) is a molecular fingerprinting technique used to analyse the composition, diversity, and dynamics of a microbial community. It separates PCR products based of differing sequence composition. The bands with high guanine and cytosine (GC) content migrate further through the gel under increasingly denaturing conditions (Green et al., 2010). A 70/30 high-low gradient gel was used in this procedure. It was run for 16 hours at 100 V in a 1x TAE buffer solution at 60 °C. The gel was incubated for 30 minutes with SYBR Gold (Thermo Fisher, Germany) in 1x TAE, under gentle agitation. The gel was then viewed in the Molecular Imager Gel Doc XR (Bio-Rad, Germany). Bands selected for subsequent sequencing were cut. DNA was extracted and amplified as described in section 2.2.2 above, then purified using the Wizard Purification Kit (Promega, Germany) before sending for sequencing.

2.2.4 Sequencing

Sequencing was done by an external laboratory, utilising the overnight Mix2Seq Kit (EuroFins, Germany) and following the manufacturer's instructions. 15 µL of purified DNA sample and 2 µL of forward and reverse primer were mixed in the tubes provided. The FASTA sequences obtained were then compared to those in the bacterial database available in National Center for Biotechnology Information (NCBI)using the Basic Local Alignment Search Tool (BLAST). Results were filtered based of maximum score. A FASTA format is a text-based representation of nucleotide or amino acid sequences.

2.2.5 Cell Counting

Cell counting was achieved through direct epifluorescence microscopic counting of SYBR-green stained cells on agarose-coated slides. A homogenous 20 µL sample from the bioreactor was incubated with 2.6 µL of 100x diluted SYBR Green stock solution for 10 minutes at room temperature, before being viewed under the microscope.

2.2.6 Protein Determination

Protein concentration was quantified by following the Bradford Protein Assay (Ernst and Zor, 2010). A calibration curve was established using Bovine Serum Albumin (BSA) and Bradford Reagent (Bio-Rad, Germany) was used to establish the protein content. The calibration curve was prepared for each measurement. Each curve had an R^2 value over 0.99 indicating low variance. A calibration curve can be seen in Appendix 6.7. Samples were taken of both the solids, and the supernatant after settling for 3 min. Cells were disrupted using a modified RIPA buffer (150 mM sodium chloride (NaCl), 1 % Tween 20, 0.2 % SDS, 50 mM Tris (pH 7.4)). Bradford reagent was then added and the absorbance at 450 and 590 nm was measured using Evolution 160 UV-Vis Spectrometer (Thermo Fischer, Germany). The ratio between the wavelengths corresponded to the protein content of the sample.

2.3 Analytical Techniques

Ammonium was determined utilising column chromatography and measured on an UltiMate 3000 High Performance Liquid Chromatography (HPLC) (Thermo Scientific, Germany). Nitrate was measured using on a Dionex DX-120 ion chromatographer (ThermoFisher, USA) equipped with an IonPac AS4A-SC (4 mm × 250 mm) column. The eluent was comprised of 0.7 mM sodium carbonate ($NaCO_3$) and 0.7 mM sodium bicarbonate ($NaHCO_3$) with a flow rate of 1 mL min^{-1}. Nitrite concentrations of the reactor could not be determined by ion chromatography due to the high chloride concentration of the medium, which resulted in overlapping peaks.

Due to inconsistencies with nitrite measurements, ammonium, nitrite and nitrate were all be determined via ion chromatography using Dionex ICS-4000 (ThermoFisher, Germany). Samples were diluted to between 1 and 20 mg L^{-1} of each compound before measurement.

2.4 Calculations

2.4.1 Anammox Process Ratios

In order to investigate the consumption and production patterns of anammox bacteria during operation, several ratios were calculated.

2.4.1.1 Nitrite and Ammonium consumption

Where initial is the number of mmol in reactor at beginning of the period and final is the number of mmol in reactor on the final day of the period. Period duration is the number of days in the period.

$$Daily\ N-Consumption = \frac{\left(NO_{2\,Initial} + NO_{2\,added} - NO_{2\,final}\right) + \left(NH_{4\,initial}^{+} + NH_{4\,added}^{+} + NH_{4\,final}^{+}\right)(mmol)}{Period\ Duration\ (days)}$$

Equation 2: Calculation of daily consumption of NO_2 and NH_4^+

The substrate (nitrite and ammonium) that was lost through exchange of the medium is not considered in this equation. It also is unable to differentiate between consumption from anammox bacteria and consumption from other nitrifiers and denitrifiers.

2.4.1.2 N-NO_2/N-NH_4^+ Ratio

Where initial is the number of mmol in reactor at beginning of the period, final is the number of mmol in reactor on the final day of the period and added is substrate added due through feeding.

$$\frac{N-NO_2}{N-NH_4^+} = \frac{(N-NO_2)_{initial} + (N-NO_2)_{added} - (N-NO_2)_{final}}{(N-NH_4^+)_{initial} + (N-NH_4^+)_{added} - (N-NH_4^+)_{final}}$$

Equation 3: Calculation of N-NO_2/ N-NH_4^+ ratio

2.4.1.3 $N\text{-}NO_3^-/N\text{-}NH_4^+$ Ratio

Where initial is the number of mmol in reactor at beginning of the period, final is the number of mmol in reactor on the final day of the period and added is substrate added due through feeding.

$$\frac{N-NO_3^-}{N-NH_4^+} = \frac{(N-NO_3^-)_{initial} + (N-NO_3^-)_{added} - (N-NO_3^-)_{final}}{(N-NH_4^+)_{initial} + (N-NH_4^+)_{added} - (N-NH_4^+)_{final}}$$

Equation 4: Calculation of $N\text{-}NO_3^-/N\text{-}NH_4^+$ ratio

2.4.2 Nitrogen Gas Production

The anammox process results in the production of nitrogen gas which accumulates in the headspace of the bioreactor. The headspace pressure was converted into an analogue electrical signal by a pressure transducer, providing an overview of the reactor's activity. The output is measured in millivolts (mV) and correlates with the pressure in the headspace.

The electrical signal must be converted to mols to determine the nitrogen produced. The pressure sensor was calibrated by removing 5 mL medium from the reactor. A pressure change of 137.5 mV was observed. 1 mL of gas therefore corresponds to 27.5 mV. From this measure it was possible to determine the volume of gas produced each day. The ideal gas law (Equation 5) was used to calculate the mmol of gas produced.

$$PV = nRT$$

Equation 5: Ideal Gas Law

Rearranged to solve for n:

$$n = \frac{PV}{RT}$$

Where:

Variable	Factor	Unit	Value
P	*Pressure*	*Pa*	*101325*
V	*Volume*	m^3	
n	*Moles*	*mol*	
R	*Gas Constant*	*J mol $^{-1}$ K^{-1}*	*8.3144*
T	*Temperature*	*K*	*305.15*

Table 1: Variables of Ideal Gas Law

To calculate the theoretical gas production of an anammox process within a period, the following calculation was solved: The molar ratio of 1.02 was derived from Equation 1, that 1 mmol nitrite should stoichiometrically produce 1.02 mmol nitrogen gas ($\frac{1.02}{1} = 1.02$). The following equation (Equation 6) was used to calculate the theoretical nitrogen gas yield.

$$N_{2\,Theoretical} = (Initial\ N - NH_4^+ + N - NH_4^+\ added\ - Final\ N - NH_4^+)(mmol) * 1.02$$

Equation 6: Calculation of theoretical nitrogen gas yield

2.4.3 Nitrogen Loading Rate

NLR was calculated and plotted in Figure 10. Equation 7 was used to calculate the NLR.

$$NLR = \frac{Inf\ N - NO_2(mM) * Q_{NO2}\left(\frac{L}{day}\right) + Inf\ N - NH_4^+(mM) * Q_{NH4+}(\frac{L}{day})}{V(L)}$$

Equation 7: Calculation of NLR

Flow (Q) is inclusive of volume added as part of the cycle and replaced during sampling. The initial concentration of the ammonium medium and nitrite medium was 160 and 105 mM, respectively. The initial flow of each medium was 20 mL day^{-1} and 30 mL day^{-1} respectively. The volume of the reactor was 1 L.

3 Results and Discussion

3.1 Operational Conditions and Microbial Activity in the Bioreactor

A lab-scale anammox CSTR was operated for 84 days treating a synthetic influent containing ammonium and nitrite in an appropriated ratio for anammox cultivation. DO concentration, pH and temperature (Figure 8) and nitrogenous compounds in the bulk liquid of the bioreactor were analysed periodically throughout the entire operation (Figure 9). The influent flow rate, and thus, the NLR, were adjusted according to the performance of the bioreactor (Figure 10). In addition, the on-line measurement of the pressure in the headspace of the bioreactor allowed for the continuous follow-up of the activity, recorded as an electrical signal (Figure 11). Gas production (Figure 11) was continuously measured and automatically recorded. The standard deviations are caused by multiple gas 'releases' per day (when the pressure exceeds 400 mV).

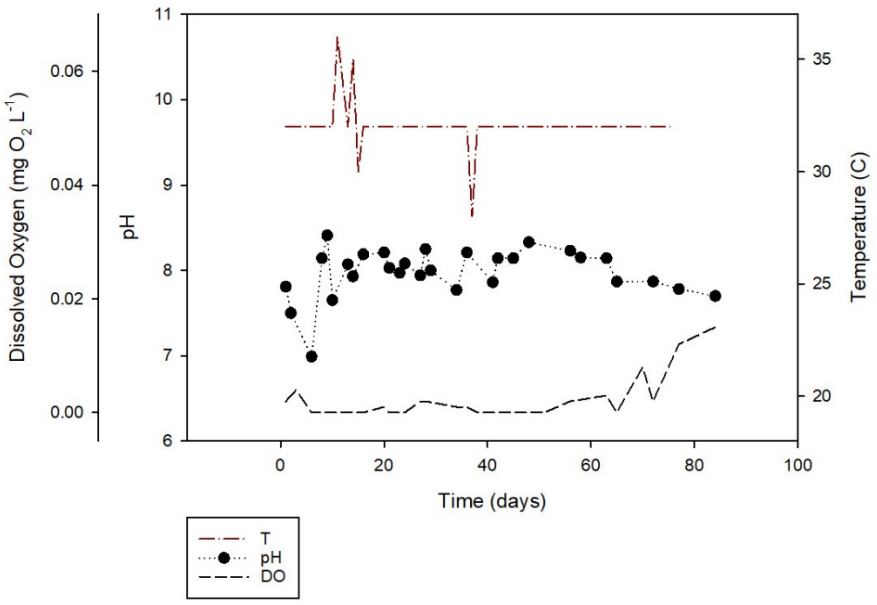

Figure 8: DO concentration, pH and Temperature during CSTR operation

During the operation of the CSTR, the temperature remained relatively constant (Figure 8). Days 11-14 saw exceptionally warm weather, and the temperature control of the system was unable to maintain the set temperature of 32 °C. However, this brief period of higher temperature was unlikely to have negatively affected the anammox cells, as they are suited to a range of 27-37 °C (Zhang and Okabe, 2020). The temperature also cooled unexpectedly

overnight on day 37 to about 28 °C, again remaining within a range suitable to anammox species, although a reduction in activity could have been expected. The pH was also maintained in appropriate values for anammox bacteria during the entire operation of the bioreactor. A sharp increase in pH was observed in the first week of operation which was consistent with denitrification and anammox activity. From then on it remained steady between 7.77 and 8.33, but from day 65 onwards, pH gradually decreased. This corresponded with a period of almost no activity (Figure 11).

DO concentration was always lower than 0.004 mg L^{-1} for the first 65 days of operation. From this point, decreasing pH and loss of reactor activity correlated to an increasing DO concentration. On days 63, 70 and 84 Na_2SO_3 (an oxygen scavenger) was added to the reactor to reduce DO concentration levels. This was only temporarily effective, as shortly after DO concentrations rose again.

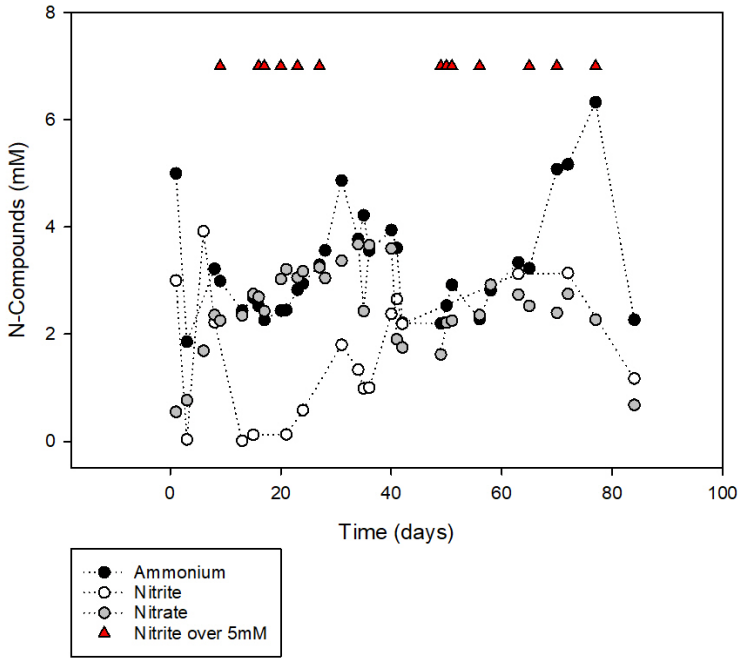

Figure 9: Concentration of nitrogenous compounds (ammonium, nitrite and nitrate) measured throughout the operation of the CSTR. Nitrite concentrations higher than 5 mM were plotted as red triangles.

The concentrations of nitrite, nitrate and ammonium were measured throughout the operation to understand the activity and manage the reactor. Due to loss of linearity, it was not possible to determine the nitrite when the concentration exceeded 5 mM. To this end, when nitrite was too high to accurately measure it has been indicated with the red triangle.

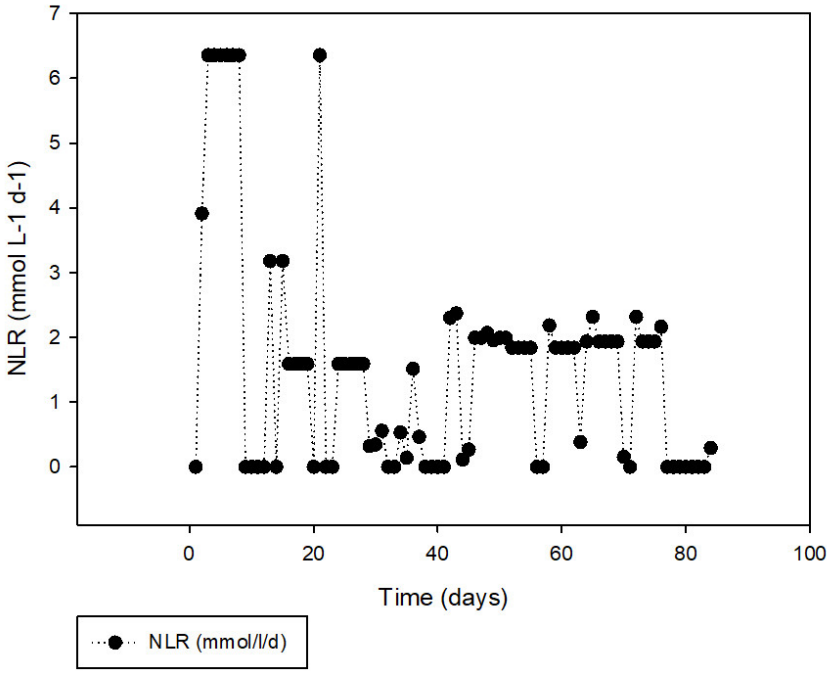

Figure 10: Nitrogen Loading Rate(NLR) during operation

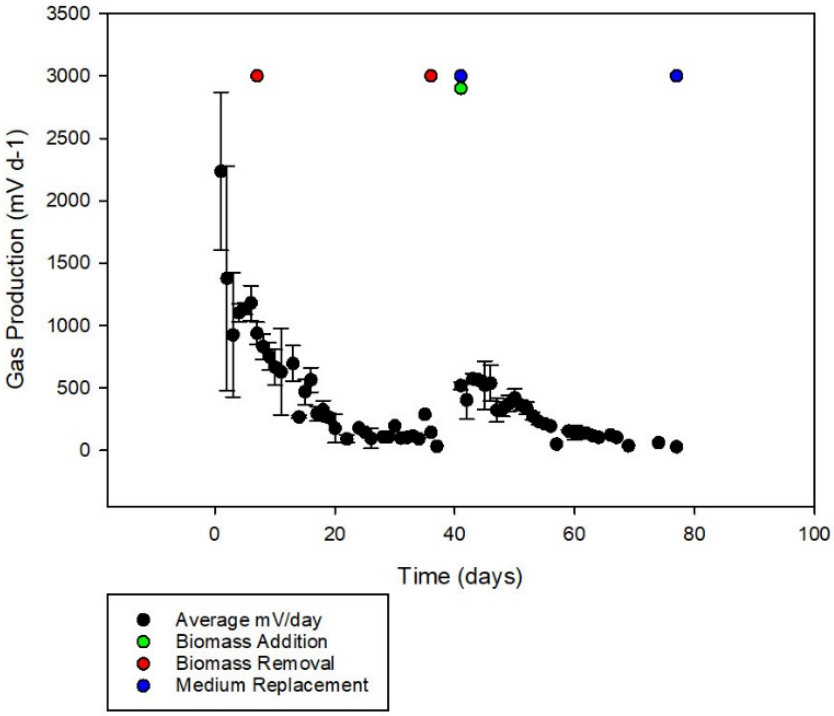

Figure 11: Reactor activity, measured by gas production (mV)

Figure 10 above shows the NLR over the operation period. NLR is dependent on the flow and concentration of influent streams and accounts for the vessel volume. The initial medium concentrations where 160 mM ammonium and 105 mM nitrite respectively. The initial flow was set to 50 mL day^{-1}, resulting in an initial NLR of 6.35 mmol L^{-1} day^{-1}. The inoculum was taken from a reactor with an NLR of 22 mmol L^{-1} day^{-1}, however, this had a high volume of biomass, so a lower initial NLR was selected. Over the operation period the flow was adjusted by increasing or decreasing the frequency of the feeding cycle from between 2 to 8 hours. Changes in the feeding cycle altered the NLR, and it varied frequently throughout the operation.

Flow was initially high, then decreased as accumulation was observed, especially when the nitrite concentration was high (Figure 10). Up to day 42, there was steady accumulation of both nitrate and ammonium in the reactor. Increasing ammonium can be explained by ammonium being in excess to nitrite reaction in an attempt to prevent nitrite inhibition. Not all the ammonium provided to the culture was expected to be consumed, and therefore slowly accumulated within the bioreactor. Nitrate levels were also expected to increase because of anammox activity.

On day 42, the ammonium and nitrite mediums were each diluted for a final concentration of 92.6 mM and 60.8 mM respectively. This was done to increase the flow while maintaining the NLR. The aim of increasing the flow (and therefore reducing the HRT) was to apply selective pressure for anammox growth. In this way anammox bacteria, which had sufficient substrates and suitable conditions for growth, would be able to multiply. They would be able to out-compete other bacteria which would be unable to replicate before being washed out of the reactor. From day 64, the flow of ammonium was increased from 1.67 to 2.10 mL $cycle^{-1}$ (due to a miscalculation). This excess ammonium was then clearly shown to accumulate rapidly (Figure 9). Feeding was stopped on day 77 and the concentration of all nitrogen compounds declines.

Figure 11 shows the removal of biomass on days 7 and 36 of the operation (2.5 mL and 1.5 mL respectively). Biomass was removed to facilitate the removal of granules from the reactor, to favour planktonic growth. On day 41, 300 mL fresh medium (with 6.6 mM nitrite and

ammonium) was exchanged. It was hypothesised that perhaps the culture had consumed all a vitamin or mineral, as there was no significant activity, despite sufficient substrates will being available. In addition, 2.5 mL of biomass was taken from the initial SBR to reinoculated the reactor. This resulted in a short-lived increase in activity, which thereafter steadily decreased. 700 mL medium without nitrogen compounds was replaced on day 77 (retaining all the settled biomass) in order to dilute the substrates as high nitrite concentrations were recorded. No significant activity was recorded after this point.

During the operation period, it was observed that pressure increased slightly after each feeding cycle. Bicarbonate was used in the medium as an inorganic carbon source. Bicarbonate (HCO_3^-) exists in equilibrium with carbon dioxide as shown in Equation 8. Changes in temperature and concentration and pressure can alter the equilibrium (McMurray and Fay, 2010).

$$CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$$

Equation 8: Equilibrium of carbon dioxide (CO_2) and bicarbonate (HCO_3^-)

In addition, the solubility of carbon dioxide in water decreases as temperature increases (Shapely, 2011). Therefore, it is possible that when additional medium was added to the reactor, this increased the concentration of bicarbonate when combined with the increased temperature of the reactor, resulted in a release of carbon dioxide into the headspace. This would have been measured as an increase in pressure, that was not nitrogen gas. However, the effects of this were minimal and it was only observed in period where the microbial activity was null, such as at the end of the operation.

In nitrogen removal reactors, the nitrogen removal rate (NRR) is typically discussed in addition to the NLR. However, this operation never reached a steady state. Without a steady operation it is not possible to calculate this parameter. Although a hydraulic steady state was achieved for days at a time, the concentration of products and substrates was constantly changing due to extremely variable microbial activity. In addition, the possible production and consumption of biomass makes the calculation of an NRR challenging. Instead, estimations of consumption were calculated to evaluate the anammox reaction, as can be seen in Table 2.

3.2 Evaluation of Anammox Process

The occurrence of anammox activity was evaluated considering the theoretical stoichiometry associated to the anammox process (Equation 1). This section assesses the consumption and production of the nitrogenous compounds involved in the anammox reaction utilising the nitrite to ammonium consumption ratio (NO_2/NH_4^+) and the nitrate produced to ammonium consumed ratio (NO_3^-/NH_4^+). The operation was divided into six periods. These non-consecutive periods were selected based on the availability of reliable data for calculations to provide an insight into reactor activity over the operation. Various levels of microbial activity were observed, and the main results associated to each period are discussed.

The NO_2/NH_4^+ molar ratio is the relationship between the consumption of the substrates. If anammox activity were present, this ratio would be close to 1.32 as was stoichiometrically calculated. Table 2 shows the nitrite to ammonium consumption ratio during the different periods of operation. For almost all periods this value was lower than expected. This indicates that either that less nitrite was consumed than expected, or a higher consumption of ammonium occurred. A higher-than-expected consumption of ammonium could be explained by the presence of ammonia oxidizing bacteria (nitrifiers) in the culture. Their growth was expected to be limited by the absence of oxygen, since nitrifiers are aerobic bacteria, however, some oxygen was expected to be present in the medium (despite flushing it periodically with nitrogen gas) allowing their growth.

In addition, in Periods 5 and 6, there as increasing oxygen in the system (Figure 9), which may have enhanced the nitrifying activity. This could also explain the low ratio at the end of the bioreactor operation (Table 2). The NO_3^-/NH_4^+ molar ratio represents the relationship between nitrate produced, for each mmol of ammonium consumed. With anammox activity, this should have been 0.26, although revised stoichiometry has placed this figure closer to 0.16 (Wu et al., 2020). A negative ratio is caused by a consumption of nitrate. This was a clear indication that there were denitrifiers within this culture, as the anammox metabolism only produces, not consumes nitrate.

Period	Operation time (day)	Period duration (days)	NH_4^+, NO_2 Consumed (mmol day^{-1})	$N\text{-}NO_2$/ $N\text{-}NH_4^+$	$N\text{-}NO_3^-$/ $N\text{-}NH_4^+$	N_2 produced (mmol day^{-1})	N_2 produced (mmol)	N_2 theoretical (mmol)	Volume Exchanged (mL)
1	3-8	5	5.64	0.93	0.12	1.53	7.67	14.93	300
2	8-13	5	0.73	2.73	-0.002	0.94	4.68	0.79	0
3	21-24	3	0.18	0.99	-0.015	0.13	0.39	2.78	50
4	31-40	9	0.39	0.55	0.180	0.22	2.02	2.33	26
5	63-72	9	1.19	0.86	0.002	0.14	1.22	5.84	161
6	72-84	12	1.26	0.66	-0.342	0.14	1.63	9.28	134

Table 2: Stoichiometric comparisons for different operation periods

3.3 Nitrogen Gas Production by Anammox Bacteria

The first period had the highest activity of the entire operation with 5.6 mmol-N being consumed each day. This period was also characterised by the highest gas production. Thereafter, activity steadily decreased until day 20. The remainder of the operation was far less active, as was confirmed by the gas production shown in Figure 11.

The anammox reaction results in the production of nitrogen gas. Hence, it was possible to calculate the expected nitrogen gas production based of ammonium consumed, according to the theoretical stoichiometry of the anammox process (Equation 1). The expected ratio was 1.02 mmol nitrogen gas for each mmol ammonium consumed. All periods had lower production than expected, except period 2. This could indicate that anammox activity was low in the reactor and other bacterial activities were taking place. It was hypothesised that some gas production was the result of heterotrophic denitrification, which also produces nitrogen gas as seen in Equation 9.

$$5HCOO^- + 2NO_3^- + 7H^+ \Rightarrow 5CO_{2(aq)} + 6H_2O + N_2$$

Equation 9: Denitrification stoichiometry, where HCOO- is organic matter

This process also produces carbon dioxide which could also contribute to increasing pressure. The experimental set up was not able to differentiate between gas types or which process produces the gas. This limits the ability to draw relationships between the anammox activity and gas production.

3.4 Loss of anammox activity

As observed in Figure 11, there were two periods when anammox activity was detected but gradually decreased (between days 0–20 and 41–60). The first one was at the starting up of the reactor, the second one when fresh cells were added. At the end of the operation the activity was null. The main hypothesis behind the loss of activity was the nitrite inhibition. Some activity was observed after adding fresh anammox cells in the reactor, but activity was

subsequently lost, and substrates accumulated. During the operation, nitrite concentrations were often so high that they likely inhibited growth (Figure 9).

When nitrite levels exceed a certain threshold, activity can be inhibited. Inhibition levels vary due operation conditions such as NLR, biomass aggregation state, length of exposure and the health of the culture. This poses a challenge to determine inhibition levels during operation (Wen et al., 2020). There is considerable debate as to whether nitrite inhibition is irreversible and at what level (Lotti et al., 2012, Wen et al., 2020). It can also be difficult to determine whether high nitrite levels resulted from inhibition and the resultant lack of nitrite consumption, or high nitrite conditions were responsible for inhibition (Lotti et al., 2012). From literature, partial inhibition has been observed when nitrite concentrations exceeded 3.57 mM and total inhibition was observed at nitrite levels over 7.14 mM (Wu et al., 2020, Zhang and Okabe, 2020). In this study, nitrite concentrations within the reactor were frequently above these values, contributing to reduced activity.

In natural environments, anammox bacteria are able to exploit low substrate concentrations (Jetten et al., 2009, Zhang and Okabe, 2020). (Oshiki et al., 2013) theorises that a low substrate concentration promotes the growth of planktonic cells, due to the limitations of substrate transport in aggregations. Therefore, the high concentrations in this operation would have been counterproductive to both growth and the establishment of a planktonic culture. However, substrates and nutrients must be present for exponential growth phase, and substrate limitation should be avoided to prevent undue stress on new cells (Zhang et al., 2017b).

At the end of the operation, the combination of decreasing pH and increasing DO concentration corresponds to a loss of activity in the reactor. The pH levels where never inhibitory for anammox bacteria, and instead are an indicator of the loss of activity and reduced consumption of H^+ ions. Anammox bacteria have reported inhibition of 50 % from just 0.03 mg O_2 L^{-1} – 3.8 mg O_2 L^{-1} (Yan et al., 2020). Inhibition by oxygen is also reversible (Yan et al., 2020). This rising oxygen level could have led to the inhibition of anammox bacteria and denitrification activity, which are inhibited 35 % with a DO concentration of 0.09 mg L^{-1}, compounding the loss of activity (Oh and Silverstein, 1999).

During the operation, it was observed that the biomass lost its colour. The inoculum was a vibrant pinkish orange but as the operation proceeded, the biomass loses its characteristic colour, appearing a greyish pink (Figure 12). Cytochrome C is the compound responsible for the pink colour. The deeper the red observed and measured in the culture's hue is found to have a positive correlation to anammox activity (Kang et al., 2018). This bright colour also been observed in the operation of highly active anammox cultures, operating in in an SBR within the laboratory (Appendix 6.8).

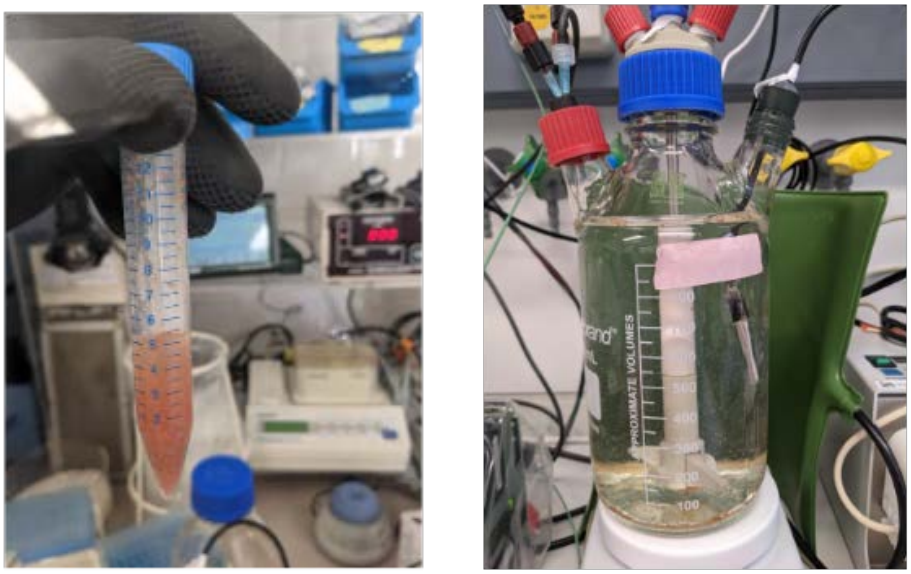

Figure 12: Biomass at re-inoculation day 42 (left) and day 70 (right)

Nitrifiers, which also have a typical orange-brown colour, were thought to be present in the culture. When oxygen is limiting, they are reported to turn brown or black (Alleman and Preston, 1992). This did not occur, possibly because the population was only minor component of the culture and there was sufficient oxygen from medium inflow, but they could have contributed overall colour of the reactor's biomass.

3.5 Bacterial Growth

Bacterial growth was monitored through the operation. The CSTR was inoculated with granular biomass. Overtime it was hoped that this would breakdown due to agitation, and when combined with the growth of new planktonic cells, the overall composition of the reactor would shift from granular biomass to planktonic. This strategy been achieved in an

MBR, as seen in Appendix 6.9. The extent of the shift from granular to planktonic aggregation states was observed by measuring the protein content of the supernatant and settled solids in combination with cell counting.

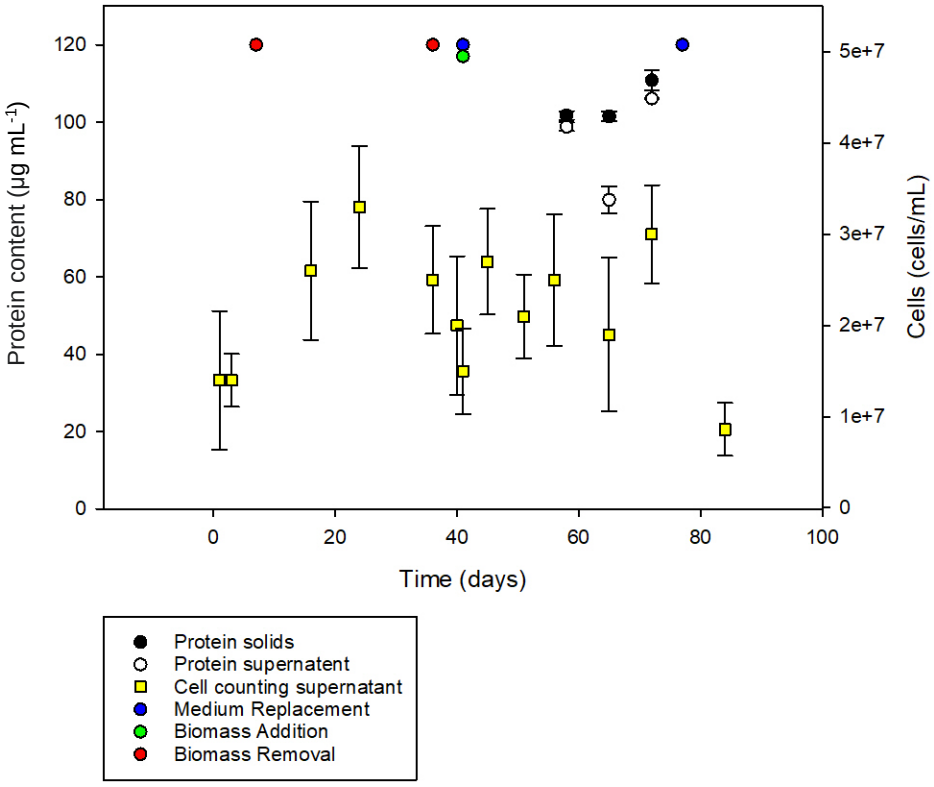

Figure 13: Cell Counting and Protein Composition

Cell counting was undertaken during operation to observe cell growth. During the first 3 weeks of the operation, there was an increase in the cell count, increasing from 1.7 x 10^7 cells mL^{-1} on day 1, to 3.3 x 10^7 cells mL^{-1} on day 24, before decreasing. After dilution (day 42), the cells had a concentration of 1.5 x 10^7 cells mL^{-1}. On day 72, 3.0 x 10^7 cells mL^{-1} was measured. On the last day of operation, the cell count had dropped to 8.6 x 10^6 cells mL^{-1}. This decrease was likely due to the medium exchange on day 77. A planktonic cell culture was not established during the operation.

One limitation of cell counting is that it is not easy to differentiate between anammox cells and other cells. Anammox cells appear after staining to be a C shape due to the presence of

the anammoxosome, which contains no DNA (van Niftrik and Jetten, 2012). This shape was not observed (Appendix 6.10). It was therefore possible that nitrifiers and denitrifiers were growing in addition to anammox bacteria. A hypothesis for the growth patterns observed could be explained by the presence of nitrifiers in the culture. They consumed any oxygen in the medium, keeping levels low for the first period of the operation. They were then unable to grow due to lack of oxygen, eventually becoming inactive, which led to an increasing DO concentration within the reactor. This increased DO concentration could have hindered planktonic anammox cells, promoting granulation, or inhibiting growth. This was supported by the observation of biofilm and flocs formation in the bioreactor.

Denitrifiers were also thought to present in the community. As fast-growing heterotrophs, they could have outcompeted the anammox species, increasing the cell count within the reactor. Once all the organic matter was consumed (from dead cells), growth would have to cease. This could explain the initial growth of planktonic cells in the culture.

Changes in the aggregation states of the biomass were also monitored (Figure 13). This was achieved by protein determination of solids, comprising of settled biomass, and supernatant. The protein content of cells that were present as granules or biomass increased slightly over a 2-week period, from 101.7 μg mL^{-1} to 110.8 μg mL^{-1}. The protein content of the supernatant also increased from 98.8 μg mL^{-1} to 106.1 μg mL^{-1}. This could indicate that growth was occurring, though whether this was anammox bacteria or other bacteria, cannot be determined. A distinct shift from granular to single cells was not observed, with granular biomass increasing at a faster rate than planktonic cells. Cell counting and supernatant protein determination on day 65 were both unexpectedly low, perhaps due to an error in sampling.

It has been theorised that the activity of anammox bacteria could depend on cell density, through quorum sensing, with increased activity being observed at higher densities (above 10^{10} cells mL^{-1} (Oshiki et al., 2020, Wu et al., 2020). The lack of a high cell density could therefore also contribute to the limited anammox activity

3.6 Microbial Characterisation

DGGE is a microbial fingerprinting technique that was used for observing changes in community diversity over time. This, in combination with sequencing allows for observations about the microbial community present in the reactor.

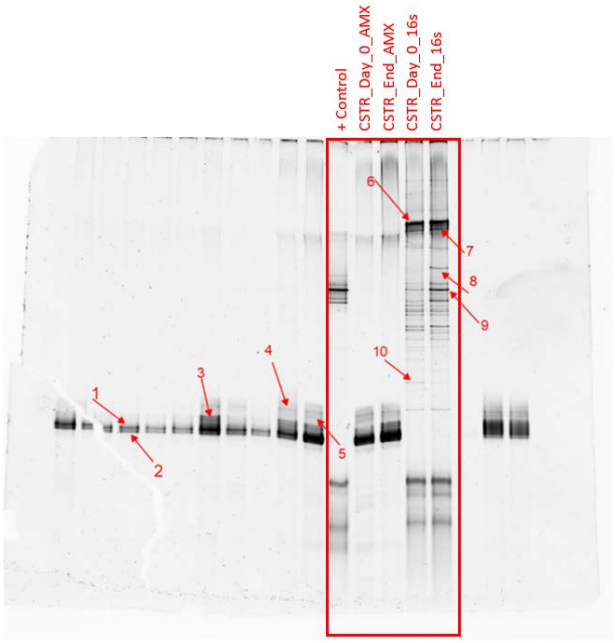

Figure 14: DGGE Bands showing population changes over time

Band	BLAST Result
6	Candidatus Brocadia sp. enrichment culture clone
7	Uncultured anaerobic ammonium-oxidizing bacterium
8	Uncultured anaerobic ammonium-oxidizing bacterium
9	Candidatus Brocadia sp. enrichment culture clone
10	Candidatus Brocadia sp. enrichment culture clone

Table 3: Sequencing results from DGGE bands

Table 3 above shows the sequencing results after cutting the gel bands indicated in Figure 14. The full FASTA sequence can be found in Appendix 6.11 The positive control was taken a culture of *Ca. Kuenenia stuttgartiensis* that was successfully enriched to 87 % (Ding et al., 2018). All parameters indicating a match between query sequence and the database are high, demonstrating a good correlation and high degree of certainty in the identification.

From the sequencing result, the dominant bands were associated with anammox bacteria, specifically *Ca*. *Brocadia*. Bands 6 and 10 were also associated with *Ca. Brocadia fulgida* EU478693.1. This was expected, as the culture was inoculated with an enriched culture of *Ca. Brocadia fulgida*. Bands 6 and 7 were the most intense bands, therefore most abundant in the culture. An enrichment of anammox DNA in bands 8 and 9 was observed over the operation period. It was thought that perhaps band 9 would indicate *Ca. Kuenenia*, as this was in a similar position to the dark band of the positive control lane, but the species of anammox was undefined. In contrast, a loss of band 10 was observed. Overall, there were limited changes in microbial community, which is consistent with slow growing anaerobic populations (Wen et al., 2020, Wu et al., 2020). A limitation of PCR and subsequent DGGE is that it assesses the DNA of the whole full population both dead and alive. This could result in the amplification of biomass that died at the beginning of the operation which was still present in the reactor on day 84. This would also contribute to the limited shift in population changes.

Although only anammox DNA was shown from the sequencing results, it was expected that denitrifiers were present in the initial inoculum. Band 3, which comes from a DNA sample of the same SBR that provided the inoculum (but from an earlier date than inoculation) corresponded to *Pseudomonas*, a common denitrifying bacteria.

The separation between bands when anammox specific primers were used was not successful. A different denaturing gradient should be applied for preparing the DGGE gel in further experiments. These bands could not be cut and used for sequence identification. They do however confirm the presence of anammox bacteria in the community. Furthermore, the position of the bands agrees with *Ca*. *Brocadia* when comparing with previous experiments performed in our labs.

4 Conclusions

A CSTR was operated for 84 days to establish a planktonic culture enriched in *Ca. Brocadia fulgida*, however, stable operation of the anammox process in a CSTR was not achieved. Due to lack of activity, the decision was made to stop reactor operation.

The culture was only active after the addition of biomass, thereafter anammox activity was lost as observed through reduced gas production and loss of biomass colour. Several factors were hypothesised as the cause of activity loss, including inhibition by high nitrite concentrations. Although a hydraulically stable operation was achieved, the complexity of a bioreactor made it impossible to fully calculate nitrogen mass balances. This presented a challenge to calculating the reactor activity and kinetic parameters.

Bacterial growth was observed, as determined by cell counting and protein determination. However, this could not be conclusively linked with anammox cells. The difficulties in enriching planktonic cultures of anammox bacteria have been well documented, due to slow growing times and complex microbial interactions. *Ca. Brocadia* also has a high aggregation tendency, perhaps in response to a lower tolerance to environmental stressors. This could also pose additional challenges to planktonic cultivation.

Sequencing showed that the culture was enriched with anammox bacteria, with very few changes in the microbial community. The DGGE procedure should also be optimised for the anammox specific primers, such as altering the denaturing gradient and shortening the running time. This could provide clearer insights into the anammox community. From consumption and production patterns of nitrogen compounds, it was highly likely that other microorganisms were also present in the reactor.

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6 Appendix

6.1 Operation Set-Up

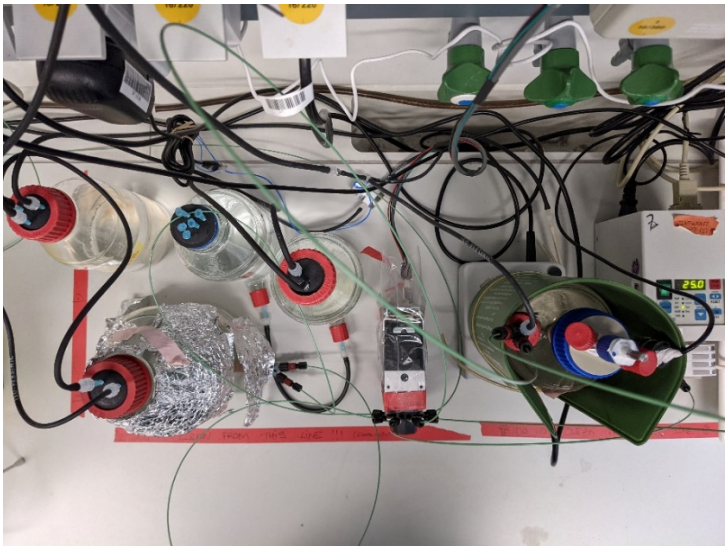

Figure 15: Reactor set up from above

6.2 Influent Composition

NH_4 and Salt Medium	**Concentration**
Trace Element 1000x	10x
Salt Solution	4x
NH_4Cl	160 mM
HCL (20 % w/w)	12 mM
NO_2 Medium	
Se/W/Mo	4x
350 mM phosphate buffer (pH 7.2)	7 mM
$NaHCO_3$	20 mM
$NaNO_2$	52.5 mM
Na_2SO_4	1.2 mM
1000x Vitamin	4x

Table 4: Influent Composition

6.3 Initial Medium Composition

Mix 1	**Final Concentration**
Salt w/o phosphate 100x	1x
Phosphate buffer 350mM	3.5 mM
1000x trace solution	5x
1000x Se/W/Mo	2x
Na_2SO_4	0.1 mM
Mix 2	
NH_4Cl	5 mM
$NaHCO_3$	10 mM
$NaNO_2$	3 mM
1000x Vitamin	2x

Table 5: Initial Medium Composition

6.4 Vortex Treatment of Granular Biomass for Inoculation

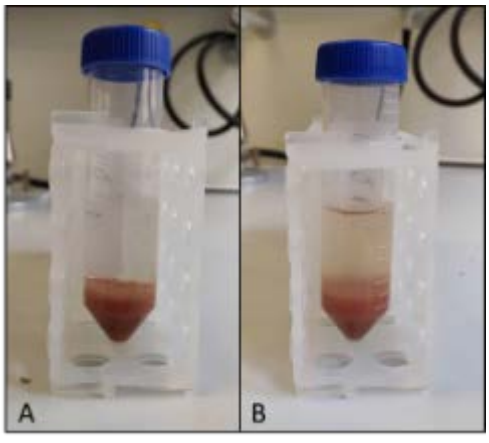

Figure 16: Inoculum before and after vortex treatment

6.5 DNA Quantification and Qualification

Sample Name	Biomass (mg)	260/280	230/260	DNZ (µg/mL)
CSTR Day 0	17.9	1.49	0.52	39.4
CSTR End	42.5	1.67	0.95	80.3

Table 6: DNA concentration and quality of biomass from initial and final days of operation

6.6 Confirmation of DNA Amplification

Figure 17 and 18 confirm the successful DNA amplification after PCR.

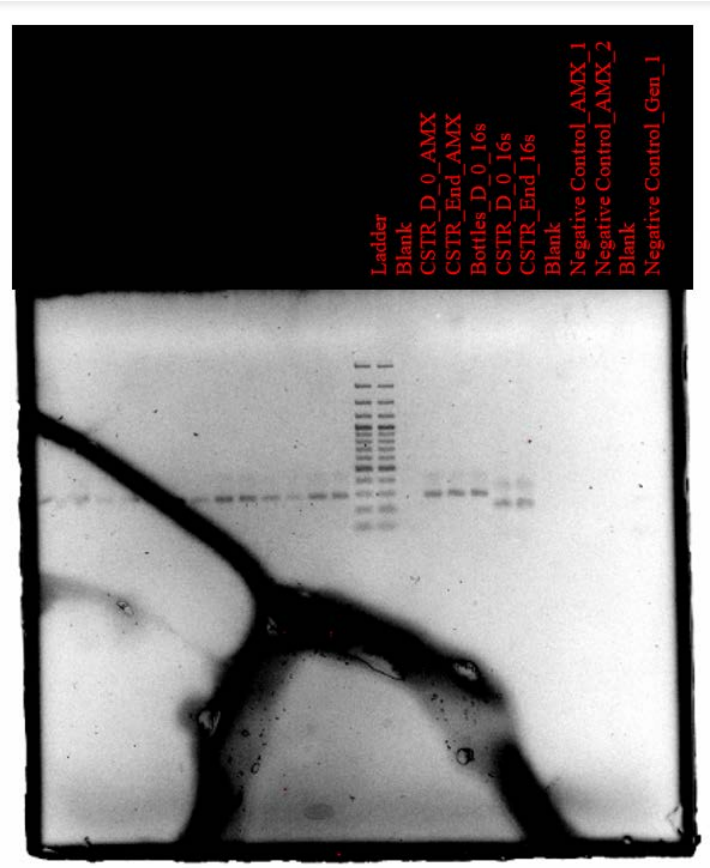

Figure 17: DNA Amplification of DNA from Biomass samples

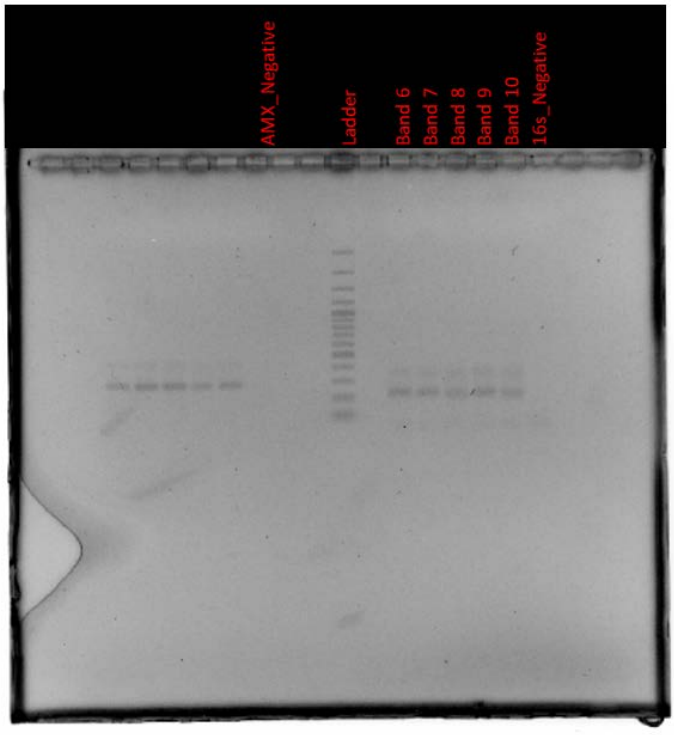

Figure 18: Amplification of DNA extracted from DGGE bands

6.7 Bradford Assay Protein Calibration Curve

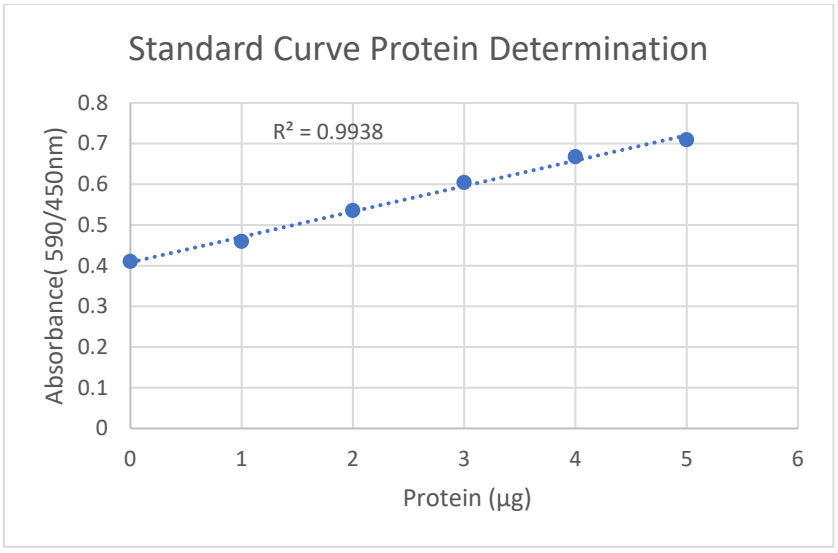

Figure 19: Standard curve for protein determination

6.8 Active Anammox Culture

Figure 20: Ca. Brocadia fulgida in an SBR, UFZ, Leipzig.

The biomass colouring of an active annamox culture is very pink, as opposed to that observed in the CSTR.

6.9 Change from Granular Biomass to Planktonic Cells

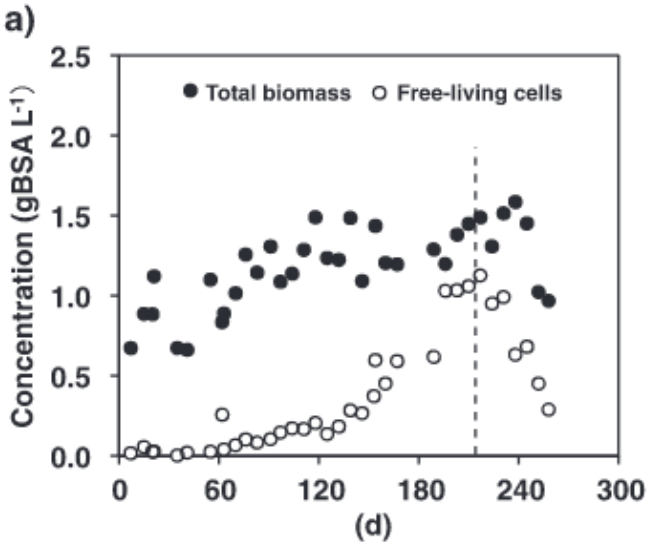

Figure 21: Growth trends. Composition of total biomass and planktonic culture in Ca. Brocadia cultivation in an MBR (Oshiki et al., 2013)

Overtime it was hoped that biomass would breakdown due to agitation. This would promote the growth of new planktonic cells, and the overall composition of the reactor would shift from granular biomass to planktonic, as can be seen in this figure from planktonic cultivation of *Ca. Brocadia* in an MBR (Oshiki et al., 2013)

6.10 Cell Counting

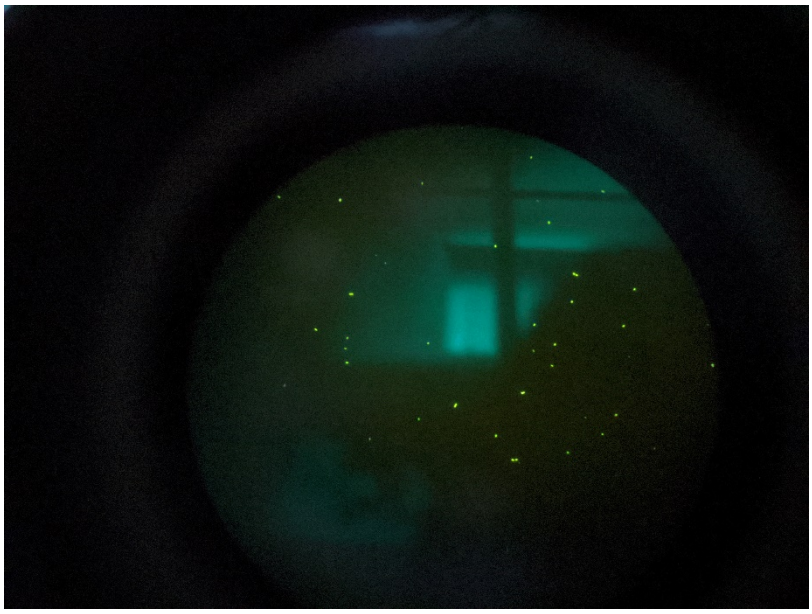

Figure 22: Cell counting

The distinctive crescent shape of anammox bacteria was not observed. It is therefore not possible to determine whether the cells were annamox or another species

6.11 FASTA Sequencing Results

Band	FASTA	BLAST Result	Score	Query Cover (%)	E Value	% Identity	Accession Number
6	ACATCGTTGCATTTCTTAACTCCCGACAGCGGTTTACAACCCGAAGGCCTTCTTCCCGCACGCGGCGTCGCTTCGTCACC CTTTCGGGCATTGCGAAAGATTCTCGACTGCTGCCTCCCGTAGGCCCCCCGTGCCCCCGCCCCGCCCGCCGCGCGCGGCG GGCGAGTGATACCACTGTACTGTATGTATCCAATCCACTCAAGACCTCGTCCAGGTGGCTGTCTCACCCAGTGCCAGCTA TAACCACTGCTGATGGTGAAGCCAGACACGGTGCAGGTCAGGCTCAGGGTCTGGCTAGGTCTCACAAGACCTGGACCGCT CTCCTGCAGCTGGACCTGGCCTTTAGTGCTACCTTCACCACTCCCGGGCTTTCCTGAACCGGAAGTGGATCCTTTGATTT CCACCTTGGTCCCTTGGCCGAACGTGGGATAACTACTCCACTGATGGCAGTAGTAGGTGGCGATGTCCTCTGGCTGGAGG CTGCTGATGGTGAAGGTGAAGTCGGTACCGCTACCGCTACCGCTGAATCTGCTTGGCACACCAGAAGCCAGGTTGGATGT GCTGTAGATCAGCAGCCTTGGAGCCTTGCCTGGCTTCTGCTGATACCAGTGCATGTAACTGACACTCGAGCTGGTACTAC AGGTGATGGTCACTCTGTCACCCACGCTGGCGCTCACGCTGCTTGGGCTCTGGGTCATCTGGATGTCACTGTGCACGCCT GTAGCTGTTGCTACCAGGAAGAGGATGATCC	Candidatus Brocadia sp. enrichment culture clone	219	16	1e-52	98.39	JQ691617.1
7	TTCTTAACCTCCCGACAGCGGTTTACAACCCGAAGGCCTTCTTCCCGCACGCGGCGTCGCTTCGTCACCCTTTCGGGCAT TGCGAAAGATTCTCGACTGCAGCCTCCCGTAGGCCCCCCGTGCCCCCGCCCCGCCCGCCGGCGG	Uncultured anaerobic ammonium-oxidizing bacterium	202	78	2e-48	99.12	LC192354.1
8	GCGGTTTACAACCCGAAGGCCTTCTTCCCGCACGCGGCGTCGCTTCGTCACCCTTTCGGGCATTGCGAAAGATTCTCGAC TGCCGCCTCCCGTAGGCCCCCCGGGCCCCCGCGCCGCCCGCCGCGCGCGGCGGGCGAAG	Uncultured anaerobic ammonium-oxidizing bacterium	172	69	1e-39	98.96	LC192378.1
9	AACAATGCTATTAACATCCTTGCATTTCTTAACTCCCGACAGCGGTTTACAACCCGAAGGCCTTCTTCCCGCACGCGGCG TCGCTTCGTCACCCTTTCGGGCATTGCGAAAGATTCTCGACTGCAGCCTCCCGTAGGCCCCCCGGGCCCCCGCCCCGCCC GCCACGCGCGGGGGGGG	Candidatus Brocadia sp. enrichment culture clone	246	75	1e-61	100	JQ691617.1
10	TTAGCATGCTTGCATTTCTTAACTCCCGACAGCGGTTTACAACCCGAAGGCCTTCTTCCCGCACGCGGCGTCGCTTCGTC ACCCTTTCGGGCATTGCGAAAGATTCTCGACTGCTGCCTCCCGTAGGCCCCCCGTGCCCCCGCCCCGCCCGCCGCGCGCG GCGGGGGACG	Candidatus Brocadia sp. enrichment culture clone	219	72	2e-53	99.64	JQ691617.1

Table 7: Complete sequencing results from DGGE bands, including the parameters indicating the match confidence

Total Scare represents the alignment with the sequence, accounting for matched and mismatched nucleotides.
Query Cover is a representation of how closely the sequences match in organisation and length. In other words, how much of the query length is covered by the target sequence.
E(xpected) Value is the number of alignments that could be expected by chance. The lower the E value, the less likely the result is a coincidence. A value of under 0.1 is considered reliable.
Percent Identity is a description of the how many nucleotides match in the sequences. This higher this value, the more significant match.
Accession Number is the identification of the sequence on record (BIOSEQ, 2018).