

Master thesis

**In partial fulfilment for the award of
Master of Science**

Topic:

Determination of specific metabolic activities of Anammox bacteria and their sensitivity to oxygen

(Untersuchungen zu spezifischen metabolischen Aktivitäten und der
Sauerstoffempfindlichkeit von Anammoxbakterien)

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Abstract

The discovery of Anammox bacteria has bridged our knowledge gap in the nitrogen cycle. This is because it explained the direct conversion of nitrite and ammonium to nitrogen gas in many reducing natural environments. However, it has been found that the presence of oxygen is an important determinant factor for certain biochemical processes and the distribution of organisms in the environment. In this work, two methodological approaches were used to investigate the specific metabolic activities of Anammox bacteria and their sensitivity to oxygen using a mixed biomass culture. Two laboratory-scale bioreactors were maintained and the biomass in the reactors served as the inoculum for the investigations. The molecular characterization of the biomass was done using the Fluorescence in situ hybridization (FISH) technique. The specific Anammox activity (SAA) and important inhibition kinetic parameter characterization were estimated while monitoring activities of the biomass using several batch experiments.

The results of the biochemical characterization revealed that several microbial activities such as denitrification, nitrification, dissimilatory nitrate reduction to ammonium and Anammox processes were competing in the bioreactors. These were observed in the analysis of effluent recirculation of the feed solutions in the bioreactors, however, the Anammox process was predominant. An optimum sonication time t_{op} of eight minutes was established used to determine the average optical density to dry mass (OD:DM) ratio of 0.87 in the biomass. The FISH characterization estimated $60.4\% \pm 14.6\%$ (mean \pm standard deviation) Anammox bacteria concentration in the biomass of Bioreactor 1. The Anammox bacteria were made up of *C. Kuenenia Stuttgartiensis* (~78%) and the remaining *C. Brocadia Fulgida*.

The maximum SAA of $0.025 \text{ mgNH}_4\text{-N (TSS}_{\text{Anammox}}^{-1} \cdot \text{d}^{-1})$ and $5.4 \text{ mgNH}_4\text{-N (}10^{11} \text{ Cell}_{\text{Anammox}}^{-1} \cdot \text{d}^{-1})$ for ammonium, and $0.04 \text{ mg N}_2\text{-N (TSS}_{\text{Anammox}}^{-1} \cdot \text{d}^{-1})$ and $9.4 \text{ mgN}_2\text{-N (}10^{11} \text{ Cell}_{\text{Anammox}}^{-1} \cdot \text{d}^{-1})$ for nitrogen gas were found in the biomass. While a binding affinity constant $K_i = 0.07 \text{ mgO}_2/\text{l}$, maximum half inhibition concentration $IC_{50} \sim 0.2 \text{ mg O}_2/\text{l}$ and $K_M = 50 \text{ mg/l}$ were estimated for the inhibition kinetics of oxygen in the biomass. The low K_i value found in this study indicates that oxygen is an efficient inhibitor for Anammox activities and concentrations between $0.4 - 0.64 \text{ mg O}_2/\text{l}$ can lead to complete inhibition of Anammox activity. However, this process is reversible. Finally, the implication of this inhibition kinetic results might be important in tracing the distribution of Anammox processes in natural environments where fixed nitrogen is present.

Zusammenfassung

Die Entdeckung der Anammox-Bakterien hat unsere Wissenslücke im Stickstoffkreislauf geschlossen. Dies liegt daran, dass es die direkte Umwandlung von Nitrit und Ammonium in Stickstoff in vielen reduzierenden natürlichen Umgebungen erklärt. Es wurde jedoch festgestellt, dass die Anwesenheit von Sauerstoff ein wichtiger bestimmender Faktor für bestimmte biochemische Prozesse und die Verteilung von Organismen in der Umwelt ist. In dieser Arbeit wurden zwei methodische Ansätze verwendet, um die spezifischen metabolischen Aktivitäten von Anammox-Bakterien und ihre Empfindlichkeit gegenüber Sauerstoff mit einer gemischten Biomassenkultur zu untersuchen. Zwei Bioreaktoren im Labormaßstab wurden unterhalten und die Biomasse in den Reaktoren diente als Inokulum für die Untersuchungen. Die molekulare Charakterisierung der Biomasse erfolgte mit der Fluoreszenz-in-situ-Hybridisierungstechnik (FISH).

Die Ergebnisse der biochemischen Charakterisierung zeigten, dass mehrere mikrobielle Aktivitäten wie Denitrifikation, Nitrifikation, dissimilatorische Nitratreduktion zu Ammonium und Anammox-Prozesse in den Bioreaktoren konkurrieren. Diese wurden bei der Analyse der Abwasserzirkulation der Feedlösungen in den Bioreaktoren beobachtet, jedoch war das Anammox-Verfahren vorherrschend. Eine optimale Beschallungszeit von höchstens acht Minuten wurde festgelegt, um das durchschnittliche Verhältnis von optischer Dichte zu Trockenmasse (OD:DM) von 0,87 in der Biomasse zu bestimmen. Die FISH-Charakterisierung schätzte $60,4 \% \pm 14,6\%$ (Mittelwert \pm Standardabweichung) Anammox-Bakterienkonzentration in der Biomasse von Bioreaktor 1. Die Anammox-Bakterien bestanden aus *C. Kuenenia stuttgartiensis* (~78%) und dem restlichen *C. Brocadia fulgida*.

Die maximale SAA von $0,025 \text{ mg NH}_4\text{-N}$ ($\text{TSS}_{\text{Anammox}}^{-1} \cdot \text{d}^{-1}$) und $5,4 \text{ mg NH}_4\text{-N}$ ($10^{11} \text{ Cell}_{\text{Anammox}}^{-1} \cdot \text{d}^{-1}$) für Ammonium und $0,04 \text{ mg N}_2\text{-N}$ ($\text{TSS}_{\text{Anammox}}^{-1} \cdot \text{d}^{-1}$) und $9,4 \text{ mg N}_2\text{-N}$ ($10^{11} \text{ Cell}_{\text{Anammox}}^{-1} \cdot \text{d}^{-1}$) für Stickstoffgas wurden in der Biomasse gefunden. Für die Hemmkinetik von Sauerstoff in der Biomasse wurden eine Bindungsaffinitätskonstante $K_i = 0,07 \text{ mg O}_2/\text{l}$, maximale halbe Hemmkonzentration $IC_{50} \sim 0,2 \text{ mg O}_2/\text{l}$ und $K_M = 50 \text{ mg/l}$ abgeschätzt. Der in dieser Studie gefundene niedrige K_i -Wert weist darauf hin, dass Sauerstoff ein wirksamer Inhibitor von Anammox-Aktivitäten und Konzentrationen zwischen $0,4 - 0,64 \text{ mg O}_2/\text{l}$ können zu einer vollständigen Hemmung der Anammox-Aktivität führen. Dieser Vorgang ist jedoch reversibel. Schließlich könnte die Implikation dieser kinetischen Ergebnisse der Hemmung wichtig sein, um die Verteilung von Anammox-Prozessen in natürlichen Umgebungen zu verfolgen, in denen fixierter Stickstoff vorhanden ist.

Table of Contents

Abstract	i
Zusammenfassung	ii
Table of Contents	iii
List of Tables	vi
List of figures	vii
Abbreviations and symbols	viii
Chapter 1: Introduction	1
1.1 Background.....	1
1.2 Study objectives	2
1.3 Research approach	2
Chapter 2: Literature review	4
2.1 Nitrogen cycle.....	4
2.2 Fixation	5
2.3 Denitrification.....	5
2.4 Nitrification.....	6
2.5 Anammox process.....	6
2.6 Anammox Bacteria	7
2.7 Parameters affecting Anammox activities	9
2.8 Influence of DO on Anammox process.....	10
2.9 Fluorescent in situ hybridization FISH	11
2.10 Activity and inhibition of biomass.....	12
2.10.1 Inhibition kinetics.....	12
Chapter 3: Methodology and Experimental design	15
3.1 Materials and methods	15
3.1.1 Anammox Bioreactors.....	15
3.1.2 Inoculum preparation for the Batch experiments	16
3.1.3 Batch experiments	17
3.1.4 Determination of dry mass of biomass.....	19

3.1.5	Microbiological Analysis	21
3.2	Analytic procedures and data analysis	23
3.2.1	Determination of Ammonium	23
3.2.2	Determination of Nitrite	24
3.2.3	Determination of Nitrate.....	24
3.2.4	Determination of Total bounded nitrogen (TNb) and Total organic carbon TOC	24
3.3	Calculation of Specific Anammox activities (SAA).....	25
3.3.1	Percentage activity and kinetics of inhibition.....	26
Chapter 4: Results		28
4.1	Bioreactors	28
4.2	Sensitivity of Anammox process to DO	30
4.2.1	Approach One.....	30
4.2.2	Approach Two	32
4.3	Kinetics of Inhibition	35
4.4	Recovery of Anammox process after oxygen inhibition.....	36
4.5	Determination of dry mass in the Biomass	37
4.6	Concentration of Anammox organisms in the biomass	38
4.7	Specific Anammox activity (SAA).....	40
Chapter 5: Discussion		42
5.1	Bioreactors	42
5.1.1	Biochemical and microbiological characterization of the biomass in the bioreactor	42
5.2	Anammox versus Denitrification processes.....	44
5.3	Fluorescence in situ hybridization (FISH).....	45
5.3.1	Taxonomic classification and quantification of Anammox bacteria	46
5.4	Determination of dry mass in the biomass.....	47
5.5	Specific Anammox activities (SAA).....	48
5.6	Sensitivity of Anammox activities to oxygen	50
5.7	Kinetics of Inhibition	52
5.8	Comparison of the methodological approaches	53

5.9	Ecological implication of influence of dissolved oxygen in Anammox process	53
Chapter 6: Conclusion		55
6.1	Limitations and some recommendations for future studies	55
List of references		57
Acknowledgement		64
Appendices		65
Appendix 1: Determination of sonication time for dry mass measurements.....		65
Appendix 2: RECIPE for reagents used in Fluorescent In situ Hybridization (FISH) analysis		67
Appendix 3: Results of FISH analysis.....		70
Appendix 4: Biochemical characterization of biomass		72
Appendix 5: Approach 1		74
Appendix 6: Approach 2 (¹⁵ N- label experiments).....		78
Appendix 7: Kinetics of inhibition		88

List of Tables

Table 1: Some Anammox species in both human and natural ecosystem.....	8
Table 2: Summary of some important results of the inhibition kinetics parameters in the batch experiments	36
Table 3: Determination of biomass concentrations obtained from experiments in Batches 1 and 2.....	38
Table 4: Summary of some important results of Specific Anammox Activities in the batch experiments	41
Table 5: Comparing thermodynamic and doubling time of Anammox and denitrification processes. .	44
Table 6: Some inhibition DO concentration found in literature	52
Table 7: Determination of Batch experiment	66
Table 8-A: 4% Paraformaldehyde (PFA).....	67
Table 9-A: 3x Phosphonate Buffered Saline (PBS)	67
Table 10-A: Dehydrating ethanol solutions	67
Table 11-A: NaCl solution	67
Table 12-A: 10% SDS (Sodium dodecyl sulfate).....	67
Table 13-A: Tris/HCl pH 7.2	67
Table 14-A: Preparation of lyophilized oligonucleotides	68
Table 15-A: Wash solution.....	68
Table 16-A: Mineral composition of Anammox feed. Source: (Krause, 2011).....	68
Table 17-A: Composition of trace element solution Source: (Krause, 2011)	69
Table 18-A: Properties of oligonucleotides probes used in the FISH analysis	70
Table 19-A: Some pictures from the FISH analysis using different probes.....	71
Table 20: Results of test for microbial activities in the bioreactor 1.....	72
Table 21: Results for test for microbial activities in biomass from terminated biomass	73

List of figures

Figure 1 Processes in biological nitrogen cycle showing the different oxidation states of nitrogenous compounds and transformation processes	4
Figure 2: Transmission electron micrograph of a 'Ca. B. Sinica' cell. A. Anammoxosome; R, Riboplasm; P, Paryphoplasm.	7
Figure 3: Graphical presentation of activity against substrate concentration.....	12
Figure 4: Simple scheme showing material flow in the Bioreactors	16
Figure 5: Illustration for detecting trace oxygen in a vial.	18
Figure 6: Results of measured substrates in effluent recirculation from the bioreactors. (a.) results of recirculations in Bioreactor 1 containing Ca. Brocadia Fulgida and (b.) Bioreactor 2 for Ca. Kuenenia Stuttgartiensis	29
Figure 7: Graphical presentation of results of the sensitivity of Anammox bacteria to oxygen for one of the batch experiments in approach one using different concentrations of oxygen in the test vial. (a.) Shows measurement of different parameters in the vial including TNb, TOC and O ₂ and (b.) curves of NH ₄ ⁺ -N and O ₂ in the test and control vial showing a trend of loss of Anammox in the test vial.	31
Figure 8: Comparison of rate of ²⁹ N ₂ production, TNb and NH ₄ losses for Batch 1	32
Figure 9: Comparison of rate of ²⁹ N ₂ produced with TNb and NH ₄ degraded in Batch 2.....	33
Figure 10: Relations between C/N ratio and NO ₃ in Batch 2 experiment	33
Figure 11: Comparison of rate of ²⁹ N ₂ produced, TNb and NH ₄ degraded in Batch 3 experiment.....	34
Figure 12: Measured nitrate degraded and C/N ratio in Batch 3 experiments	34
Figure 13: A graphical presentation of a sigmoid plot for the calculation of IC ₅₀ for the combined batch experiments.	35
Figure 14: Determination of important inhibition kinetic parameter: Ki(app) calculated from the combined results of batches 1 and 2.....	35
Figure 15: Graphical presentation of recovery activities of Anammox organisms in the biomass for Batch 2 experiments after purging with Ar/CO ₂	36
Figure 16: Graphical presentation for the determination of the length of sonication during dry mass measurement.....	38
Figure 17: Capture image of biomass stained with DAPI after counting. Viewed @20x magnification from NiKON Eclipse LV 100 microscope using the NIS Element program.	40
Figure 18: Images from the FISH analysis stained with (a.) DAPI and (b.) PLA 46, a general oligonucleotide probe for planctomycete phylum.....	40
Figure 19: Turn-over rate of ammonium and TNb in experiment Batch 3	50
Figure 20-A: Curve of IC ₅₀ estimation for Batch 1 experiment.	80
Figure 21-A: Curve of IC ₅₀ estimation for Batch 2 experiment	84

Abbreviations and symbols

AeAOB	Aerobic ammonium oxidizing bacteria
ANAMMOX	Anaerobic ammonium Oxidizing/Oxidation
AnAOB	Anaerobic ammonium oxidizing bacteria
AOA	Ammonium Oxidation by Archaea
ATU	Allylthiourea
C: N	carbon-nitrogen ratio
DAPI	4', 6-diamido-2-phenylindole
DM	Dry matter
DNA	Deoxyribonucleic acid
DNRA/N	Dissimilatory nitrate reduction to ammonium / nitrite
DO	dissolved oxygen
DOC	Dissolved organic carbon
FISH	Fluorescent in situ hybridization
HAO	Hydroxylamine oxidoreductase
IC_{50}	Half maximum inhibitory concentration
K_i	Binding affinity constant
K_M	Michaelis constant
M_{wt}	Molar mass
N_2	Nitrogen gas
NO_3^- -N	Nitrate
NH_4^+ -N	Ammonium nitrogen concentration
NO_2^- -N	Nitrite nitrogen concentration
NOB	Nitrite Oxidizing Bacteria
O_2	oxygen gas
OD	Optical density
OMZ	Oxygen minimum zone
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid

SAA	Specific Anammox Activity
SBR	Sequence batch reactor
TOC	Total organic carbon
TN _b	Total inorganic bound nitrogen
TSS	Total suspended solid
UV /VIS	Ultraviolet / Visible spectrophotometer
v	Maximum specific substrate / turnover rate
VSS	Volatile Suspended Solid
WWTP	Wastewater treatment plant

Chapter 1

1 Introduction

1.1 Background

Nitrogen is one of the major nutrients required by plants for normal growth and survival. It is an important element that makes up the basic biological system (Environmental literacy council, 2008). It is the most abundant constituent in the atmosphere and it is seen as an important nutrient for regulating primary production and species diversity in both aquatic and terrestrial ecosystems (Cabello *et al.*, 2009).

It is no news that the influence of human activities is creating an imbalance in the natural nitrogen cycle. Activities such as the burning of fossil fuels for energy production, treatment of waste from animal husbandry production (piggery) and the application of nitrogen-based fertilizers for agriculture purposes, discharge of effluents from WWTPs and the petroleum industries and other human activities lead to the release of activated nitrogen to the atmosphere and ultimately the hydrosphere; the most common destination of nitrogenous waste (NADP, 2000). Thus, there are far-reaching consequences that will result from the indiscriminate release of activated nitrogen into the environment due to human activities.

Oğuz, (2005) in his work stressed that some of the nitrogen nutrients released into the aquatic ecosystem from effluents of WWTP are toxic to aquatic life, causing oxygen depletion in water, which controls different respiratory activities in the environment (e.g. Eutrophication and expansion of OMZ) in receiving waters. Bernhard, (2012) reported in his work that changes in the nitrogen cycle induced by human activities can lead to an overall modification in the ecosystem function such as changes in food-web structure and habitat degradation if not checked.

Anammox process is also one of the latest microbial discoveries, which influences the nitrogen cycle. The activities of Anammox are performed by recently discovered “impossible bacteria” as described by (Kartal, *et al.*, 2013) classified as lithotrophs and it is beginning to gain very high environmental importance since it has been linked largely to loss of nitrogen in the marine environment (Strous, *et al.*, 1999). They are mostly found in most oxygen-deficient environments where fixed nitrogen is present. Anammox process involves the oxidation of mineralized organic nitrogen in the form of ammonium using nitrate as an electron acceptor to directly produce nitrogen gas in both natural and man-made systems. Investigation to determine the nitrogen pathway during Anammox activities revealed that one N-atom of the nitrogen gas produced during the process comes from nitrite and the other originates from ammonium (van de Graaf, *et al.*, 1997, Egli, *et al.*, 2001).

Many researchers have found and reported Anammox bacteria and their activities in several both natural and man-made ecosystems (Jensen *et al.*, 2008; Thamdrup and Dalsgaard, 2002, Galán, *et al.*, 2009). In the marine ecosystem, before the discovery of Anammox bacteria, the heterotrophic denitrification

process has been considered solely responsible for the nitrogen loss in the oxygen minimum zone OMZ but today, more and more convincing investigations of Anammox activities in the marine environment have shown these Anammox organisms are responsible for the removal of substantially fixed nitrogen making them a very important player in the global nitrogen cycle (Kalvelage, et al., 2011).

Anammox process currently presents an alternative to the traditional nitrification/denitrification processes in the removal of nitrogen nutrients in wastewater having a low degradable organic carbon under anaerobic conditions (Arrojo *et al.*, 2006). The Anammox organisms are found in a symbiotic microbial community in nature which offer them a survival strategy for nourishment and protection from harsh environmental conditions (Liu, *et al.*, 2008, Kuai and Verstraete, 1998). One of the most challenging features in the understanding of the Anammox process in both natural and engineered systems has been the inability to cultivate pure Anammox organisms (Egli *et al.*, 2001).

1.2 Study objectives

In this work, we intended to investigate and characterize two genera of Anammox bacteria species; *Candidatus Brocadia Fulgidas* and *Candidatus Kuenenia Stutgartiensis*. The biomass cultures used in this work were obtained from enrichment cultures maintained at the Chair of Biotechnology of Water treatment at the Brandenburg University of Technology Cottbus-Senftenberg. These biomass cultures were maintained and characterized in two labelled laboratory-scale bioreactors for both genera of Anammox bacteria already mentioned for these investigations.

Thus, our interest will be to establish to what extent oxygen can inhibit the biochemical activities of Anammox bacteria and/or does oxygen have toxic effects on these organisms and at what concentrations? Therefore, the influence of different concentrations of oxygen will be used to ascertain what concentration of oxygen can completely inhibit the biomass or will be toxic to the Anammox bacteria. This result might be interesting for technological application purposes.

The second aspect of this study is to calculate the nitrogen turnover rate per cell by counting the cell number of Anammox in the batch cultures. The biological molecular technique, Fluorescence in-situ hybridization (FISH) technique will be used to targets the 16S rRNA gene phylogeny of the Anammox organisms in the biomass to identify and estimate the number of Anammox bacteria present in the microbial community.

Part of the task is to establish support, characterize and the kinetics of the two Anammox species used in this work. We also investigated and established a method for determining the maximum activity of the bacteria at the varying oxygen concentrations

1.3 Research approach

It has been reported that the Anammox process is one of the major processes leading to the loss of nitrogen in oxygen-deficient environments where fixed nitrogen is present in most environments. But this statement is also true for heterotrophic denitrification in the presence of degradable organic carbon

and both processes can occur if conditions are favourable. We made some basic research assumptions in this work to ascertain the sensitivity of the Anammox process to oxygen concentration. Since there are hardly pure Anammox cultures due to the nature of the organisms, the following assumptions were put forward to help us monitor the activities of Anammox bacteria in the course of this investigation.

- Inhibit the activities of aerobic ammonium oxidizing bacteria (AeAOB) which can lead to ammonium degradation if present in the biomass by use of suitable inhibitors which has little or no effect on Anammox activities.
- Anammox activities were estimated in terms of $^{29}\text{N}_2$ produced (using tracer studies) during the experiments according to the approach of van de Graaf and his colleagues (1996) and also the degradation of ammonium substrate. This process confirms the activities of Anammox organisms and their contributions to N loss in the system.
- The chemistry of ammonium degradation, total bounded nitrogen TNb and total organic content TOC were used to monitor the accuracy of the method and to keep track of the activities of Anammox organisms during the batch experiments since inhibition of denitrification was not considered.
- Based on the stoichiometry of Anammox chemistry proposed by Strous *et al.*, (1998) turnover measurements of ammonium degraded and the estimated total nitrogen gas produced were used to estimate the accuracy of the methods. The expression given in (1) defines the relationship between the ammonium degraded and nitrogen gas ($\text{N}_2\text{-N}$) produced and it was deduced from the equation of the Anammox process according to Strous, *et al.*, (1998):

$$1 \text{ mass unit } \text{NH}_4^+\text{-N degraded} \equiv 2.04 \text{ mass units of } \text{N}_2\text{-N produced} \quad (1)$$

- The biological molecular technique FISH will be used for the qualitative characterization and quantitative estimation of Anammox bacteria in the biomass using taxon-specific Anammox oligonucleotide probes. The total biomass population can be estimated by using DAPI stained counting method. With this estimated ratio the specific Anammox activities (SAA) can be calculated.

Chapter 2

2 Literature review

2.1 Nitrogen cycle

The nitrogen cycle is seen as the natural interlink between the three main nitrogen sinks on earth: atmosphere, soil/groundwater and the biosphere and the complex exchange among them according to Cabello et al. (2009). It is the total sum of a complex biogeochemical inter-conversion of nitrogenous compounds starting with the conversion of inert atmospheric nitrogen gas. The cycle involves diversities of microbial and other naturally occurring physical activities. The main nitrogen species in the cycle are predominantly dissolved fixed inorganic nitrogen (NH_4^+ , NO_2^- , and NO_3^-), dissolved organic nitrogen and molecular nitrogen gas which constitutes about 80% of the atmosphere. These nitrogenous species show different oxidation states, from -3 in its most reduced state i.e. NH_4^+ to +5 in its highest oxidation state i.e. NO_3^- . The processes involved in the nitrogen cycle have several intermediate stages which are accompanied by different microbial activities that produce several nitrogenous species under different oxygen conditions as shown in Figure 1

Traditionally, the nitrogen cycle consists of 5 main reactions as shown in Figure 1. These include fixation, nitrification, denitrification, mineralization or ammonification and assimilation (Burt, *et al*, 1993). Recent studies have shown discoveries of many other processes which are filling the gap in our knowledge of the nitrogen cycle (Strous *et al.*, 2008). Within the context of this study, we shall discuss just a few of these processes in the Nitrogen cycle, to highlight some of their activities in nature.

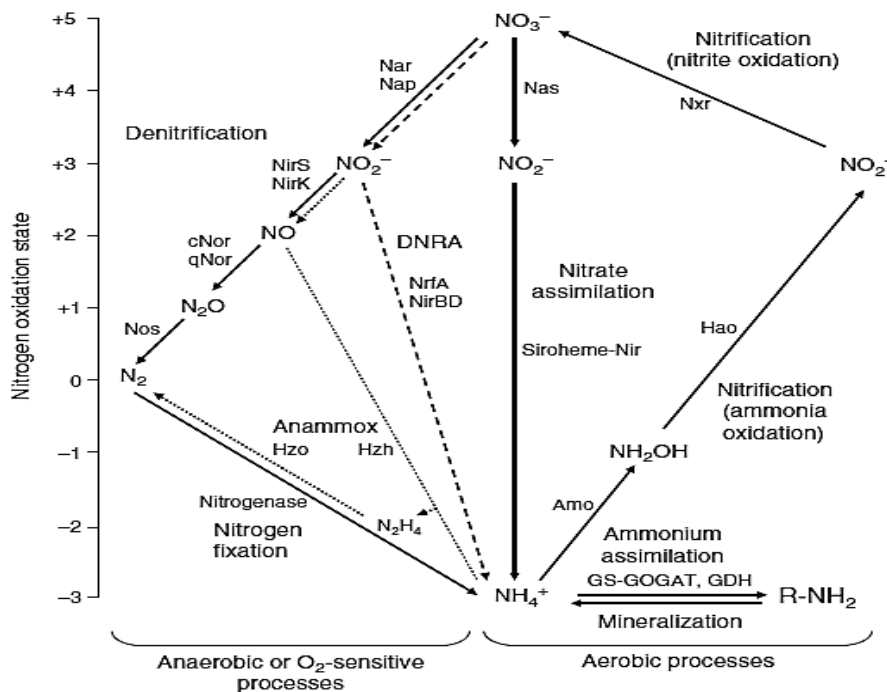


Figure 1 Processes in biological nitrogen cycle showing the different oxidation states of nitrogenous compounds and transformation processes (Source: Copella et al., 2009)

2.2 Fixation

Nitrogen fixation is an important part of the N cycle as it converts atmospheric dinitrogen gas to ammonia and subsequently other N-form, therefore providing fixed nitrogen and also making up for nitrogen losses taking place through denitrification and Anammox processes (Fernandez, *et al.*, 2010). Atmospheric nitrogen is not very useful to organisms, although it is the most abundant component of the atmosphere. Nitrogen fixation involves the metabolism of atmospheric nitrogen gas to the forms needed by plants (primary producers) for their growth. This process is made possible through the activities of nitrogen-fixing bacteria and it is crucial to ensure that biological productivity is sustained in the ecosystems. The bacteria could be freely living or symbiotic. They include bacteria like *Rhizobium*, *Azotobacter* for fixation in the soil; cyanobacteria and associated cyanobacteria are responsible for the aquatic and marine environment (Howarth, *et al.*, 1988). It also maintains the availability of nitrogen that is lost through other processes in the cycle (Jetten, 2008). Furthermore, there could also be contributions from high energy phenomena like lightning, forest fire and hot lava which can also fix nitrogen in the soil leading to fixation of nitrogen.

2.3 Denitrification

De-nitrification is carried out by a wide spectrum of respiratory bacteria representing many genera and physiological types (Kuenen and Robertson, 1994). This process takes place under anaerobic conditions in which nitrate ions were used as an oxygen source by denitrifying bacteria for their metabolic processes. Some common denitrifying bacteria are facultative while others obligate anaerobes. They include species like *Pseudomonas*, *Achromobacter*, *Bacillus* and *Micrococcus* (Mechalas, *et al.*, 1970, p. 2). Until recently, de-nitrification was believed to be the only process in the nitrogen cycle that could produce molecular dinitrogen gas. The denitrification process has been closely linked to the emission of the environmental pollutant nitrous oxide N_2O and carbon dioxide CO_2 through the activities of the heterotrophic denitrifying bacteria which uses nitrates and nitrites as electron acceptors. This is the reason why the process is sometimes considered nitrate respiration because, under anaerobic conditions, organic substrates are degraded using nitrate. However, the mechanisms of the reaction paths are not yet fully understood (Wunderlin, *et al.*, 2012). It is believed that the reactions go through these intermediate nitrogenous compounds as shown in the scheme below and finally to nitrogen gas under certain conditions.



Recently de-nitrification and Anammox processes are seen in the marine ecosystem as a sink for fixed nitrogen and these processes are ways by which nature carries out self-cleansing of different ecosystems to maintain balance and thereby preventing the problem of eutrophication in many aquatic environments. Some authors argued these processes can lead to a situation of poor primary production in these environments. This is because their processes remove fixed nitrite and nitrate required by autotrophs in the form of gaseous molecular nitrogen to the atmosphere (Fernandez, *et al.*, 2010).

2.4 Nitrification

Nitrification is a microbial process, which involves the oxidation of ammonium to nitrate. This process mostly occurs under aerobic conditions and it is catalyzed in the presence of consortia of chemoautotrophic nitrifying bacteria in a two-stage: nitritation and nitrification processes, in which ammonium is converted to nitrite in the first stage by aerobic ammonium oxidizing bacteria (AeAOB) and in the second stage, nitrite produced in the first stage is converted to nitrate as the end product by Nitrite oxidizing bacteria NOB. (Güell, 2008, Cabello *et al.*, 2009). Hanke and Strous, (2010) identified some environmental important nitrifying bacteria in their review in the nitrification step. Aerobic ammonia-oxidizing bacteria affiliated with beta proteobacteria like *Nitrosomonas Europaea*, Gammaproteobacteria such as *Nitrosococcus Oceani* and Crenarchaea *Nitrosopumilus Maritimus* are known. At the moment, five different groups of nitrite oxidizers are known from different phyla of bacteria. In natural surface water, an uncontrolled nitrification process can also lead to situations such as acidification, depletion of oxygen level and subsequently eutrophication (Choi, 2005).

2.5 Anammox process

Anammox is an acronym used to represent Anaerobic AMMonium Oxidation which is simply a process performed by anaerobic ammonium oxidizing bacteria (AnAOB). It is one of the few recently discovered drivers of the microbial nitrogen cycles that have currently been explored, filling an important gap in our knowledge of the global nitrogen cycle. The process offered new technological opportunities in the area of treatment of ammonium-rich wastewater and the ecological explanation of the interaction of the nitrogen and the carbon cycles (Strous, et al., (1999), Koeve and Kahler, (2010)).

Anammox activity was first reported in a man-made ecosystem i.e. wastewater treatment plant WWTP and subsequent studies revealed the activities of Anammox organisms in various natural habitats. The natural habitats include anoxic marine sediments and water columns, freshwater sediments and water columns, terrestrial ecosystems and some special ecosystems, such as petroleum reservoirs. In these environments, reviews showed that the Anammox process led to different percentages of loss of fixed nitrogen in these environments. For instance, the Anammox process accounted for 50% of nitrogen loss in the marine, between 9-40% and 4-37% of inland lakes and agricultural soils respectively. (Hu *et al.*, 2011, Terada *et al.*, 2011). Other newly explored autotrophic processes that can lead to nitrogen removal include ammonium oxidation by archaea (AOA), nitrate reduction by foraminifera and nitrite-oxidizing prototroph i.e. an organism capable of synthesizing all its essential proteins (Jetten, 2008).

Oshiki *et al.*, (2011) studied the physiological characterisation of the Anammox process and they found optimum temperature and pH conditions in the ranges of $32 \pm 12^\circ\text{C}$ and 7.5 ± 0.8 were suitable for the Anammox process and the doubling period of Anammox bacteria lies between 11-19 days (Dapena-Mora *et al.*, 2007). The growth rate of 0.0041 h^{-1} , the degradation half-saturation K_s of NH_4 , and NO_2 substrates of $28 \pm 4 \mu\text{M}$, and $86 \pm 4 \mu\text{M}$ respectively, inhibition occurred with NO_2 , ethanol, methanol, phosphorus and salinity at different concentrations. Pilcher, (2005) reported the process offers an

economic and environmentally friendly method for biological nitrogen removal from wastewater and other high ammonium laden liquids as compared to the traditional nitrogen removal processes of nitrification-denitrification. The merit of Anammox application in biological wastewater treatment have been widely reported by many investigators and it has been found that the process reduces the operational cost in terms of energy and material saving (100% C- sources and 60% aeration O₂) as compared to the traditional biological method of nitrogen nutrient removal from WWTPs (Tao and Gao, 2012, Hu et al., 2010, Joss *et al.*, 2011, Ryzhakov *et al.*, 2010) but the slow growth rate of the bacteria makes it difficult for large scale application. Ryzhakov *et al.* (2010) recommended the use of special reactors which maintain high biomass concentration to overcome the problem of growth of Anammox biomass in large scale nitrogen removal

2.6 Anammox Bacteria

Anammox is a chemoautolithotrophic and physiologically unconventional bacteria. This is because the organisms are seen as a hybrid of prokaryotes and eukaryotes due to the presence of an internal organelle which is not common for simple celled prokaryotes. The tiny sac-like structures organelle is packed with toxic hydrazine, which gives the organism its unique status (Pilcher, 2005). The bulk of the Anammox bacteria cytoplasm consists ribosome-free compartment also known as the Anammoxosome. This is the metabolic site in the Anammox process. Due to this physical feature, epi-fluorescent examination of the environmental samples containing Anammox bacteria is used for the identification of the bacteria which is visualized as ring-shaped or sickle-shaped structure after fluorescent in situ hybridization (FISH) analysis (Strous *et al.*, 1999b).

Electron microscopy (EM) and phylogenetic analysis of 16S rRNA sequence of Anammox enriched culture identified the bacteria as deep branching *Planctomycetes* which are capable of carbon dioxide fixation (Kuenen, 2008, Strous *et al.*, 1999a). The Anammox process is carried out by bacteria belonging

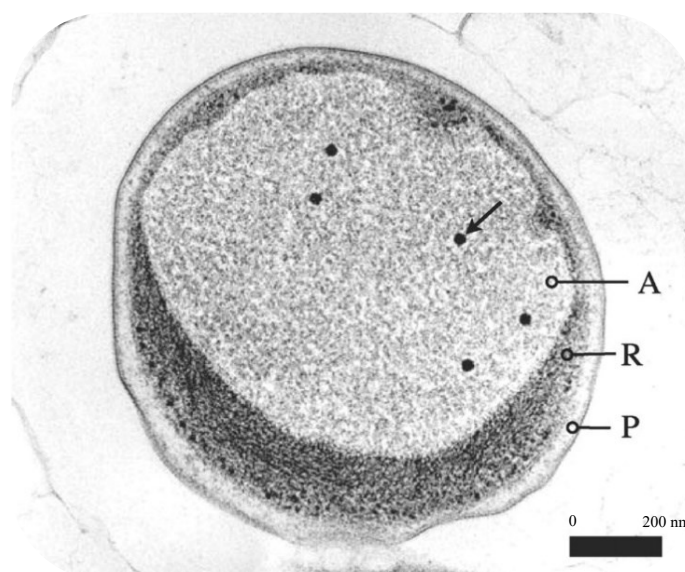


Figure 2: Transmission electron micrograph of a 'Ca. B. sinica' cell. A, anammoxosome; R, riboplasm; P, paraphoplasm. The arrow indicates condensed and electron-dense particles. (Source: Oshik, *et al.*, 2011)

to a deep-branching monophyletic group within the Planctomycetes phylum, and can thus be studied through 16S rRNA gene surveys using widely available molecular techniques (Schmid *et al.*, 2005).

Jetten and colleagues (2009) in their review of Anammox bacteria have submitted that Anammox bacteria are obligate anaerobes and are reversibly inhibited above $2\mu\text{M}$ ($<0.1\text{ mg l}^{-1}$) DO. The size of the observed diameter of Anammox cells was estimated to be approximately $0.6\text{ - }1\ \mu\text{m}$ for *Ca. Kuenenia stuttgartiensis* and *Ca. Brocadia fulgida* (Duan *et al.*, 2012, Jetten *et al.*, 2009).

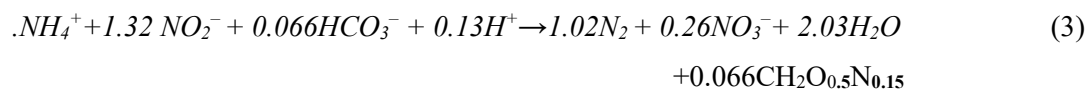
Nozhevnikov *et al.* (2012) reported that Anammox bacteria are attributed to five different genera. At the moment, there are several biodiversities of Anammox species that have been identified (in the Candidatus status). Dapena-Mora *et al.*, (2007) had earlier identified three genera of the bacteria: *Candidatus Brocadia*, *Candidatus Kuenenia* and *Candidatus Scalindua*. See Table 1 for the biodiversity of Anammox bacteria found in both man-made and natural ecosystems. The full characterization of the bacteria was published and the causative organism was named in the work of Strous *et al.*, (1999b.). The bacteria are usually found in symbiotic association in the environment; in a kind of association that offers Anammox organisms' benefit from participating organism in terms of growth factors like right oxygen condition and nourishment and the organisms benefit from the byproduct of Anammox anabolic activities. The image in Figure 2 showed the specialized organelle called Anammoxosome in *Ca. B. Sinica*. The Anammoxosome is the Anammox specific site and it contains about 10-15% hydroxylamine oxidoreductase (HAO) responsible for the oxidation of hydrazine to nitrogen gas in the cell.

Kuenen, (2008) described the biochemical mass balance analysis of the Anammox process and he showed that the organism metabolized carbon dioxide and utilizes nitrite as an electron acceptor for ammonium oxidation. The nitrite is used as an electron donor for the reduction of carbon dioxide or hydrogen carbonate as the source of carbon for the metabolism of biomass ($\text{CH}_2\text{O}_{0.5}\text{N}_{0.15}$) used for the growth of the organism according to equation (3)

Table 1: Some Anammox species in both human and natural ecosystem

Genus	Species	Source
<i>Brocadia</i>	<i>Candidatus Brocadia Anammoxidans</i>	Wastewater
	<i>Candidatus Brocadia fulgida</i>	Wastewater
<i>Kuenenia</i>	<i>Candidatus Kuenenia stuttgartiensis</i>	Wastewater
<i>Scalindua</i>	<i>Candidatus Scalindua brodae</i>	Wastewater
	<i>Candidatus Scalindua wagneri</i>	Wastewater
	<i>Candidatus Scalindua Sorokinii</i>	Seawater
	<i>Candidatus Scalindua Arabica</i>	Seawater
Others	<i>Candidatus Jettenia Asiatica</i>	Not reported
	<i>Candidatus Anammoxoglobus Propionicus</i>	Wastewater

Modified from Van Hulle *et al.*, (2010)



The stoichiometry of Anammox substrate shown in equation (3) was first proposed by van de Graaf *et al.* (1996) and supported by Strous *et al.* (1998) in two independent investigations. The theoretically proposed stoichiometry showed that the Anammox process removes about 88.8% of total N, while nitrite is partially oxidized to nitrate which makes up about 10% of the total N as a by-product of the process (Yang, 2012). Attempts to isolate pure breed of bacteria have been hampered because of the slow growth rate. This is one of the demerits of applying the Anammox process on a large scale for wastewater treatment (Ryzhakov *et al.*, 2010). The presence of other bacteria in the community is important to remove toxic products or provide essential nutrients for the Anammox bacteria. This makes it difficult to obtain pure cultures of Anammox bacteria.

2.7 Parameters affecting Anammox activities

Malovanyy *et al.* (2010) in their work grouped parameters influencing Anammox activities into three. These reflected on the main factors that generally influence the performance of most biological processes and they include:

- Inhibiting factors
- Substrate availability and
- Temperature.

Inhibiting factors have been studied under the subject ecological factors (EF) which influences Anammox activities by many investigators. These factors include DO and organic compounds of low molecular size. They are reported to have high inhibitory effects on the Anammox activity. Other substances identified to have an inhibiting effect include nitrite, sulfide, phosphate, acetate and some antibiotic substances (e.g. chloramphenicol, Oxytetracycline) both in lower or higher concentrations in the environment (Güven *et al.*, 2004; Isaka *et al.*, 2008, Strous *et al.*, 1997, Strous *et al.*, 1999a; Dapena-Mora *et al.*, 2007, Jung *et al.*, 2007, Tao and Gao, 2012, van de Graaf *et al.*, 1996).

Ammonium and nitrite are the main substrates for Anammox bacteria, so increased availability of these substrates leads to increasing bacterial activities when diffusion limiting. Anammox activities can be inhibited at very high concentrations on its substrate. Strous *et al.* (1999) and Dapena-Mora *et al.* (2007) submitted in their work that nitrite can inhibit Anammox activity but at different concentrations of NO₂-N. Changes in concentrations of NO₂ substrate has also been reported to influence the stoichiometry of the Anammox reaction. Many studies on SAA have been made by different investigators. Dapena-Mora *et al.* (2007) found that SAA in their investigation that there was a 50 % decrease in activity when concentrations of NH₄-N/l and NO₂-N/l rose to 770 and 350 mg/l respectively. On the other hand, some investigators in their work, Strous *et al.*, (1999) made a different observation, no inhibition was observed for ammonium at concentrations up to 980 mg NH₄-N/l and a total loss of activity of nitrite concentration

of 98 mg NO₂-N/l. Fux and Siegrist, (2004) also reported serious inhibition of Anammox activity when the concentration of nitrite stayed at 30-50 mg NO₂-N/l for six days.

Arrojo and colleagues, (2006) were able to demonstrate the effect of mechanical stress on Anammox granules in a sequence batch reactor (SBR). They established that with increasing agitation speed Anammox are inhibited and can lead to the loss of Anammox activity.

Generally, temperature increases the rate of both chemical and biological processes. With increasing temperatures most reactions and biochemical transformations are driven faster, but for biological systems, there are limits to which increasing temperature can influence the metabolic activities of organisms. Biological systems show retarded activity to an increasing temperature above certain temperature thresholds. This is because, at this threshold temperature, enzymes, which catalyses the metabolic activities of the organisms become inhibited since they are proteins. More so high temperatures are harmful to Anammox organisms and might lead to the lysis of the cells above 45°C. An earlier review of the effect of temperature on the rate of Anammox activities with increasing temperature not greater than 40°C by different scholars showed a corresponding rise in Anammox rates (Malovanyy *et al.*, 2010). Dosta *et al.*, (2008) found optimum temperature conditions for Anammox processes in the range between 35-40°C. But at higher temperatures, the rate starts to decrease. Anammox activities have been observed in both marine environment and wastewater treatment plants during seasons of cold temperatures, this indicates the Anammox process takes place at low temperatures.

2.8 Influence of DO on Anammox process

DO is an important environmental factor in different natural ecosystems. It detects the pace of many microbial activities and influence most biogeochemical processes. Biochemically, the pathway of the Anammox process proceeds via nitric oxide and hydrazine (N₂H₄) can be inhibited by the presence of oxygen (Hanke and Strous, 2010). In the marine ecosystem, different studies have reported the presence and activities of Anammox bacteria in the depths of the ocean of the world (Galán *et al.*, 2009, Kalvelage *et al.*, 2011). In the deep marine waters, DO concentrations have been found to differ with depth since the main source of oxygen is via the exchange between the top layer of the waters and the lower atmosphere, through advective processes, such as photosynthesis and respiration which play a major role of regulating the distribution of DO and organisms within the ocean.

Murray *et al.*, (2005) defined the vertical stratification of dissolved oxygen in the ocean. Three zones were identified namely; oxic, suboxic and anoxic zones respectively. Oxic zone is well oxygenated and it covers between 0 to 50m deep, whereas the depths between 100 – 2000m are defined as anoxic also known as Oxygen minimum zone (OMZ). The suboxic zone can be found in the boundary between the oxic surface and anoxic deep layers approximately 50 to 100 m depth. Galán *et al.* (2009) reported on their work the abundance of Anammox cells and highest activities were found in the upper part of the suboxic OMZ. At this depth, there are still traces of DO and Anammox substrates present metabolized by the activities of autotrophic nitrification.

As already mentioned Anammox process is found in an oxygen-deficient environment and environmental parameters such as DO helps to regulate the activities taking place in this stratum. Many scholarly works have shown that the presence of oxygen can reversibly inhibit the effect on microbial activity (Strous *et al.*, 1999, Jensen *et al.*, 2008, Galán, *et al.*, 2009). Strous *et al.* (1997), reported that a very low concentration of DO ($>0.1 \text{ mg L}^{-1}$) can inhibit the Anammox process but this process is reversibly inhibited. Bräue *et al.* (2004) reported that the presence of oxygen can be toxic to most anaerobic microorganisms and its presence can also lead to an appreciable reduction in digestion activities in most anaerobic reactors. However, facultative anaerobes may metabolize dissolved oxygen before the toxic effects become noticeable. The activities of Anammox bacteria when inhibited by oxygen can be revitalized by vigorously purging the system with inert gases to reverse the inhibition (Jetten, 2001).

2.9 Fluorescent in situ hybridization FISH

Nowadays molecular biology offers readily available techniques for the identification and classification of Anammox bacteria in environmental samples. This includes a range of suitable techniques available for the detection of Anammox bacteria in environmental samples and samples from wastewater treatment sludge.

PCR amplification approaches can be used to discover previously unknown organisms by studying the phylogeny of the detected organism using gene primers, which target the 16S rRNA and this technique has been successfully used to detect Anammox bacteria in both man-made and natural ecosystems (Jetten *et al.*, 2009). Recently, more specific PCR primers have been developed to amplify the Anammox genes encoding hydroxylamine/hydrazine oxidoreductase (HAO/HZO) proteins. This method showed that these genes are suitable targets for molecular ecological studies on both aerobic and anaerobic ammonium-oxidizing bacteria.

The FISH technique can be an alternative molecular technique to PCR for the quantitative assessment of microbial communities on environmental samples. Although no molecular approach offers complete access to the genetic and functional diversity of complex microbial communities. The PCR technique requires a more advance DNA/RNA extraction technique for environmental samples (Gurdeep and Rajesh, 2011). FISH offers both qualitative and quantitative valuation methods for Anammox bacteria and this approach has recently been used to validate the clone library. Jetten *et al.* (2009) reported that the FISH technique could be improved in combination with more advanced techniques to raise its precision as an identification and quantification method. The FISH technique is a molecular biological technique used to visualize selected microorganisms after staining the cell with fluorescent genetic probes (oligonucleotides). It is most effective for detecting organisms' concentrations greater than $\sim 10^3$ - 10^5 cells/ml. It can be used to characterize targeted organisms in combination with epifluorescence microscopy (Bennett *et al.*, 2006).

FISH technique investigates and retrieves information from rRNA genes within the cell of targeted individual microbes. This molecular procedure uses a specific oligonucleotide probe (short sequences

of single-stranded DNA) to label the rRNA in the targeted organism to produce fluorochrome without the prior need for cultivation. For most FISH applications, the probes target one copy of 5S, 16S, or 23S rRNA within the cells but the probes do not hybridize the DNA. Sufficiently high ribosome content (i.e., of actively growing populations), and high rRNA concentrations in the fixated biomass will produce a strong fluorescent signal (intensity) detectable during the epi-fluorescent examination.

2.10 Activity and inhibition of biomass

The activity of the microbial population, either by the total biomass (e.g. Volatile suspended solids) or the targeted microbial population: is represented by the inherent ability to degrade the test substrate. The activity is usually measured as the specific rate of substrate consumption or end-product generated. It is usually expressed by the slope of the curve of substrate degraded or product generated. The unit is expressed as the mass of substrate removed per unit mass biomass per unit time (Rozzi and Remigi, 2004).

Inhibition indicates a detrimental effect that a test substance causes on the activity of a microbial population. An inhibiting substance generally decreases the rate of measured activities for a given substrate in enzyme-catalyzed reactions. The typical inhibitory activity is done when an inhibiting substance mimics a substrate and binds to the regulatory centre in an enzyme-catalyzed reaction thereby preventing the biochemical activity in the biomass (Nagase and Salvesen, 2001).

Inhibitory activities could be *reversible* i.e. the inhibitor can be released or displaced from the enzyme regulatory centre or *irreversible*, meaning that the inhibitor binds strongly that it cannot be easily detached or even bind to the enzyme when the enzyme is denatured. The situation results in the formation of a covalent bond with the functional group of the active site. For both modes of inhibitions, partial inhibition may occur, if the enzyme-inhibitor complex remains active or complete inhibition takes

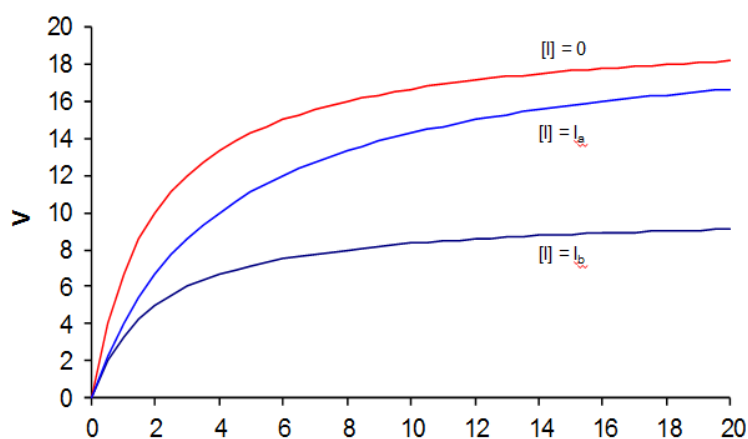


Figure 3: Graphical presentation of activity against substrate concentration.

2.10.1 Inhibition kinetics

The General rate equation for the inhibition process is given by the scheme in (4)



E, S, I and P represent enzyme, substrate, inhibitor and product concentrations for an enzyme-catalyzed equation. ES and ESI are the transition complexes of enzyme-substrate and enzyme-substrate-inhibitor interactions in (4).

Equation (4) represents a general reaction scheme for substrate-enzyme-inhibition showing different interaction mechanisms for enzyme kinetics. Two important kinetic constants define enzyme-inhibitor interactions: Speed (association rate constant k) and strength (binding constant, K_i) (Nagase and Salvesen, 2001).

k_2 is the rate constant of the uninhibited reaction and the binding of the inhibitor from the ESI complex reduces activity and k_6 becomes the new rate constant. The equation contains two dissociation constants for the uninhibited substrate S and the inhibitor I as shown in (5) and (6):

$$K_A = \frac{k_{-1}}{k_1} = \frac{[E][A]}{[EA]} ; K_{Ai} = \frac{k_{-5}}{k_5} = \frac{[EI][A]}{[EAI]} \quad (5)$$

$$K_{ic} = \frac{k_{-3}}{k_3} = \frac{[E][I]}{[EI]} ; K_{iu} = \frac{k_{-4}}{k_4} = \frac{[EA][I]}{[EAI]} \quad (6)$$

The terms in (5) and (6) are related by (7)

$$\frac{K_A}{K_{Ai}} = \frac{K_{ic}}{K_{iu}} \quad (7)$$

The amount of enzyme used can be expressed as enzyme complexes as shown in the reaction scheme and defined by (8) if the enzyme-inhibitor complexes are replaced by inhibition constants.

$$[E]_o = [E] + [EA] + \frac{[E][I]}{K_{ic}} + \frac{[EA][I]}{K_{iu}} \quad (8)$$

For reversibility and under the steady-state conditions, the enzyme inhibited kinetics can also be likened to the Michaelis-Menten equation.

$$v = \frac{d[P]}{dt} = \frac{V_{max}[S]}{K_m[S]} \quad (9)$$

Assuming steady-state, the differential equations for the enzyme and enzyme-inhibitor complexes can be considered equals zero and manipulating these rate equations for the above relationships

$$v = \frac{(V_o)[A]}{K_m \left(1 + \frac{[I]}{K_{ic}}\right) + \left(1 + \frac{[I]}{K_{iu}}\right)[A]} \quad (10)$$

This equation can be compared to the basic Michaelis–Menten equation. The linearization of (9) can be transformed according to Lineweaver-Burk (double reciprocal) plot, Hanes plot and Eadie-Hofstee plot. But in this work, only the double reciprocal linearization plot was considered for some of the calculations.

Generally, the inhibition mechanism can be described as competitive, non-competitive or uncompetitive based on the interaction of the enzyme-substrate-interaction. Mathematically these modes of inhibition can be easily classified according to the pattern of straight lines of the linearized diagram. Competitive inhibition is characterized by a straight-line intercept on the ordinate while an intercept on the abscissa is characteristic of non-competitive. A parallel line intercept represents an uncompetitive and a line in between both axes defines mixed inhibition (Bisswanger, 2008). See details in the reference text.

The kinetics of the interaction of inhibitors with the enzymes defines its efficiency in substrate inhibition. The half-maximum inhibitory concentration IC_{50} is used to describe the potency of an inhibitor (i.e. The concentration of the inhibitor that gives 50% inhibition of the target enzymatic activity or the percentage inhibition of the enzymatic activity of a fixed concentration of inhibitor). This parameter is most useful when working with enzyme concentration less than K_i (the inhibitor affinity constant) and therefore only useful for poor inhibitors since most good inhibitors have very low K_i s. IC_{50} depends on parameters such as substrate concentration [S], binding affinity constant K_I and Michaelis-Menten constant K_M along with other experimental conditions (Cer *et al.*, 2009).

Chapter 3

3 Methodology and Experimental design

3.1 Materials and methods

In this section, we shall cover different activities and procedures used that led to the successful completion of this investigation. These activities include characterization, maintenance and analyses of effluent recirculations of the bioreactors. Then setting up of different batch experiments aimed to determine the specific activity of Anammox bacteria and their sensitivity to Oxygen will be described under the following sub-sections.

3.1.1 Anammox Bioreactors

Two laboratory-scale bioreactors labelled 1 and 2 were maintained as sources for two different species of Anammox bacteria (*Candidatus Brocadia Fulgida* and *Candidatus Kuenenia Stuttgartiensis*) being investigated in the Anammox enrichment cultures used in the batch experiments respectively. These reactors were monitored throughout the study. The idea was to maintain a steady source of biomass for characterization in the batch experiments and also to study the response of the Anammox process to oxygen concentrations

Another objective was to investigate the level of enrichment of the Anammox species in the biomass. The source biomass in the bioreactors was taken from a large bioreactor maintained at the chair of biotechnology for water treatment, at the Brandenburg University of Technology Cottbus. See Figure 3 for a simple schematic sketch of the flow chart of the Bioreactors.

3.1.1.1 Features of the bioreactor

The bioreactor was fabricated from a cylindrical iron steel pipe with an internal diameter of 0.812 m and a height of 0.12 m. The volume of each of the two bioreactors was calculated as $6.2 \times 10^{-4} \text{m}^3$. The bioreactors were designed to allow the biomass to settle at the bottom of the reactor, a similar situation we find in the most natural aquatic environment and this design objective was attainable since the design does not include any agitator. To achieve this to a reasonable degree the mixing of Anammox medium in the Bioreactors with dosed substrates was realized through diffusion and recirculation of the reactors' content using the recirculation pump. The bioreactors can maintain airtight anoxic conditions by ensuring that all fittings were well sealed. The reactor design included an oxygen sensor spot (optode) to check the presence of trace oxygen in the recirculation at the top of the bioreactors. Anammox feeds for the bioreactors were modified according to the recipe proposed by van de Graaf *et al.*, (1996) containing appropriate quantity Anammox substrates stock prepared in the stoichiometry ratio suggested by Strous *et al.*, (1998) and dose into the Bioreactors at feed input volumetric rate of $2.5 \times 10^{-5} \text{m}^3/\text{d}$.

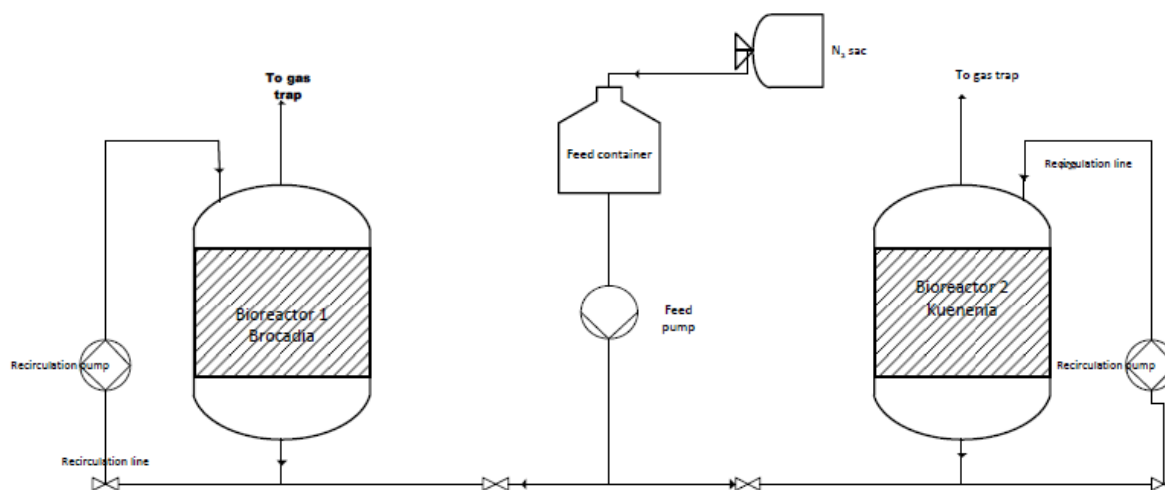


Figure 4: Simple scheme showing material flow in the Bioreactors

Tables 14-A and 15-A in Appendix 2 described the recipes of the synthetic medium used to maintain and nourish the Anammox organisms prepared according to van de Graaf et al., (1996). The bioreactors' feed was prepared with a sterilized 500-ml bottle and the pH of the medium was adjusted to between 7.1 -7.4 maximum. The feed was purged with nitrogen gas for 30 minutes and carbon dioxide for 30 seconds to remove DO and reintroduce CO₂ in the medium as an inorganic carbon source for Anammox respectively. The pressure in the feed bottle was maintained above the standard pressure at prevailing room temperature using a nitrogen gas filled-sac connected to the feed to maintain anoxic conditions in the bottle and making it easier to feed the bioreactors. The transfer of Anammox feed to the bioreactors was controlled with the help of a pump connected to the feed bottle at a steady rate.

The gas produced in the bioreactors was collected in gas traps as an indicator of the activity of the bioreactors but no further analysis was carried out on the gas composition. The gas collected if analyzed can be used for material balancing for the bioreactors. More so the microbial activity of the biomass was monitored by the daily sampling of recirculating feed for analyses. Total inorganic nitrogen species (ammonium, nitrite and nitrate) in the recirculation and gas produced through the biochemical activities of the biomass in the bioreactors were measured and recorded.

These activities were maintained for six months, starting from November 2012 till March 2013. Chemical analyses of the substrates from the recirculation effluents were carried out at least three times weekly. The concentrations of substrates in the Anammox feed were 0.34 g NH₄-N/l and 0.45 g NO₂-N/l and this was prepared at NH₄-N: NO₂-N ratio of 1:1.32 based on the theoretical stoichiometry and a measured daily loading rate of substrates in both reactors were 0.018 g NO₂-N /l/d and 0.02g NH₄-N/l/d summing up to total N load of 0.038 g -N/day at a feed input volumetric rate of 0.025 l/day.

3.1.2 Inoculum preparation for the Batch experiments

The inoculum preparation covered both batch experiments for the determination of specific metabolic activities of Anammox bacteria in the biomass and their sensitivity to DO. Biomass was taken directly from the bioreactors under anaerobic conditions to avoid exposing the biomass to oxygen. The

concentrations of the substrate in suspension were ascertained by analysis. For cases where the concentrations of the Anammox substrate were low in the bioreactors, appropriate concentrations of the substrates were prepared in degassed Anammox medium to bring the substrate concentrations to about 40 mg-N/l for both ammonium NH_4 and nitrite NO_2 substrates respectively.

The biomass suspension from the bioreactors was first collected into a 50-mL sealed vial, (vials used for the experiments were sterilized and purged with inert gases – N_2 or Ar/CO_2 depending on the approach), then the appropriate volume of N-allythiourea was added and concentrations of the Anammox substrates in the feed can be adjusted to suit the experimental plans. Adjusting the concentration of the substrates in the Anammox medium can be done by preparing appropriate stock concentration in the corresponding volume of degassed Anammox medium without the substrates. The initial sample conditions were established such as prevailing temperature, pH adjusted to 7.4 and dry mass concentration of inoculum was measured. The dry mass of the inoculum depends on the concentration of the biomass suspensions in the bioreactor and an average range of 0.6 -1g/l dry mass of inoculum was used during the batch experiments.

3.1.3 Batch experiments

The effect of oxygen on the Anammox process was investigated using two approaches following several batch experiments. The first approach looks at the effect of oxygen concentrations on Anammox organisms in the biomass by observing how the Anammox species in the batch culture respond to the gradual increase in concentrations of oxygen over a time interval and then purging with inert gas to reestablish complete anaerobic condition. The influence of oxygen on the Anammox process was monitored as a measure of ammonium degradation in the Anammox medium after the activities of aerobic ammonium oxidizing bacteria (AeAOB) was inhibited with a suitable inhibitor.

The second approach involves tracer studies by labelling a portion of one of the substrates. The effect of oxygen at different concentrations can be investigated on the test vials containing a fixed amount of Anammox species by simultaneously measuring the amount of nitrogen produced containing the trace isotope and other parameters to ensure the accuracy of this method.

This was done using a known labelled portion of heavy ammonium substrate in the pool of normal substrates of known concentrations in the synthetic Anammox medium and the Anammox activities were monitored as $^{29}\text{N}_2$ produced.

3.1.3.1 Approach one: Chemical analysis based on nitrogen turnover

Several batch experiments were set up in this approach. The experiments were performed in two 50-ml vial bottles. The vials contain calibrated oxygen sensor spot – optode: used to measure trace DO concentration in the test vial. One of the vials serve as a control, and thus no oxygen was added. The vials were filled halfway with Anammox medium containing appropriate concentrations of the Anammox substrates (ammonium and nitrite - N) and ATU was added to inhibit activities of AeAOB. The volume of biomass suspension used was considered to allow sufficient head space for gas exchange

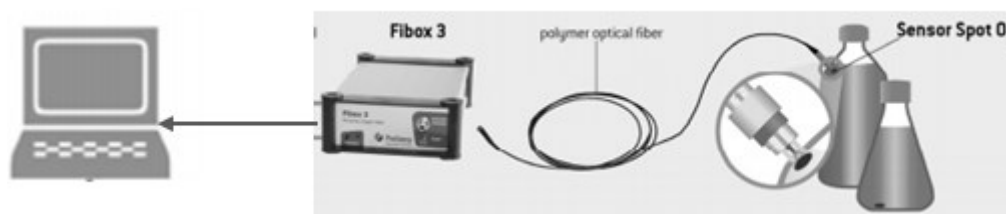


Figure 5: Illustration for detecting trace oxygen in vial (modified from Precision Sensing GmbH, 2011).

between the liquid and gas phase in the vials. The pH of the medium was corrected to pH range 7.0-7.4 after preparation and the experiments were performed at the prevailing room temperature. The vials were stopped with butyl rubber and analuminum crimp seal.

The biomass suspension taken from the bioreactor under anaerobic conditions was distributed between the test and control vials and the concentrations of the substrates adjusted appropriately. The headspace in the vials was exchanged by purging the suspension with N_2 gas for 3 minutes. With this approach, the source of oxygen in the test vial was achieved by introducing an appropriate quantity of air into the test vial and maintained over the time for which the vials were observed. The volume of the oxygen concentration in the test vial was measured using Fibox 3 trace v3 mini-sensor trace oxygen meter and the accompanying software PST3v701 (PreSens GmbH, Regensburg, Germany). The phase value of oxygen concentration in air was obtained and this can be converted to manageable units of O_2 concentration using a conversion spreadsheet calculator provided at the chair of biotechnology of water treatment in BTU Cottbus-Senftenberg.

The illustration in Figure 5 describes a typical setup for detecting oxygen in a sample using the oxygen trace meter. After each analysis, the vials were kept in a shaker to ensure continuous mixing of the headspace with the suspension. After the oxygen treatment and analysis of the test vial, a recovery test was carried out by purging the biomass in suspension with inert gas. Then the vials were allowed to stand overnight in a shaker. Assays were taken from the vials for analyses and the activity of biomass in the test vial compared with the activities in the control vial. This procedure applies to both approaches one and two. The following parameters were also measured in the samples: total bounded nitrogen (TNb), total organic carbon (TOC) and NH_4^+ in the assay taken from the vials after the observation period.

3.1.3.2 Approach Two: Tracer studies (^{15}N labelling experiment).

In this approach, the working principle was to use some labelled portion of the ammonium substrate as $^{15}NH_4^+$ to trace the activities of Anammox organisms in the biomass. Anammox activity was measured in terms of heavy nitrogen gas $^{29}N_2$ produced after inhibition of aerobic ammonium oxidizing bacteria (AeAOB). The heavy nitrogen gas detected and measured in the vials after the oxygen treatment was used as a measure of Anammox activity in this approach.

The procedure used in this work was modified according to ^{15}N label experiments as described by Kuypers *et al.* (2005) and Kalvelage, *et al.* (2011). Three different batch experiments were performed. In each of the batch experiments, four test vials and positive control were set up. Each batch experiment

was performed in a 20-ml vial (each containing calibrated with an oxygen sensor spot (optode)). The 10 ml of biomass suspension after preparation, taken from the bioreactor under anaerobic conditions as already explained for approach one, were distributed in each of the different test vials. Ar/CO₂ was used to purge the biomass suspension to remove traces of air and also prevent the introduction of N₂ in the background. The purging period lasts for approximately 3 minutes.

Pure O₂ was used as the source of the DO at different amounts of O₂ were added to the test vials according to the experimental plan with the aid of a gas-tight syringe. Afterwards, 10% tracer heavy ammonium (¹⁵NH₄⁺) of the concentrations of normal ammonium pool in the suspension was added to each vial and allowed to stand on a shaker over an incubation period of 5-6 hours. The effect of oxygen on the Anammox process was measured by measuring the amount of ²⁹N₂ produced in the head-space in each test vial determined using the GC-MS QP 2010 plus, (SHIMADZU, USA). The ²⁹N₂ produced were presented as integrals of the peaks generated in the detector of the GC-MS. The integrals are the peak signal obtained in the different test vials produce by the detector. These integrals were converted to moles of N₂ using the calibration curve of ²⁹N₂ and expressed in N₂-N ppm.

The assays were taken from the test vials and simultaneously analyzed for the following parameters: total bounded nitrogen (TNb), total organic carbon (TOC) and complete analyses of the inorganic nitrogen (NH₄⁺, NO₂⁻ and NO₃⁻) in the biomass suspension in the assay in the biomass suspension contained in the vials after the observation period. These analyses serve as a check for the accuracy of this method when compared to the total amount of nitrogen gas produced via the Anammox process.

3.1.4 Determination of dry mass of biomass

Vacková *et al.*, (2012) reviewed some of the methods used for the determination of concentrations of microorganisms in the biomass. In their work, the methods of determination of the dry mass of biomass reviewed and used in this work include:

- a.) Measuring the optical density.
- b.) Dry weight determination.
- c.) Direct counting under a microscope
- d.) Determination of specific substances like DNA, protein and/or lipopolysaccharide extracted from the organism. Three of the methods were employed in this work but the last method mentioned have already been described and used in Ijioma, (2012) and it will not be repeated in this work.

3.1.4.1 Determination of dry mass via spectrophotometric Optical density

The Optical density measurement is also known as the turbidimetric measurement. The principle of this method is based on light scattering or absorbance at a particular wavelength to determine the concentration of the microbes in a sample. This light scattered is closely related to the dry mass of the cells. This method has the advantages of being rapid, cheaper and also a non-destructive method compared to the direct method of dry mass determination (Sutton, 2011).

The biomass suspension was sonicated using the ultrasonic bath SONOREX super RK 510H (Bandelin electronic GmbH and Co: KG Berlin, Germany) to break apart soft biomass agglomeration or clusters to single cells as much as possible. This step is important because if particulate matter is present in the biomass this can lead to inaccurate measurement of the dry mass of biomass. Hence it is necessary to establish an optimum sonication time to help improve the light absorbance or scattering of the biomass suspension after appropriate dilution the suspension was made. The biomass suspensions were sonicated at different times and the corresponding dry mass of the biomass was measured via optical density at each sonication time using the UV-DU 640 spectrophotometer (BECKMAN, USA) at 580 nm.

From these measurements, the optimum sonication time (t_{op}) was established. Subsequent ultrasonic treatment of the biomass in the batch experiments was run at this time. The optical density was taken as the dry mass of the biomass at the determined t_{op} . The accuracy of the method is maximum when the absorbance measurement in the suspensions is between 0.1 and 0.5 as a rule of thumb (Wang).

3.1.4.2 Dry mass determination in the biomass

The dry mass of the biomass was also determined gravimetrically using the moisture analyzer (SARTORIUS AG., Göttingen, Germany). This is done by taking a known volume of the biomass suspension V (ml). The biomass suspension has already been washed with deionized water to remove mineral matter in the suspension solution and transferred into an accurately weighed aluminium pan, W_p in the moisture analyzer. The water in the sample was evaporated and the dry mass DM of the biomass was determined by weighing the aluminium pan and its residue. The dry matter can be calculated by the mathematical expression shown in (11)

$$DM = \frac{W_{DM} - W_p}{V(ml)} \times 1000 \quad (mg \text{ TSS } / l) \quad (11)$$

Where W_{DM} = mass of dried biomass in the Aluminium pan

W_p = mass of the aluminium pan lone.

3.1.4.3 Cell concentration by direct counting

This method is used to calculate the cell concentration in the biomass expressed in terms of total cell per volume used in the batch experiments. It involves direct counting of cells after staining with DAPI under an epifluorescent microscope.

With this method, the biomass suspension was sonicated using an ultrasonic bath, SONOREX super RK 510H for 5 minutes. Two dilutions (10 and 100-fold) of the biomass suspensions taken from the test vials were prepared used, after the termination of the batch experiments in PBS solution. Then, the appropriately diluted biomass suspensions were stained with 1: 20 100 μ g/ml DAPI solution and allowed to incubate for 5 minutes in the dark. 125- μ l of DAPI stained biomass suspensions were filtered under vacuum with 5-ml of PBS over 0.2 μ m pore filter. The stained residual biomass cells on the filter were viewed under the microscope using a Nikon eclipse LV-100, microscope (NIKON, Japan), and the images were digitally captured. The cells on the filter were counted manually with the assistance of

the NIS Element program. The cell concentrations N_t was calculated based on the relationship given in (5).

$$N_t = \frac{N_{ave}}{A_r} * A_{filter} * \frac{1}{Vol} * \frac{1}{DF} \quad (12)$$

Where N_{ave} = Average number of cells counted in the raster per image captured

A_r , and A_{filter} = Area of the raster in μm and filter respectively in

$Vol.$ = Volume of biomass in (ml)

$DF.$ = Dilution factor.

3.1.5 Microbiological Analysis

3.1.5.1 Fluorescent in situ hybridization (FISH)

FISH technique was used for the characterization and quantification of Anammox organisms present in the biomass both in the Bioreactors and for the batch experiments. This technique combines staining targeted organisms' 16S rRNA with appropriate fluorophore dye embedded in the oligonucleotide probes coupled with epi-fluorescence microscopic examination of the hybridized biomass. One of the major demerits of this molecular ecological technique is low signal intensity, background fluorescence, and target inaccessibility in FISH analysis (Gurdeep and Rajesh, 2011). This molecular procedure was carried out with sterilized wares to avoid interference and contamination of the biomass with other microbes in the course of experiments. We describe FISH procedures under the following subsections according to the protocols obtained from the chair of biotechnology for water treatment at BTU Cottbus.

3.1.5.1.1 Sample preparation

Fresh biomass was taken either from the Bioreactors or from the test vials after the batch experiments were preserved chemically (fixated) before the FISH analysis by experience, it was observed that the biomass produces a lot of extracellular polysaccharides (EPS) which does not allow the probes to penetrate the cells during hybridization. Fixated biomass suspension was sonicated to realize single cells for easy identification of Anammox cells and makes cell counting much easier.

3.1.5.1.2 Chemical preservation / Fixation of biomass

The chemical preservation technique used in this work represents one of many preservation techniques that can be used to preserve biomass to get good images during the microscopic examination and it is exclusive for FISH (Bennett et al., 2006). This step is important because it helps us to preserve viable cells in the biomass. 4% Paraformaldehyde in phosphate buffer saline (1xPBS) solution was used to fixate the biomass. (See Appendix 2 for recipes of 3xPBS and 4% PFA). It is recommended that the fixation time in PFA does not last for more than 24 hours and freshly prepared 4% PFA solution or 4% PFA solution stored under N_2 atmosphere at 4°C could be used). After 12 hours of biomass fixation in the PFA solution, the biomass suspension was washed 3 times with 1x PBS solution to remove the 4%

PFA solution in the suspension. The biomass was re-suspended in 1ml of 1:1 absolute ethanol in PBS and stored at -20°C. The preserved biomass can be stored for as long as 2 years at this temperature.

3.1.5.1.3 Enzymatic degradation of biomass

The enzymatic degradation of the biomass was required to remove the extracellular polymeric substance layer produced by the biomass which prevents the oligonucleotide from getting to the targeted 16S rRNA in the biomass. The preserved biomass was treated with 1 mg Trypsin inhibitor [57U/mg] (this prevents degradation of translators in the biomass) and 2.8mg Lysozyme (glycoside hydrolase) [140000U/mg] suspended in 1mL probe buffer (See recipe in Appendix 2). The biomass suspension and the enzymes were subjected to sonication at 37°C for 30mins in SONOREX super RK 510H. After the enzymatic treatment, the biomass was re-suspended in 1-ml (1:1) absolute ethanol in PBS solution after washing 3x times with 1xPBS solution. The biomass can be stored back at -20°C in the freezer.

3.1.5.1.4 Fixing biomass in the slide

Special non-fluorescence Teflon coated FISH slides with 8 sample wells of diameter 10mm were used. 10µl of the fixated biomass were placed in the wells and allowed to air dry to ensure that cells are strongly attached to the slide. It is recommended that samples in the wells should be controlled by uniformly distributing the suspended cells in the well to avoid clustering of cells at intervals. The biomass in wells was further dehydrated by serial washing steps of the slides in 50%, 80%, and 98% (v/v) ethanol for 3 min and allowed the slide to air dry.

3.1.5.1.5 Hybridization

1ml of the master mix hybridization buffer solution was prepared according to the formamide stringency of the probe used (See Appendix for the recipe). Formamide in the recipe was used to help the oligonucleotide probe attach to the rRNA structure in the cells of biomass. The appropriate oligonucleotide probes used were prepared by making a 1:6 dilution of 300ng/µl probe (50ng) in the hybridization buffer. The multi-welled Teflon-coated slides already fixed with biomass were spotted with 10µl of 1:10 appropriate probes in the hybridization solution or mix, preferably in the dark. Then, the hybridization was performed in a pre-warmed Eppendorf thermostat hybridization block at 46°C and allowed to incubate/hybridized for 3 hours. The incubation/hybridization times can vary between 2 to 24 hours preferably in the dark. 30 minutes before the end of the hybridization time, 50 ml of the wash buffer was prepared according to the stringency of the probe (See recipe in the Appendix under FISH) and preheated to 48°C in a water bath. At the end of the hybridization, the slides were carefully rinsed with 1-mL of the pre-warmed hybridization wash buffer in the dark and then the slides gently dipped into the 50ml Eppendorf tube and allowed to stand for 15mins. Care must be taken to avoid the loss of the sample from the slides. The slides were removed and allowed to air-dry in the dark

3.1.5.1.6 Post hybridization

10 μL of 5 $\mu\text{g}/\text{mL}$ 4', 6-diamino-2-phenylindole (DAPI) was used to counterstain the hybridized biomass on the wells and allowed for 5 minutes to incubate at room temperature preferably in the dark. The DAPI was rinsed off the wells with 1 mL of deionized water and allowed to air dry. 10 μL of the mounting oil was used to cover the hybridized and DAPI counter-stained biomass on wells, then cover the slide with cover glass. The slides were carefully pressed to remove excess mounting oil on the slide and then the sample is ready for microscopic examination.

3.1.5.1.7 Epi-fluorescent microscopy

The examination and evaluation of the hybridized samples were done using NIKON Eclipse LV 100 microscope (NIKON, Tokyo, Japan) under the epi-fluorescent light. The digital images and scans of the examined biomass were captured and processed with Nikon digital sight, Nikon intensilight C-HGFI (NIKON, JAPAN), Prior Pro scan II and NIS-Elements software. The images of hybridized biomass counter-stained DAPI were captured and also images of the biomass corresponding to oligonucleotide probes were used for the FISH analysis. Anammox cells in images were identified by their conspicuous sickle shapes and subsequently quantified.

3.2 Analytic procedures and data analysis

The analyses for the determination of the Anammox substrates ammonium, nitrite and nitrate; and the determination of dry mass via optical density were all performed using a spectrophotometer (UV-DU 640 spectrophotometer BECKMAN, USA). Other parameters analyzed include TNb and TOC. These parameters were determined with the DIMA TOC 2000 (DIMATEC Analysentechnik GmbH, Essen, Germany). The standard operating procedure for the methods used in the analyses were all based on the protocols used at the Chair of biotechnology for water treatment at the BTU as described below. The assays for chemical analyses are suspensions of biomass taken from the bioreactors or the test vials. Before the analyses, the biomass in the suspensions was centrifuged to form pellets as described in Ijioma, (2012) to get the supernatants, which were being analyzed for different parameters.

3.2.1 Determination of Ammonium

The analysis is based on the principle of ammonium determination described by Höll, (1968). The principle is based on the formation of Potassium iodide amalgam. The ammonium concentrations in the sample are determined photometrically. The limit of detection of NH_4 for this spectrophotometer is in the range of 0.1 to 5 mg/l.

5 ml of appropriately diluted sample was mixed with 200 μl of potassium sodium tartrate solution and 200 μl Nessler's reagents and thoroughly stirred. The samples were allowed to stand for five to eight minutes leaving a golden-yellow colouration and then measured photometrically at 425 nm with the UV /VIS spectrophotometer.

3.2.2 Determination of Nitrite

The assay was analyzed based on the method described by Snell and Snell, (1949) for the determination of nitrite. The basic principle of this method is based on the diazotization of aromatic amine in an acid solution, followed by coupling of the diazo compound with an aromatic amine to form an intensely colour azo dye (Jander and Blasius, 1995).

1% sulfanilamide solution (SA) in 1.5M hydrochloric acid was mixed in a 1:1 ratio with 0.02% sodium N (-1naphthyl) -ethylenediamine dihydrochloride solution (NED). From this SA/NED reagent, a 1:2 dilution was made with distilled water. Then, 2 ml of the diluted SA/NED solution was added to 5 ml of the appropriately diluted sample. The sample solution was allowed to stand for 20 min leaving a pale pink colouration. The measurement detection range NO_2 in the spectrophotometer for this method is 0.005 - 1 mg NO_2 / L and it is measured at a wavelength of 540 nm.

3.2.3 Determination of Nitrate

The assays taken from the test vials were analyzed for nitrate in the substrate medium according to Cataldo and colleagues (1975). This method is based on the principle of the formation of a 5-nitrosalicylic acid complex under highly acidic conditions. The complex formed absorbs maximally at a wavelength of 410 nm in a basic solution ($\text{pH} > 12$). The absorbance of the chromophore is directly proportional to the amount of nitrate-N present in the solution.

2 The procedure for the analysis follows: 50 μL of the sample was mixed with 200 μL of freshly prepared salicylic acid (0.2g Salicylic acid / 4ml concentrated sulphuric acid) in a test-tube and gently shake to dissolve the precipitate. Allow the solution to stand for 20 minutes. Carefully add 4 ml of 2N NaOH solution to the sample solution and shake properly to mix leaving a golden yellow colour solution. Allow the solution to gradually cool at room temperature. The NO_3^- in the sample was determined spectrophotometrically at a wavelength of 410 nm.

3.2.4 Determination of Total bounded nitrogen (TNb) and Total organic carbon TOC

The determination of Total bounded nitrogen (TNb) in the assay samples is an indicator that presents different inorganic nitrogen in the medium. Generally, a loss in TNb is a result of microbial activities of Anammox bacteria and/or heterotrophic denitrifying bacteria if decomposable organic carbon is present in the medium in the samples.

TNb in the assays were measured with DIMA-N connected to DIMA TOC 2000 allow the simultaneous measurement of TNb and TOC in the samples. DIMA-N runs on the principle of chemiluminescence at the following operating conditions: Oxygen pressure of 1 bar and temperature of 330°C. The signals generated from the samples were evaluated in the seamlessly integrated control software (DIMA TOC 2000 program) linked to the TOC analyzer (Dimatec Analysentechnik GmbH, 2013). During the analysis, supernatants from the biomass suspension were diluted ten times to get the measurement range of the equipment. The diluted sample was transferred to the sample rag of DIMA TOC 2000 analyzer

and it was programmed for the measurement. The results of TNb and TOC in assays were printed out after measurements.

3.3 Calculation of Specific Anammox activities (SAA)

The maximum activity of Anammox bacteria can be obtained at optimum conditions as already discussed in the reviewed section. The influence of certain environmental compounds on Anammox organisms can be directly monitored relative to the maximum metabolic activities when such compounds are not present in the medium. This depends on whether the compounds enhance or inhibit the activities of the organisms in the biomass. The maximum turnover rate ν_o of Anammox organisms was calculated based on the activity of the biomass in the control vial under the prevailing experimental conditions.

Since it was believed that several microbial activities could be responsible for the production of nitrogen gas in this work, we calculated the traced $^{29}\text{N}_2$ produced by Anammox bacteria as already described in approach two. The method of calculation of SAA in terms of $\text{N}_2\text{-N}$ production has already been described by Dapena-Mora, *et al.*, (2007), but in this work, we modified their approach to suit our hypothesis and the method of $^{29}\text{N}_2$ estimation.

The following equations and expressions were used to estimate and calculate the quantity of $^{29}\text{N}_2$ produced in g $\text{N}_2\text{-N}$. This term was derived from the concentration in ppm calculated from the calibration curve which was used to convert the GC-MS detector integral peak read out by taking the following steps:

$$C_{ppm} [^{29}\text{N}_2] = \text{measured concentration in ppm} * c.f \quad (13)$$

c.f = correction factor, which is the quotient of $^{29}\text{N}_2$ measured in the air to the calculated $^{29}\text{N}_2$ in a vial containing only air during the batch experiments.

The amount of mole $^{29}\text{N}_2$ produced is estimated by Equation (14):

$$n [\text{moles } ^{29}\text{N}_2] = \frac{V_g}{24.5} \times \frac{C_{ppm}}{10^6} \quad (14)$$

where V_g = gas volume equals volume (L) of the headspace in the vial and

24.5 = molar volume (l/ mole) of ideal gas @25°C.

Since the labelled ^{15}N used in the experiments was only 10% of the total NH_4 pool, we can calculate the total N_2 produced due to Anammox activities by the expression.

$$\text{N}_2 [\text{g } \text{N}_2\text{-N/l}] = \frac{28 \times 10 \times n}{V_1} \quad (15)$$

Where V_1 = volume (l) of suspension in the vials,

The constants 28 = molecular mass of Nitrogen gas (g/mole)

10 = multiplying factors for total N_2 produced by Anammox activities

The accuracy of the N_2-N calculated in this approach can be tested by comparing the calculated values to the amount of NH_4-N degraded in the medium. The comparison is based on the relationship proposed in the research assumptions.

The specific metabolic activities of Anammox bacteria can be calculated in terms of nitrogen gas produced per dry mass or Anammox cell concentration or by the degradation rate of ammonium per dry mass or per Anammox cell concentration in the biomass defined by the relationship in Equation (16) and (17).

$$SSA = \frac{v}{W_b * \alpha} \quad [gN_2-N. (g TS_{Anammox})^{-1}.d^{-1}] \quad (16)$$

$$= \frac{v}{N_t * \alpha} \quad [gN_2-N. (Cell_{Anammox})^{-1}.d^{-1}] \quad (17)$$

Where $v = d \frac{[^{29}N_2]}{dt} = d \frac{[NH_4]}{dt}$

W_b = dry mass of biomass in the sample.

α = estimated proportion of Anammox cells in the biomass

N_t = total cells per unit volume of the biomass.

The α -value was calculated as the proportion of Anammox cells in the biomass estimated from the images acquired from the FISH analysis expressed in percentage.

3.3.1 Percentage activity and kinetics of inhibition

This is the ratio of Specific Anammox activity in the test vials relative to the specific Anammox activity of the biomass in the control vial expressed as a percentage. The activity is a measure of ammonium degradation rate in terms of substrate or rate of N_2-N produced. The presence of an inhibitory substance reduces the rate in the test vials. The inhibitory substance in this work is oxygen. Mathematically, the percentage activity r_i of Anammox organisms in the biomass can be expressed as by the relation in (18):

$$r_i = \frac{SAA}{SAA_o} \times 100 \quad (18)$$

Where r_i = % of activity when the inhibitory compound was introduced

SAA_o = specific Anammox activity in the control vial

SAA = specific Anammox activity in the test vials for the different concentrations of oxygen.

The IC_{50} can be estimated by plotting r_i against corresponding inhibitory concentrations $[I]$, where DO is the inhibitory substance. IC_{50} was calculated using a dose-response curve derived from a four-parameter logistic (4PL) equation. The curve has a variable slope parameter and it is calculated from a commercial curve fitting program sigma plot 12.5.

However, K_i is an intrinsic and thermodynamic property of the inhibitor and depends on the enzyme and inhibitor concentrations but is independent of the substrate concentration. The lower K_i value indicates better efficiency and strong affinity of the inhibitor to the enzyme. In this work, the K_i was determined using the methods described by Nagase and Salvesen, (2001), pp. 142, according to the double reciprocal equation.

Chapter 4

4 Results

The various methods used for the determination of different quantities in the batch experiments have already been described in the previous section. In this section, we shall present some of the most important results obtained from the analyses and observations made and tried to highlight some of their significance under the different sub-headings.

4.1 Bioreactors

The operations and analysis of the laboratory scale Bioreactors 1 and 2 have already been explained in the previous section. Figures 6 (a) and (b) sums up the trend of substrate removal in the effluent recirculation assays of the Bioreactors over the time for which the reactors were maintained.

From the recirculation analyses, both Bioreactors showed an approximate equal capacity to remove nitrogen substrates from the study, based on the initial measured substrate input. The average rates of $\text{NH}_4\text{-N}$ and $\text{NO}_2\text{-N}$ removal in Bioreactor 1 were calculated as 0.0148 g-N/l/day and 0.0152 g-N/l/d respectively representing a mean removal efficiency of 87.3% and 91.2% and mean $\text{NO}_2\text{: NH}_4$ ratio of 1.02. For Bioreactor 2, the average substrates removal rate of $\text{NH}_4\text{-N}$ and $\text{NO}_2\text{-N}$ were calculated at 0.013 g-N/l/day and 0.0158 g-N/l/day respectively which represented a mean removal efficiency of 61% and 95% respectively and a mean $\text{NO}_2\text{: NH}_4$ ratio of 1.21. Since the Bioreactors were in a continuous flow, a retention time of 26 days was calculated at a recirculation rate of 0.067 $\text{m}^3\text{/day}$. In Figure 6b, the accumulation of NH_4 was depicted in Bioreactor 2 in the recirculation curve. Ammonium was accumulated with continuous removal of Nitrite as seen in the recirculation effluent plot. This observation was also seen in Bioreactor 1 but in this case, this is due to a higher dosing rate of the feed input when a new stock of the Anammox feed with higher concentrations of substrates in the medium. During this time, Bioreactor 2 was connected to this feed. The feed dosing rate was adjusted accordingly to the volumetric load mentioned in the previous section. In the later stage of Bioreactor 2, accumulation of NH_4^+ was observed with a corresponding degradation of nitrite in the reactor. During this period, the feed pump was switched off and no feed was dosed into Bioreactor 2. Then appropriate feedstock containing only NO_2^- in degassed Anammox medium was introduced into the bioreactor to raise allow the biomass to balance the stoichiometry of Anammox reaction and enhance the removal of N substrates in the medium leading to a sharp increase in nitrite concentrations in the recirculation.

The FISH analysis of the biomass taken from Bioreactor 1 was used for the characterization and quantification of the extent of enrichment of Anammox organisms (*Candidatus Brocadia Fulgida*) in the biomass. The results of the FISH analysis revealed that the biomass was enriched in Anammox organisms but the Anammox organism composition was high in *Candidatus Kuenenia Stuttgartiensis*.

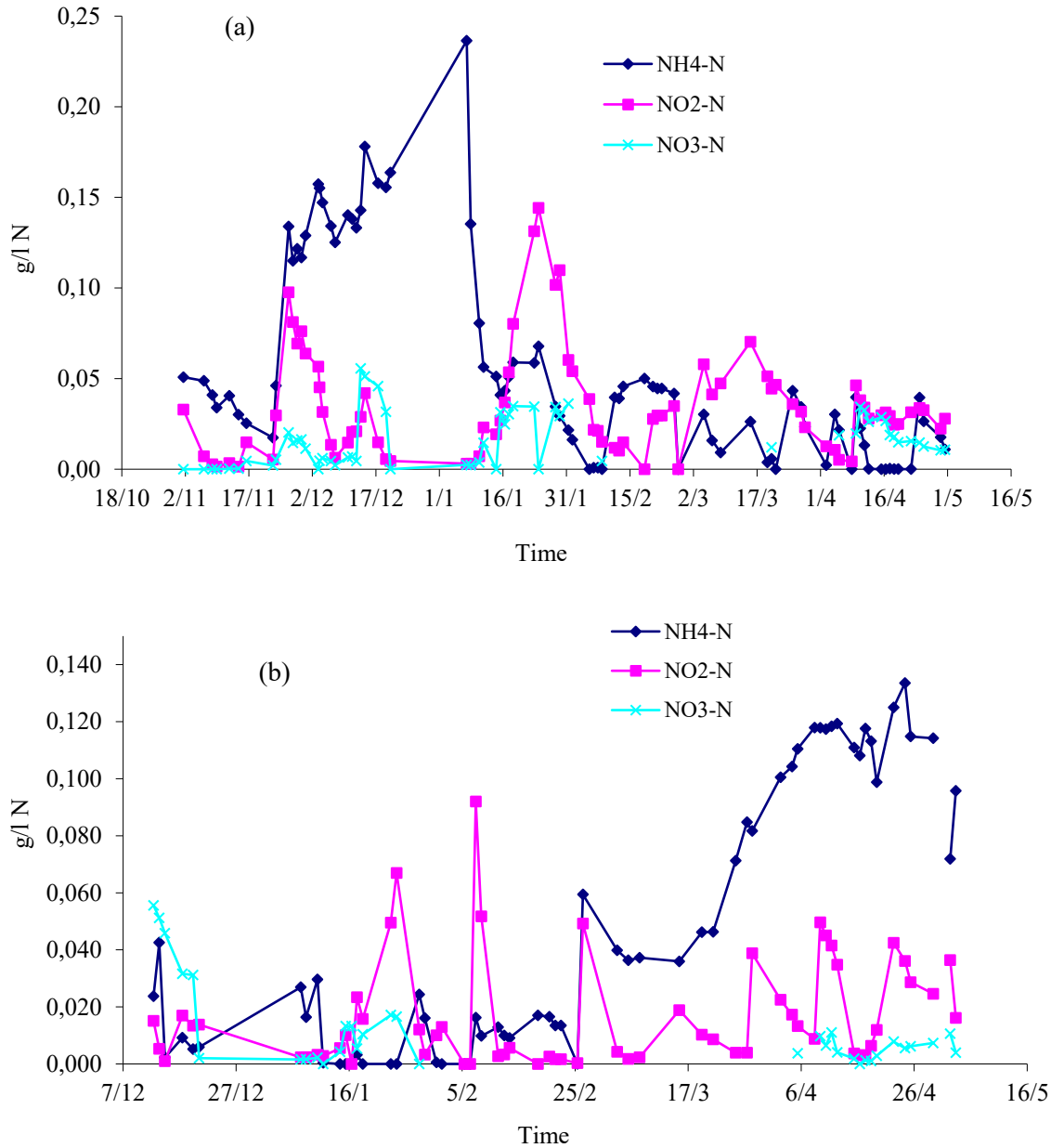


Figure 6: Results of measured substrates in effluent recirculation from the bioreactors. (a.) results of recirculations in Bioreactor 1 containing *Ca. Brocadia Fulgida* and (b.) Bioreactor 2 for *Ca. Kuenenia Stuttgariensis*.

Consequently, we concentrated our investigation on Bioreactor 1. Several microbial activities such as nitrification and denitrification processes were observed to be capable of taking place in the biomass obtained from Bioreactor 1 when conditions are right for these processes. This observation was made when biomass taken from Bioreactor 1 was examined on termination of batch experiments from one of the test vials used in Batch 1 experiment. But an examination of biomass taken directly from Bioreactor 1, there were no positive signs of denitrification activities. This effect might be as a result of the lack of degradable organic carbon in the medium but testing for nitrifying activities in the same biomass gave a positive result. See Figure 16-A and 17-A in Appendix 4 for some graphical presentation of the natural processes of nitrification and denitrification processes in the biomass taken from Bioreactor 1. Another evidence of microbial activities other than Anammox activities was the rapid growth of bacteria in the

biomass observed through changes in optical density of the biomass in Bioreactor 1. The fast increase in the absorbance indicated the growth of organisms other Anammox species in the biomass.

4.2 Sensitivity of Anammox process to DO

In the previous section, we identified two main approaches used for the investigation of the influence of oxygen on Anammox organisms in the biomass. Here are some of the important results found in the approaches.

4.2.1 Approach One

In this approach, degradation of ammonium in the suspension was used as the parameter of choice for the investigation in the study of the influence of oxygen on Anammox bacteria. By extension, inhibition of AeAOB activities and any other microbial activities that could influence the amount of ammonium in the biomass suspension, outside activities of Anammox organisms was necessary. Other parameters monitored include: TOC and TNb in the medium were also evaluated in parallel. It was found that the biomass was able to metabolize DO particularly in the absence of ATU.

At the beginning of the experiment, the TOC in the assay was analysed and found to be approximately 40 mg-C/l, with the maximum C/N ratio equals 0.33 for both the test and control vials. But the results of the analysis showed that in less than 4 hours from the beginning of the experiment, at oxygen concentration 0.3 mg-O₂/l, the TOC in both vials were completely consumed. See table in the Appendix 5 pp.87. The depletion of TOC in the vial suggested activities of denitrifying bacteria. In the test vial containing oxygen, the biomass was observed to have little or no activity when the concentration of DO was ≥ 0.4 mg O₂/l. The turnover rates of TNb and NH₄-N in the test vial were calculated. TNb was found to be - 4.2 mg-N/l/d whereas NH₄ in the medium was found to have increased in concentration, higher than the initial concentration (i.e. Change in turnover carries a positive sign). This positive sign indicates there was no microbial activity leading to the degradation of ammonium and probably suggested that the intracellular pool of ammonium substrate in the biomass might have been released in the medium.

The turnover rate for the TNb and the ammonium degradation plots in the control vial were compared by calculating the slopes of the curves in Figures 7 (a) and (b). -28 mg-N/l/day TNb was calculated against -13.6 mg-NH₄-N/l/day which gives a TNb: NH₄-N ratio of 2.06. This ratio when compared with the stoichiometry of the Anammox equation proposed by Strous, *et al.*, (1998) from where we derived the relationship between NH₄ degraded and the TNb in our research assumption, supported the fact that the Anammox process was the dominant microbial process taking place in the vials.

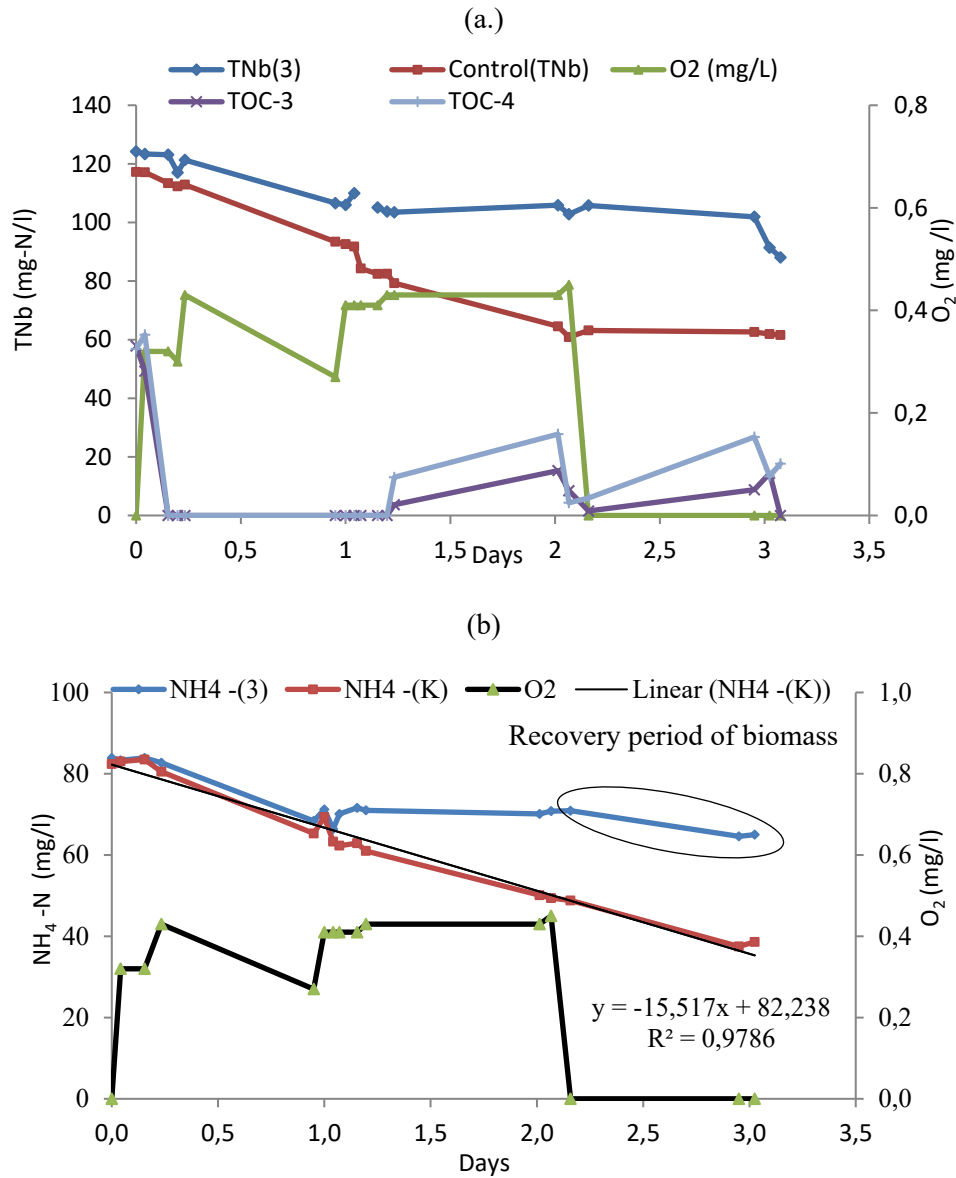


Figure 7: Graphical presentation of results of the sensitivity of Anammox bacteria to oxygen for one of the batch experiments in approach one using different concentrations of oxygen in the test vial. (a.) Shows measurement of different parameters in the vial including TNb, TOC and O₂ and (b.) curves of NH₄⁺-N and O₂ in the test and control vial showing a trend of loss of Anammox in the test vial.

The percentage recovery activity of Anammox organisms in the biomass contained in the test vial was calculated after purging the vial with nitrogen gas and allowed to stand overnight. The percentage of activity of Anammox recovery in the test vial treated with oxygen was compared with Anammox activity in the control vial. The result showed that the biomass recovered after oxygen treatment at 44.3% Anammox activity in the test vial measured against activity in the control vial based on the values of the slope as shown in figure (7b). This calculation was based on the NH₄-N degradation rates considered after the period of inactivity and purge with N₂ to reverse the inhibition as shown in Figure (7b).

4.2.2 Approach Two

With the second approach, the exact contributions of Anammox activities in the biomass were isolated through the use of tracer isotopes in one of the substrates. The influence of oxygen on the test vials was assessed by simultaneous quantifying $^{29}\text{N}_2$ produced and carry out chemical analyses on different parameters in the vials as already described in the previous sections to compare the accuracy of the method. The combined analyses will help us establish the exact activities of Anammox species in the biomass.

Since different conditions applied to the different Batch experiments, we shall consider the results on a case by case basis for the three batches of experiments considered in this approach. For the different batch experiments, the conditions were slightly modified to investigate the certain effects on the biomass and hence some variables were changed such as oxygen concentrations or biomass concentration or temperature changes in the batches.

4.2.2.1 Batch 1

The experiment consists of four test vials and a control vial. This batch used the lowest amount of oxygen as shown in Figure 8. Different amounts of pure O_2 were added into the vials (See the amounts of pure O_2 used in Appendix 6 pp. 88), but the control vial was completely purged with Ar/CO_2 gas to remove every trace of oxygen as much as possible. The maximum DO concentration used in this batch was $63 \text{ mg O}_2 / \text{l}$ and the biomass dry mass concentration of 580 mg TSS/ L (optical density) was used in this batch.

The chart in Figure 8 shows a graphical representation of results of TNb, NH_4 and N_2 turnover rates due to microbial activities taking place in the test vials measured after a 5-hour interval. The vial with the lowest concentration of oxygen represents the control vial. All the vials showed clearly Anammox activities taking place in the vials as indicated by $^{29}\text{N}_2$ production and also a corresponding decrease in the TNb and NH_4^+ in the medium.

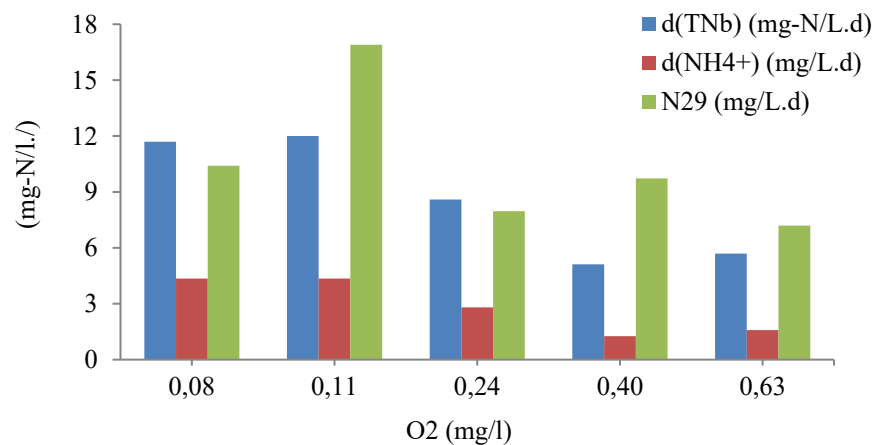


Figure 8: Comparison of rate of $^{29}\text{N}_2$ production, TNb and NH_4 losses for Batch 1

Comparing the results of microbial activities in the test vial 0.1mg/l O₂ concentration and the control vial, there were no appreciable differences in both since the concentrations of O₂ in both were almost the same. The influence of DO in the vials was mostly observed in the results of NH₄⁺ degradation and TNb obtained in the different vials but when compared with results of N₂ produced, we find some variations, but Figure 8 shows a decreasing trend line for N₂ produced

4.2.2.2 Batch 2

The same procedure was also followed as in batch 1 experiments. The difference lies in the concentrations for both the oxygen and the biomass suspension used. The same substrate concentration was used in all the vials. The maximum DO concentration in the vials was increased to 0.73 mg O₂/l and the dry mass concentration of the biomass was almost doubled to 920 mg TSS/l. After 5 hours interval from the beginning of the experiment, the results of the analyses of samples taken from the vials is represented in Figure 9. This figure presents a pictorial representation of the rate of activities of the different parameters measured to monitor the influence of oxygen on the Anammox organisms. The tables of results are enclosed in Appendix 6 for more clarity on the calculations. From these results, the rate of loss of TNb and rate of NH₄⁺ degraded agrees well with the stoichiometry and the working assumption of this work in most of the test vials. The regression trend line indicates the declining rate of Anammox activity as the concentration of oxygen increases. All the test vials show Anammox activity in respective of the concentrations of oxygen in the vials.

By normalizing and analyzing the results of nitrates and TOC, measured in the control vial during the experiments, Figure 10 present a quick trend of activities microbial activities NO₃ and TOC in the vial in this batch. The curves suggested rapid degradation of NO₃ momentarily and a sharp rise in the concentration of NO₃ until it reaches a constant value while the TOC continues to reduce until the experiment was terminated. The increase and steady level of NO₃ might be as a result of little or no TOC in the medium since these observations indicated competing activities of Anammox and denitrification processes in the biomass.

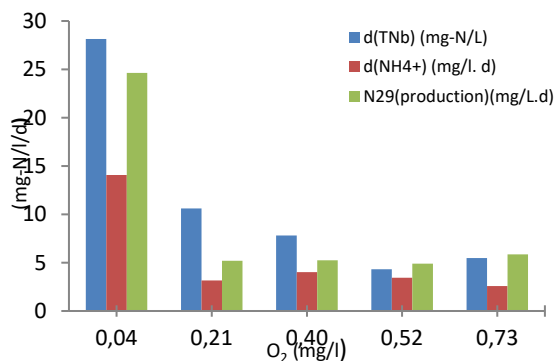


Figure 9: Comparison of rate of ²⁹N₂ produced with TNb and NH₄ degraded in Batch 2

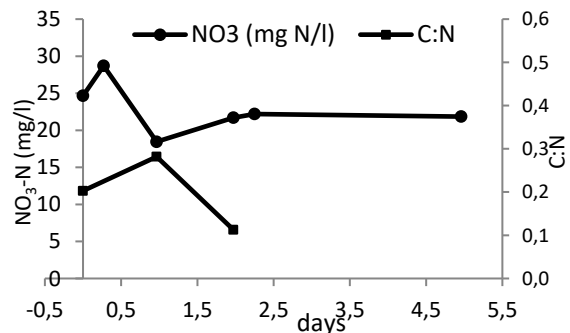


Figure 10: Relations between C/N ratio and NO₃ in Batch 2 experiment

4.2.2.3 Batch 3

In this batch experiment, we intended to investigate the toxicological effect of oxygen on the activities of the biomass, particularly on the Anammox species. Just like the other batch experiments, four test vials and the control vial were set up. Here, a larger amount of pure O₂ was introduced to inhibit the Anammox activities in the batch throughout the test vials (See Appendix 6 for the table of results).

The lowest and maximum oxygen concentrations measured in the test vials lie between 0.64 mg O₂/l and 1.02 mg O₂/l respectively. The pictorial presentation of the results is presented in figure 11. After 5 hours from the beginning of the experiments, the rate of microbial activities in the biomass was measured as usual based on the same parameters measured in the previous batches. Microbial activities were most visible in only one of the test vials with the lowest concentration of oxygen and the control vial as shown in Figure 11. At the concentration of oxygen above 0.6 mg O₂/l, no microbial activity was observed as shown in the activity bar plot of Figure 11. This is indicated by the parameter bars in the negative ordinate axis for most of the parameters measured. The recovery or reversibility test on the biomass in the test vials gave positive results as the biomass were once again active after purging the vials with Ar/CO₂ (See the results of the calculations in Appendix 6).

The analysis of data obtained from the control vial for concentrations of TOC and NO₃ in batch 3 experiments can be depicted in Figure 12. The continuous declining trend curves indicated denitrifying activities in the biomass since TOC and nitrates produced in the medium were degraded. Complete removal of biodegradable organic carbon from the medium may also lead to lysis of denitrifying bacteria in the medium and leading to a subsequent loss in biomass but increase in TOC in the medium. The normalized results of the activities of the biomass in the different batch experiments were used to estimate the sensitivity of Anammox bacteria to oxygen by calculating the IC₅₀ of oxygen in the combined experiments. The method of calculation was based on the inhibitor dose-biomass response calculated from the sigmoid plot in Figure 13 generated in sigma plot 12.5. The IC₅₀ calculated was 0.2 mg O₂/l. This implies that 0.2 mg/l of dissolved oxygen was sufficient to inhibit by 50% percentage of Anammox activities in the biomass.

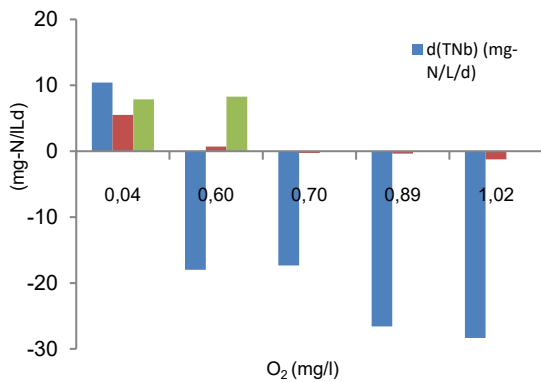


Figure 11: Comparison of rate of ²⁹N₂ produced, TNb and NH₄ degraded in Batch 3 experiment

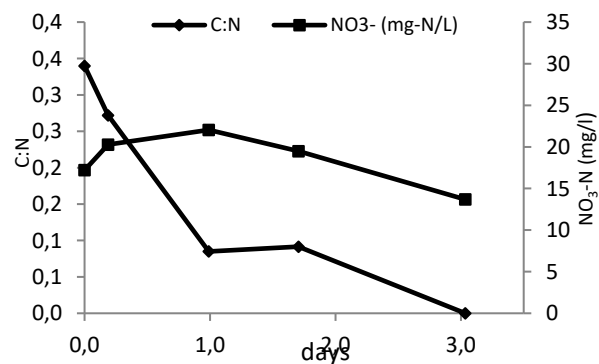


Figure 12: Measured nitrate degraded and C: N ratio in Batch 3 experiments

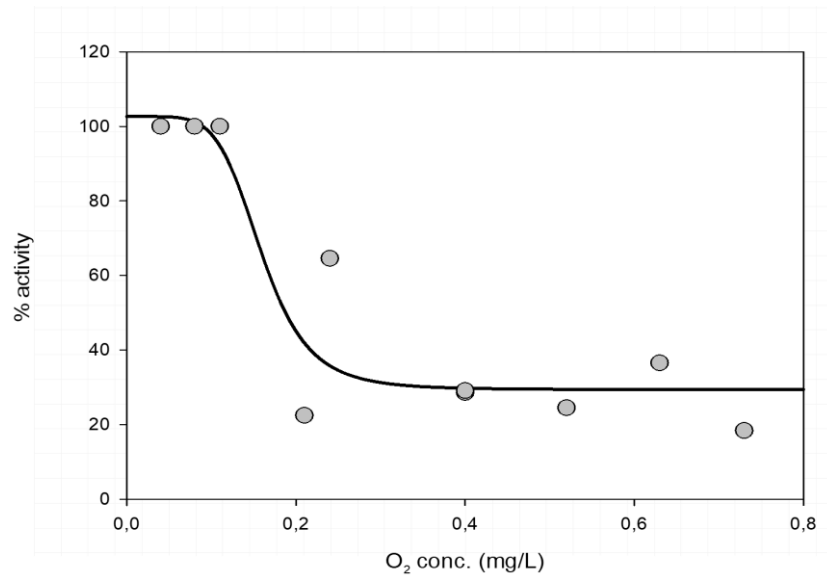


Figure 13: A graphical presentation of a sigmoid plot for the calculation of IC₅₀ for the combined batch experiments.

4.3 Kinetics of Inhibition

The introduction of oxygen in the test vials inhibits activities of Anammox organisms, thereby reducing the rate of activities in the batch experiments. The metabolic activities of uninhibited Anammox organisms in the biomass were measured from the activities taking place in the control vials of the batch experiments, hence the activities in the test vials could be normalized. The data generated from the normalized activities were used to calculate important kinetic parameters in this investigation such as the inhibition affinity constant K_i . The Michaelis-Menten constant K_M required for the estimation of the K_i was taken from Ijioma, (2012) which dealt with the influence of substrate concentrations on the kinetics of Anammox biomass. The mean K_{M-NH_4} value (0.08 ± 0.009 mg NH₄-N/l) was used for the calculation.

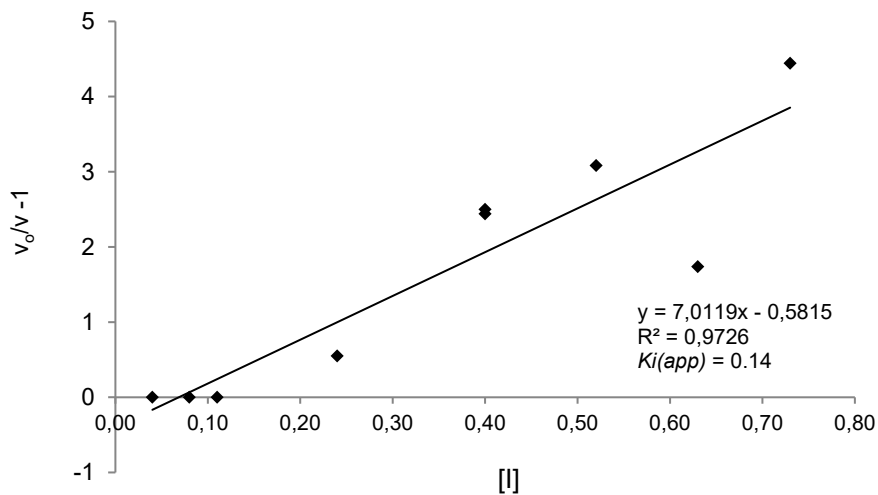


Figure 14: Determination of important inhibition kinetic parameter: $K_i(app)$ calculated from the combined results of batches 1 and 2.

Table 2: Summary of some important results of the inhibition kinetics parameters in the batch experiments

Parameters	Batch		
	1	2	Combined 1 and 2
$[I]_o$ [mg O ₂ l ⁻¹]	0.63	0.73	0.73
$[S]_o$ [mg NH ₄ -N /l]	40.1	49.2	49.2
IC_{50} [mg O ₂ l ⁻¹]	0.24	0.05	0.16
K_M (mg/l) *	0.08	0.08	0.08
K_i (μg/l)	1.00	0.30	0.20

(*) The K_M was obtained from Ijioma, (2012).

Figure 14 demonstrated the linear transformation Michaelis-Menten plot according to Lineweaver-Burk for the inhibition kinetic parameters for the combined batch 1 and 2. The plot in Figure 14 was used to obtain the apparent binding affinity constant ($K_{i(app)}$). The K_i was calculated based on the relationship shown in equation 19.

$$K_i = \frac{K_{i(app)}}{\left(1 + \frac{[S]_o}{K_M}\right)} \quad (19)$$

When the regression line of the plot intercepts with the abscissa (i.e. Horizontal axis), then this shows that the type of inhibition oxygen has on Anammox activity is non-competitive as shown in Figure 14 (Bisswanger, 2008). See some important results of the kinetics of inhibition in batch experiments 1 and 2 summarized in Table 2.

4.4 Recovery of Anammox process after oxygen inhibition

The 3 batches of experiments shown that DO can lead to a loss in the Anammox activity at different degrees and this confirms the ability of biomass to recover and show activity after purging the biomass with inert gas.

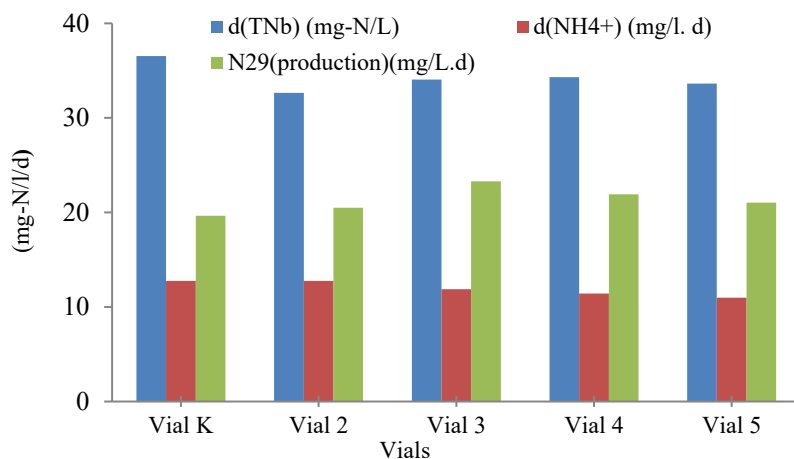


Figure 15: Graphical presentation of recovery activities of Anammox organisms in the biomass for Batch 2 experiments after purging with Ar/CO₂. Vial K is the control vial while vials 2-5 represents the test vials initially treated with oxygen between 0.2-0.73 mg/l.

A typical recovery activity by the biomass can be demonstrated using Figure 15. It was observed that in this batch experiments, despite the concentrations of oxygen used in the different test vials the reversibility occurred at almost the same strength. The rate of recovery was for all as shown in Figure 15. More so, it is worthy of mention that the turnover rates of TNb observed in the different vials exceeded the deduced hypothetical proposed stoichiometry for Anammox reaction as proposed by Strous *et al*, (1998) as shown in the figure. This observation suggested possible heterotrophic denitrification activity in the biomass leading to N loss.

4.5 Determination of dry mass in the Biomass

The different methods that were described and used for the determination of dry mass or total suspended solids in the biomass for the batch experiments have already been described in the previous section.

In this section, we shall compare the results obtained from these methods. The Optical density measurement using the spectrophotometer and the direct estimation of dry mass using the moisture analyzer both gave the dry mass in the biomass and expressed as mass concentrations (mg TSS/l). We observed a relationship between the length of sonication and the dry mass of the biomass during spectrophotometric optical density measurements at absorbance < 0.5 . The sonication time was obtained by combining different experimental batch results of biomass taken from the Bioreactors 1 and 2.

The polynomial equation of the second order for the trend line was solved to arrive at the optimum time t_{op} as shown in Figure 16. The t_{op} represents the maximum point on the curve and the optimum sonication time of approximately eight (8) minutes was established. The established t_{op} was used to treat the biomass suspensions during the OD measurements for the batch experiments.

The dry mass of biomass calculated from the direct determination of the dry matter in the biomass using the moisture analyzer was compared to the optical density dry mass. From the results obtained with both methods, direct estimation of dry mass from the moisture analyzer gave higher values than the values obtained from optical density measurement for the same sample. The (O.D/DM) ratio between the values of optical density and direct dry mass measurements gave 0.87 on the average for the different batch experiments.

The third method used for the determination of biomass concentration is the direct cell counting method which expresses the biomass concentration in terms of cells per unit volume. This method is important in the determination of the specific turnover rate per cell in the biomass. The results of biomass concentration obtained with this method by manually counting the DAPI stained cells gave an average value of 2.73×10^{11} cells /l. These results were computed from different capture images at the termination of the batch experiments. See Table 3 for results related to concentrations and quantification of biomass of batch 1 and 2 experiments.

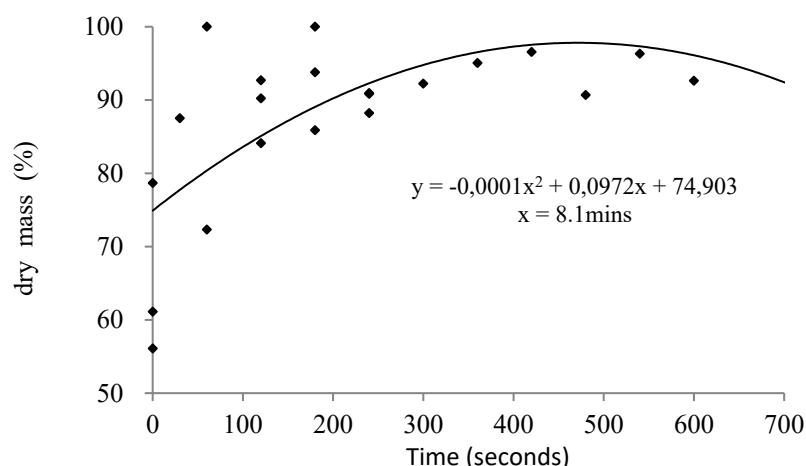


Figure 16: Graphical presentation for the determination of the length of sonication during dry mass measurement.

4.6 Concentrations of Anammox organisms in the biomass

As already described, the biomass used in this investigation were enriched Anammox culture. To estimate the number of Anammox bacteria present in the biomass was achieved by selective staining the Anammox organisms from the rest of the microbial community using the FISH technique. General oligonucleotide probes such as PLA 46 (probe specific for planctomycete phylum) and Anammox taxon-specific oligonucleotide probes such as BAN 162 and KST 157 were used to target the 16S rRNA of Anammox cells in the biomass to identify these cells from the rest of the bacterial organism present in the biomass. BAN 162 and KST 157 were used for the identification and quantification of species of *Brocadia* and *Kuenenia* in the biomass samples respectively. EUB 338 (I) used was as a control in the FISH analysis since the oligonucleotide probe is used for the identification of most bacteria. See Table 16-A in Appendix 3 for details of oligonucleotide probes used in the FISH analysis. The oligonucleotide probes are labelled with a fluorescence dye which when attached to the targeted 16S rRNA of the Anammox organisms after hybridization produced fluorescence under an epifluorescent microscope when excited with the appropriate wavelength of fluorescent light.

Table 3: Determination of biomass concentrations obtained from experiments in Batches 1 and 2.

Anammox bacteria	Batch 1		Batch 2	
	Cell concentration [cells/l]	Cell dry weight [mg-TS/l]	Cell concentration [cells /l]	Cell dry weight [mg-TS/l]
Biomass	2.73×10^{11}	580	4.35×10^{11}	924
Anammox Total	1.65×10^{11}	350	2.63×10^{11}	558.1
<i>Candidatus Brocadia Fulgida</i>	1.5×10^{10}	32	3.3×10^{10}	50.5
<i>Candidatus Kuenenia stuttgartiensis</i>	1.50×10^{11}	318	2.3×10^{11}	507.3

The positive FISH fluorescence Anammox species in the biomass are seen conspicuously as the sickle or ring-shaped cells in clusters. This shape distinguished Anammox cells from the rest of the bacteria in the microbial community.

The results of the FISH analysis revealed that all the probes gave positive signals for the biomass taken from Bioreactor 1. This result was unexpected from the two taxon-specific probes since the biomass was believed to be enriched only in *Brocardia Fulgida* as the only taxonomy of Anammox species in the biomass. To calculate the concentration of Anammox organisms present in the biomass, the Anammox cells in the images of positively stained biomass hybridized with the FISH probes were manually counted after matching them against images of corresponding DAPI stained total bacterial population in the sample for the same batch experiment. See Figure 18 (a) and (b) for epi-fluorescent images of some portions of FISH stained Anammox bacteria and the corresponding DAPI stained total bacteria population before counting. The results of the counting were presented as percentage average \pm percentage standard deviation ($\bar{x} \pm SD$) in the estimations. We estimated from these images high quite enriched Anammox species. Approximately $60.4\% \pm 14.6\%$ Anammox cells were counted from images captured in the biomass sample hybridized with PLA 46 probe. About $16.3\% \pm 12.2\%$ of the total cells represents *Ca. Brocardia Fulgida* and $54.9 \pm 13.3\%$. *Ca. Kuenenia Stuttgartiensis* were estimated to be present in the biomass based on the counted cells in images captured from the analyses of hybridized biomass using BAN 162 and KST 157 probes respectively. See Figure 17 for the picture of DAPI stained captured biomass obtained from a terminated batch experiments and used for the estimation of the total bacterial population in the biomass.

This percentage represents the α -value already mentioned in the previous section described in the SAA calculation. The concentration of Anammox organisms present in the biomass used in the different batch experiments was used for the calculation of SAA in the biomass in terms of $\text{gTSS}^{-1} \text{ l}^{-1}$ or Anammox cells. L^{-1} by multiplying the presented percentage of each species of Anammox bacteria in the biomass by the already determined dry mass or counted cells l^{-1} in each batch experiment as shown in table 4 for the estimated concentrations of the Anammox organisms in the biomass in Batch experiments 1 and 2. The dry mass of biomass used for the calculations of SAA was taken from the measurement obtained from optical density. This method was assumed to be more accurate than the determination of dry mass by direct estimation using the moisture analyser.

This is because dry mass determination using the moisture analyser comes with some inherent demerits which include: inclusion of mass of debris, sometimes intracellular minerals released into the solution after washing and possible loss of biomass during the washing step (Sutton, 2011).

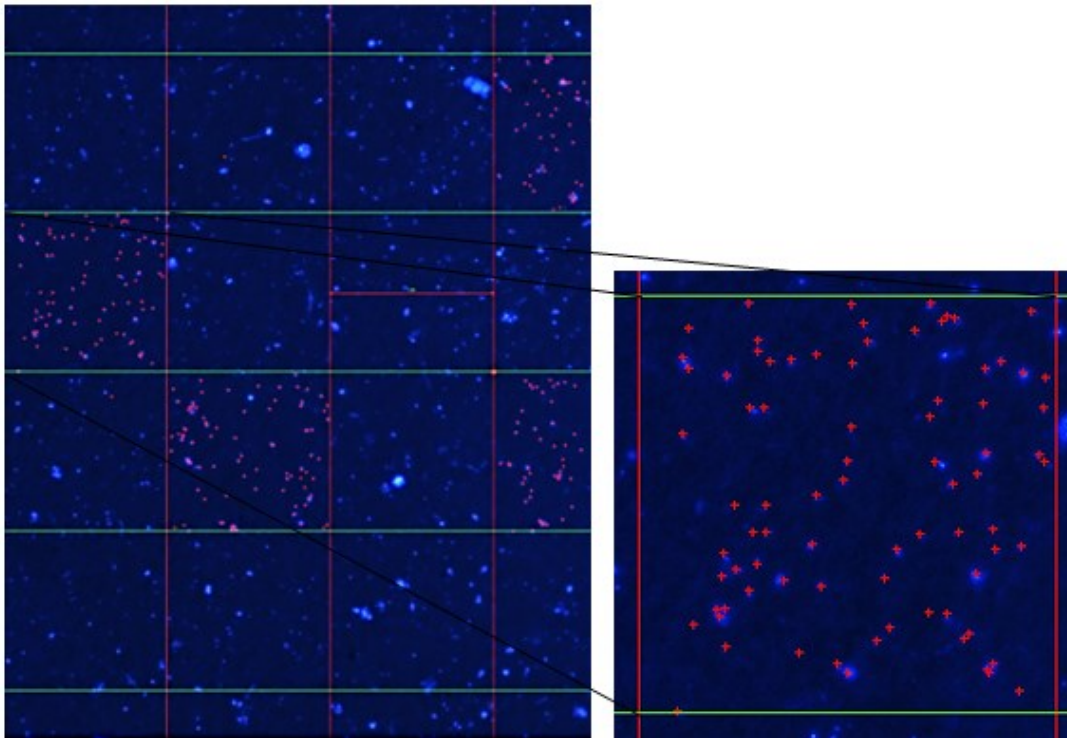
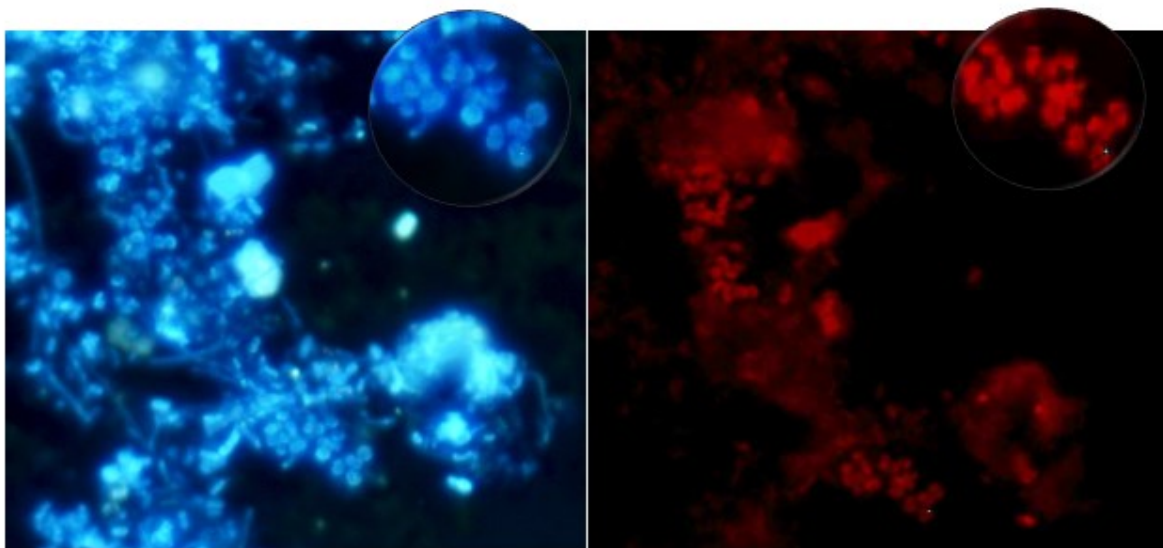


Figure 17: Capture image of biomass stained with DAPI after counting. Viewed @20x magnification from NiKON Eclipse LV 100 microscope using the NIS Element program.



a.) Counter-stained hybridized biomass with DAPI viewed @ 100x magnification

b.) Hybridized biomass stained with PLA 46 viewed @ 100x magnification

Figure 18: Images from the FISH analysis stained with (a.) DAPI and (b.) PLA 46, a general oligonucleotide probe for planctomycete phylum

4.7 Specific Anammox activity (SAA)

The maximum specific activities of the Anammox organisms in the biomass were calculated based on the activities of the control vial in the batch experiments. The result of the FISH analysis in estimating the amount of the total Anammox population present in the biomass was based on pictures from PLA 46 cells counting. The percentage of Anammox organisms was used for the calculation of the specific activities. The SAA calculated is an expression of the rate of N_2 -N produced or the turnover rate of NH_4 -

N degraded by the Anammox organism in the biomass to dry mass or the estimated number of Anammox cells in the biomass as presented in Table 3. Specific Anammox activities in the biomass in Table 4 were presented as both turnover rate per dry mass (i.e. total suspended solids (TSS)) in $[\text{mg-N (g. TSS}_{\text{total Anammox}})^{-1}\text{d}^{-1}]$ and the turnover rate per cell number of Anammox species in the biomass $[\text{mgN}_2\text{-N (cell}_{\text{total Anammox}})^{-1}\text{d}^{-1}]$ for both $\text{N}_2\text{-N}$ produced and $\text{NH}_4\text{-N}$ degradation in the medium. The increase in the specific activities from batch experiments 1 to 2 is caused by the increase in biomass used.

Table 4: Summary of some important results of Specific Anammox Activities in the batch experiments

Parameters	Batch	
	1	2
r $[\text{mg N}_2\text{-N}(\text{l.d})^{-1}]$	8.4	24.6
r $[\text{NH}_4\text{-N}(\text{l.d})^{-1}]$	4.3	14.1
$[\text{mgN}_2\text{-N (}10^{11}\text{cells}_{\text{total Anammox}})^{-1}\text{d}^{-1}]$	5.07	9.37
$[\text{mgNH}_4\text{-N (}10^{11}\text{cells}_{\text{total Anammox}})^{-1}\text{d}^{-1}]$	2.64	5.36
$[\text{mgN}_2\text{-N (g.TS}_{\text{total Anammox}})^{-1}\text{d}^{-1}]$	0.024	0.044
$[\text{mgNH}_4\text{-N (g.TS}_{\text{total Anammox}})^{-1}\text{d}^{-1}]$	0.012	0.025

Chapter 5

5 Discussion

Due to the complexity of some of the results presented in the previous section, it is important to discuss some of these results and make comments on some of the observations. This will shed more light on the results and make them more understandable. The biomass culture was taken without the knowledge of the degree of enrichment of Anammox organisms in the culture. We expected higher enrichment of the Anammox organisms in the bioreactor over time. Our focus is to reduce other side microbial activities taking place in the biomass as much as possible to concentrate only on the Anammox activities. To enhance Anammox activities in the batch experiments, good knowledge or full characterization of the biomass might be required and the operating condition should be optimized for the Anammox process. Hence the results shall be discussed under several sub-sections to support some of the results already presented in the previous section.

5.1 Bioreactors

5.1.1 Biochemical and microbiological characterization of the biomass in the bioreactor

Most of the discussions on the microbial characterization of the biomass in the bioreactor will be based on Bioreactor 1 since the FISH analysis revealed a logical discrepancy from what was thought initially. The FISH and epifluorescent examination of the biomass in Bioreactor 1, revealed that the biomass was a mixed culture of two species of Anammox bacteria and other different bacterial organisms. However, the estimation of Anammox organisms in the biomass showed that they constituted approximately 60.4 % of the microbial population at the time of batch experiments as already presented.

The bioreactors were operated under anoxic conditions; hence it was expected that microbial activities that thrive under aerobic conditions will be inhibited and probably may not survive in the bioreactors. But the test for nitrifying activities in Bioreactor 1, revealed nitrifying activities were possible in the biomass. The results of this observation were demonstrated in Appendix 4 for biomass obtained from bioreactor 1 and the biomass from the terminated batch experiments. Several investigators have shown that some nitrifying organisms such as *Nitrosomonas spp* can survive long anaerobic conditions and they have been found to coexist in Anammox culture under poorly aerated autotrophic conditions (Noophan *et al.*, 2009, Liu *et al.*, 2008, Bae *et al.*, 2010). These works suggested that these nitrifying bacteria in the biomass are in a symbiotic association with the Anammox organisms and offers protection to Anammox bacteria from the influence of DO while performing their autotrophic activities. There might be some possibility of ways oxygen might get into the Bioreactors. These possibilities could include the diffusion of air through the micro-pores of the recirculation hose and the joints of the valves and hose into the bioreactors. Another possible way for which the bioreactors was poorly aerated could be diffusion of air during periods the bioreactors' feeds were being replaced thereby keeping nitrifying organisms in the Bioreactors active for some time.

Denitrifying activities were also found in the Bioreactor. The first proof of denitrifying activities was found after the termination of the Batch 1 experiment. Biomass suspension from one of the test vials taken from Bioreactor 1, was used to test for denitrifying activities and the result was positive for denitrification activity. See results in Appendix 4. Nonetheless, this was not observed in fresh biomass taken directly from Bioreactor 1. The possible explanation for this observation might be, at the time of assessment there was no degradable organic carbon in the medium and organic carbon is known to be a limiting factor in denitrifying activities. The synthetic Anammox medium carrying the biomass in suspension was prepared without any organic carbon source, and this situation is common in both cases. According to Dapena-Mora *et al.*, (2004), the probable source of organic carbon for denitrification activities in this mixed Anammox biomass could be via the lysis of cells in the biomass. This result can occur at the time when some organisms in the biomass experience unfavourable conditions in the bioreactors. Thus, the presence of denitrifying bacteria in the Bioreactors, removes degradable any *DOC* generated in the Bioreactors and such *DOC* cannot accumulate in the bioreactors. Thus, *TOC* in the bioreactor was most often depleted in most of the measurements. Denitrifying bacteria can metabolize organic carbon to produce CO_2 .

The operating conditions in the bioreactors favour both denitrification and Anammox processes since the medium contains nitrite and nitrate which is also a by-product of the Anammox process. Sometimes when *DOC* was measured in the Bioreactors, *C: N* ratio in the Bioreactors is much more < 1 , with little or no, *DO* in the Bioreactors. Anammox and denitrifying activities can simultaneously take place in the bioreactors since both processes have some common substrates (NO_2 and NO_3) and require an anaerobic condition (Koeve and Kahler, 2010). This might explain why the Bioreactors have shown very good ability to remove NO_2 at very fast rates in the two Bioreactors, even when the ammonium was accumulating in the bioreactors. There was high removal efficiency of NO_2 substrate observed in both bioreactors from the effluent recirculation.

The trace isotope investigation as described in ^{15}N labelling in the batch experiments revealed the activities of Anammox bacteria in the biomass taken from Bioreactor 1. The activities of Anammox organisms were observed in terms of $^{29}\text{N}_2$ produced and degradation of ammonium in the experiments. Also, the positive FISH signal obtained with the Anammox specific probes on the biomass ultimately confirms their presence in the biomass. More so, concerning the possibility of having two species of Anammox organisms revealed by the FISH analysis in the biomass of Bioreactor 1, examination of the source biomass also confirmed the presence of *Ca. Kuenenia Stuttgartiensis* in the biomass is believed to be enriched only in *Ca. Brocadia Fulgida*. But at the time of this analysis the level of enrichment of *Ca. Kuenenia Stuttgartiensis* in the biomass was not known as compared with the results already reported in the previous section. If the genus *Kuenenia* was mistakenly introduced into the biomass *Brocadia* culture, population shift might be a possible clue to the high enrichment of the *Kuenenia* genus in the biomass. Oshiki *et al.* (2011) in their review of the physiological characteristic of *Brocadia Sinica*. They found that at stable Anammox conditions or long term reactor operations could lead to population shifts in the Anammox species in a mixed Anammox biomass. They attributed the reason for the population

shift to the difference in physiological characteristics between the species. For instance, the difference in affinity constant for substrates and the maximum growth rates in the species might differ.

The results of the effluent recirculation analyses of substrates in both bioreactors $\text{NO}_2\text{-N}:\text{NH}_4\text{-N}$ ratios gave a ratio of 1.21 on average. This result shows that the ratio is comparatively close to the theoretically expected stoichiometry (1.32) for the Anammox reaction. This result indicates that the Anammox process dominates the other microbial activities taking place in both Bioreactors. But this observation failed to explain in concrete terms the accumulation of ammonium the particularly in Bioreactor 2. Ammonium accumulation in the Bioreactor 2 could be explained by the activities of dissimilatory nitrate reduction to ammonium organisms – Anammox and denitrifying organisms in the biomass. Koeve and Kahler, (2010) in their work reviewed the activities of heterotrophic de-nitrification under sub-oxic conditions. They explained that ammonium can be released from organic matter via heterotrophic consumption by denitrification and dissimilatory nitrate reduction to ammonium in an oxygen-free environment.

5.2 Anammox versus Denitrification processes

Anammox and heterotrophic denitrification processes have been demonstrated in the biomass of Bioreactor 1 as we have shown in our arguments in the previous sub-heading in both the batch experiments and the Bioreactors. Anammox bacteria and heterotrophic denitrifying bacteria are anaerobes and thus, both bacterial organisms utilize nitrite as substrate and remove this inorganic nitrogen in their medium to produce N_2 although their biochemical pathway differs. The activities of these bacteria can easily be mixed up. In a man-made ecosystem, Rusalleda and his colleagues (2008) found that Anammox and denitrifiers could co-exist and they are used for the treatment of influent streams with a large amount of slow biodegradable carbon. They found that heterotrophic denitrification was limited by the low availability of easily biodegradable organic carbon and Anammox process out-compete denitrification in this system.

Since degradable organic carbon limits heterotrophic denitrification, the synthetic Anammox medium used in this work, contains no organic carbon that could support denitrification.

Table 5: Comparing thermodynamic and doubling time of Anammox and denitrification processes.

N-cycle process	Species	ΔG° (kJ/mol)	Doubling period
Anammox	Candidatus Brocadi A.	-297	11-19 days
Denitrification	Bacillus, Pseudomonas	-843	<12 hours *

(*) (Komor and Fox, 2002), the doubling period for Anammox bacteria is author dependent, for this work, we reported according to Dapena–Mora et al. (2007).

Lysis of cells of organisms in the biomass due to harsh conditions (starvation and suffocation resulting from anaerobic conditions for aerobic nitrifying organisms) can result in the release of *DOC* in the medium. This phenomenon explains the increase in the *TOC* content of the biomass suspension

sometimes in the Bioreactors and the test vials during the batch experiments. This explanation was supported by Dapena-Mora, *et al.*, (2004).

Van Hulle *et al.* (2010) in their review reported that the presence of easily degradable organic carbon does not favour Anammox activities at a high C/N ratio based on the findings of different investigators. In a mixed Anammox – denitrification biomass culture containing C/N ratio >1 , the Anammox process will not be able to out-compete denitrifying process for N removal (Nabizadeh *et al.*, 2012). In this work, the maximum C/N ratio measured in the control vial was 0.4, for such ratio, Anammox and denitrification processes can take place simultaneously and the organic carbon content in the medium was removed. Kumar and Lin (2010) argued that at very high C/N, dissimilatory activities are favoured instead of nitrogen gas release. This means that conversion of NO_3^- into NH_4^+ is favoured with the removal of organic carbon by the organisms that support dissimilatory nitrate reduction to ammonium (DNRA). But in general, under suitable conditions, the tendency of the denitrification process to out-compete the Anammox process is higher. This is because the denitrification process is thermodynamically more favoured than the Anammox process. Denitrifying organisms grow faster than Anammox bacteria and thus they are more active. See Table 4 for more details found from the literature comparing both processes. In this work, the Anammox process will out-compete denitrification since the carbon source in the medium is limiting.

5.3 Fluorescence in situ hybridization (FISH)

The results of the FISH analysis demonstrated that the biomass contains a high composition of Anammox organisms in the biomass. The epifluorescent images of the biomass stained with DAPI revealed that the bacterial composition in the bioreactor 1 and the biomass suspension used in the batch experiments comprise different microbial organisms ranging from single-celled to multi-cell filamentous bacteria. This can be easily seen from the images of biomass captured stained with DAPI. See Figure 18 (a) and more pictures in Appendix 3 under FISH results. In the pictures of the FISH analysis stained with Anammox specific probes, Anammox bacteria were generally found in clusters identified by their conspicuous moon shape and the diameter of the cells. The intensity of the FISH signals during microscopic examinations as well as indicated a high concentration of Anammox organisms in the biomass.

In the different ecosystems where Anammox bacteria have been found, it is believed by many researchers that Anammox bacteria were found in symbiotic association with other microbial organisms as already explained (Stevens and Ulloa, 2008, Han *et al.*, 2012), either receiving nourishment or protection by organisms from eventual harsh environmental factors that might harm or adversely affect their activities. For example, the presence of nitrifying bacteria co-habiting with Anammox bacteria will offer protection to Anammox organisms by metabolizing dissolved oxygen in the environment and also the nitrifying bacteria can also metabolize ammonium into nitrite at such low oxygen concentration which can be utilized Anammox organisms. For example, denitrification and Anammox bacteria have common substrates (NO_2 and NO_3) (Dapena-Mora *et al.*, 2007). Denitrification activities can be

supported in an environment cohabiting with Anammox bacteria, in which environmental conditions contain easily degradable carbon for which C/N ratio is < 1 . In such an environment, both organisms could be supported by each other.

In the batch experiments, because we have observed various microbial activities in our Bioreactors, it becomes necessary for optimized Anammox conditions to enhance activities of Anammox organism in the biomass specify a condition that will inhibit the actions of organisms other Anammox bacteria during the investigations. Anammox bacteria and AeAOB have the capabilities to utilize ammonium as a substrate but through different biochemical pathways. Since NH_4 degradation was used as an important parameter for evaluating the activity Anammox in the vials in both approaches used monitoring activities of the sensitivity of Anammox to DO, it was necessary to suppress the actions of the AeAOB. Some researchers have shown inhibition of nitrifying bacteria in mixed culture containing Anammox species with ATU, it was found that ATU has no effect on the activities of Anammox bacteria at a concentration that it is capable of inhibiting the nitrification process (Kalvelage *et al.*, 2011, Dapena-Mora *et al.*, 2007). ATU was used to achieve this objective in these experiments.

Denitrifying bacteria were assumed to be technically inactive or occurred temporarily only when conditions are right in the bioreactors since there is no steady degradable organic carbon source in the mineral medium. But some results of the batch experiments suggested that denitrifying activities in the vials were possible. See Figure 15, showing the recovery activities of Anammox bacteria in the batch experiments after oxygen treatment. The rate of turnover TNb measured shown more than double the rate of NH_4^+ -N or the other way around, it is greater than the rate of production of N_2 in this batch experiment. Under anaerobic or within the permissible limits of DO in the batch experiments, facultative denitrifying activities occur in the presence of a degradable organic carbon source. The amount of degradable carbon generated by this process might not be sufficient to induce harm to Anammox bacteria in the biomass as suggested by some investigators. Some investigators revealed that the presence of organic carbon can inhibit the Anammox process but this assertion depends on how easily degradable the organic carbon is and the C/N ratio in the conducting medium (Nabizadeh *et al.*, 2012). Generally, it has been reported that easily degradable organic carbon can inhibit Anammox bacteria but the presence of denitrifying bacteria can offer protection to Anammox bacteria in this regard.

5.3.1 Taxonomic classification and quantification of Anammox bacteria

The FISH analyses in our investigation of the biomass of Bioreactor 1 revealed the presence of two taxonomical classes of Anammox species (*Candidatus Brocadia Fulgida* and *Candidatus Kuenenia Stuttgariensis*) in the biomass. The analysis of the hybridized biomass using the epi-fluorescent microscopic examination revealed that stronger fluorescence was observed with KST 157 than BAN 162. This observation can be linked to higher concentrations of rRNA of *Ca. Kuenenia Stuttgariensis* in the fixated biomass than *Ca. Brocadia Fulgida* as confirmed in their percentage abundance estimated. The intensity of fluorescence of the FISH signal is dependent on the concentration of the rRNA in the Biomass (Gurdeep and Rajesh, 2011).

As already pointed out earlier, the background fluorescence of the biomass is one of the disadvantages of this method, especially at the low 16S rRNA concentration of the targeted organisms in the biomass. The inability of the probe to penetrate the target organisms can also be a cause for low signal intensity during the epifluorescent examination of the hybridized biomass. These situations were most prevalent while examining the biomass hybridized with BAN162. Successful FISH signal with BAN 162 reveals weak signal meaning a low abundance of *Brocadia Fulgida* in the biomass. Picture of FISH can be seen in Appendix 3.

The acquired picture of hybridized biomass with weak FISH signals can be challenging to differentiate from background fluorescence of biomass without probe particularly if the colour of the labelling dye in the probe and the background fluorescence of the biomass without probe is the same. The biomass when examined under epi-fluorescent light gives red auto-fluorescence without the distinct presence of single cells in the image or the distinctive sickle or ring-shaped of Anammox organisms in the biomass. The biomass when hybridized are stained with probes, the probes contain labelling red dyes such as Texas red and Cy 3 common in probes like PLA 46, KST 157, BAN 162 typical Texas red and EUB 388 (I), the latter, Cy3. Thus, if low concentrations of 16S rRNA for the appropriate species of Anammox bacteria are hybridized in the biomass with such probes, it will be difficult to differentiate between the biomass and the hybridized biomass under epi-fluorescent examination.

All the probes gave strong fluorescent signals which can be easily differentiated from the red auto-fluorescence of the biomass without a probe. The identification of Anammox organisms in the biomass was confirmed by conspicuous moon or sickle-shaped of the Anammox cells found in the clusters and widely scattered around the image of the sample under examination. See results of picture of FISH for the different probes in Appendix 3 under FISH results.

In a parallel study undertaken by Viehweger, (2013), her microscopic analysis of the same biomass samples obtained from Bioreactor 1 in a comparative study of kinetic activities of two genera of Anammox species. She estimated a mean 10.2 % composition of *Brocadia Fulgida* in the biomass as the Anammox species in the biomass. But in this present work, we estimated the mean Anammox bacteria compositions of about 60.4% of the biomass of Bioreactor 1 and we have shown it constituted a mix of two species of Anammox bacteria. *Ca. Brocadia Fulgida* composed an average of about 27% of the total Anammox population in the biomass. At the time of this investigation, we believed that the Anammox bacteria composition in the biomass has been further enriched and also there is a possibility of a population shift in the taxonomy of Anammox organisms present in the biomass. More so, her method of evaluation of Anammox organisms in the biomass was based on personal judgment in identifying Anammox cells in the biomass population from images obtained from the epifluorescent examination.

5.4 Determination of dry mass in the biomass

By comparative analysis, the dry mass of biomass obtained via optical density was believed to be more reliable and as such, they were used for the calculations of the SAA in the batch experiments. The results

obtained from both methods (optical density and direct estimation) gave an average ratio of OD/DM equals 0.87 which implied that direct estimation gives a higher value than optical density. This is a result of the inherent errors associated with the direct estimation method described earlier.

More so, optical density also suffers from its disadvantages. The values of dry mass obtained from the spectrophotometric measurements depend on the slope of the calibration curve. This means that the accuracy of the methods depends on several factors that could affect the calibration curve. These factors include the intensity of the lamp of the spectrophotometer and the morphology of the biomass. The intensity of the lamp depends on time, as the lamp ages with time, the more the intensity fades. Also when a new change lamp is installed, the new calibration slope is required (Sutton, 2011). In addition, the calibration slope for this procedure is performed with single cells and agglomerate or cluster cells are measured in most experiments. Generally, every method used for the determination of dry mass has its faults, but for the results obtained from the O.D measurements, the errors are within the acceptable limits as can be ascertained from the OD: DM ratio. Hence the dry mass used was by extension expressed as total suspended solid TSS in the biomass suspension as seen in the results.

DAPI is one the most commonly used DNA stains for effectively counting the cells of organisms using epifluorescent microscopy to determine the cell concentration in biomass. Bennett et al., (2006) in their work submitted that the DAPI counting method has its limitation, since DAPI stains every natural DNA and cannot distinguish between viable and non-viable cells in the biomass. This can lead to over-estimation of the total number of active cells in the biomass that can lead to an error when used for other calculations. This disadvantage of DAPI as the dye can be overcome by using a similar staining compound; 5-cyano-2, 3-ditoly tetrazolium chloride (CTC) which stains only viable and actively respiring cells. Since at the time of investigation CTC was not available, we could not compare the results from both dyes.

5.5 Specific Anammox activities (SAA)

Comparing the performance of the SAA presented in the previous section with the results of other studies might be difficult since there are hardly pure cultures of Anammox bacteria. Anammox culture at different degrees of enrichment in the biomass will give different levels of metabolic activities. Also, the activities of other microbial organisms in the biomass can influence the outcome of the investigation. We shall review SAA in other literature and compare them with SAA of our biomass in the batch experiments as shown in Table.3.

In the batch experiments, the results of SAA were presented in two different units: turnover rate per unit dry mass (TSS) and the turnover rate per unit cell of Anammox organism as found in most literature. On the one hand, the common unit of SAA expression found in most literature is presented as the turnover rates per volatile solid (g- N. (g VS⁻¹d⁻¹)) but in this work, the unit (g-N (g TSS_{Anammox}⁻¹. d⁻¹) was used and on the other hand, the turnover rate per cell of Anammox in the biomass.

The maximum calculated SAA was found in the batch 2 experiments. Here higher biomass concentrations used in this batch resulted in higher turnover rates: But in both batch experiments, approximately the same initial starting concentration of ammonium was used, but in the Batch 2 experiment, the concentration of the biomass suspension was almost doubled. The influence of the double concentration of biomass was conspicuous felt as the SAA was doubled per unit time correspondingly.

In the same parallel study already mentioned carried out by Viehweger, (2013) on Bioreactor 1, she determined the maximum turnover rate in Bioreactor 1. The mean V_{\max} calculated for the biomass was $8.7 \text{ mgNH}_4\text{-N (TSS}^{-1}\cdot\text{d}^{-1})$ and $3.8\text{-}5.4 \text{ mgNH}_4\text{-N ((}10^8 \text{ cells)}^{-1}\cdot\text{d}^{-1})$ respectively. The results of Viehweger were high when compared to the results presented in this work. This is because both works were based on different assumptions and the approach of the investigation also differs. While in her work, she worked on the assumption that the biomass was composed mainly of Anammox organisms (*Brocadia*) which on the contrary we tried to eliminate or inhibit the activities of other autotrophic AeAOB in the biomass and also, we found the biomass to contain a mix of Anammox species (*Kuenenia and Brocadia*).

In addition, the other possible basis for disagreement in these results was that FISH analysis was used for the identification and quantification of Anammox bacteria in the biomass. The quantification of enrichment of Anammox organisms in biomass was carried out when the Anammox organisms in Bioreactor 1 have been enriched further based on the doubling period of the Anammox organisms.

Other contemporary studies on the same biomass at the department, on the kinetics of Anammox process independently performed by Krause, (2011), and Ijioma, (2012), calculated turnover of nitrogen from Anammox medium based on the total solids (TS) in the biomass and found turnover of $29 \text{ mg NH}_4\text{-N (TS}^{-1}\cdot\text{d}^{-1})$ and $176 \text{ mg NH}_4\text{-N (TS}^{-1}\cdot\text{d}^{-1})$ respectively. Part of the methods used in this work was adopted and modified from these two previous studies. But the difference in these studies was derived from the assumption that the biomass composition has different degrees of Anammox and other microbial activities taking place in the biomass that was not taken into consideration. Again, these results were maximum turnover rates calculated based on the kinetics of all biochemical activities compared to the turnover rate from our experiments.

Another important observation was during the experiments in this work, a higher turnover rate was measured after overnight standing particularly during the recovery test of biomass. This fact could be likened to the experimental measurements of the former studies for which daily measurements were used resulting in such observations. But in this work, analyses were made after 5-6 hours, which were used to estimate the turnover rates. Thus, some of these measurements might have been carried out when the Anammox organisms in the biomass just acclimatized with the inhibitor and they are slowly recovering. This might be responsible for the slow turnover rates as reported in this work.

In a further review of the SAA from studies of Anammox organisms, based on rates of nitrogen gas production per Anammox cells in the biomass, Galán *et al.*, (2009), in their investigation estimated a specific turnover rate of $5.4 \text{ mg N}_2\text{-N (}10^{11} \text{ cells)}^{-1} \text{ d}^{-1}$ in the sub-oxic OMZ of northern Chile.

Hamersley *et al.*, (2009) found a specific turnover rate of N_2 produced by microbial mechanisms of Anammox and denitrification processes in an anoxic water column of the restored mining pit of Lake Rassnitzer in Germany and estimated that Anammox activity was responsible for $3.2 \text{ mgN}_2\text{-N} (10^{12} \text{ cells})^{-1} \cdot \text{d}^{-1}$ produced. This result was calculated using the absolute Anammox bacterial cell population in their sample. Kartal *et al.*, (2007) estimated Anammox turnover activity of $8.4 \text{ mg N} (10^{11} \text{ cells})^{-1} \cdot \text{d}^{-1}$ and Noophan, *et al.*, (2009) in their work, they calculated the SAA of Anammox activities of $1.9 \text{ mg-N} ((10^9 \text{ cells})^{-1} \cdot \text{d}^{-1})$. From these reviews, the results of the investigations of Galán *et al.* (2009), and Kartal *et al.* (2007) which resulted from environmental samples were quite close to the results obtained in this work.

5.6 Sensitivity of Anammox activities to oxygen

Anammox bacteria are obligate anaerobes (Kawagoshi *et al.*, 2010); therefore, require little or no oxygen for their metabolic activities. The results of the influence of DO on Anammox activities were presented on a batch by a batch basis in the batch experiments demonstrated in terms of over rate of ammonium and $^{29}N_2$ produced as shown in the result section for the approaches described in this work.

By numerical observation, approach one which monitors the influence of oxygen on the Anammox process done through biochemical degradation of NH_4 substrate in the Anammox medium. Monitoring parameters such as a change in TNb and C/N ratio in the Anammox medium sometimes might not be sufficient to explain the results obtained during the analyses for all microbial activities taking place in the biomass. Although ATU was used to inhibit activities AeAOB in the biomass, this may not guarantee 100% inhibition in the biomass. This approach can be considered as an approximation in terms of accuracy. In some other cases, deviations from the expected theoretical trend are observed due to certain responses of the biomass relative to the expected trend. But this approach can serve as a very good indicator and method for testing the accuracy of the assumption of this work and compare the activities of the biomass in the test vials as can be demonstrated in Approach 2. It was observed that if the biomass is stressed due to the influence of DO at certain high concentrations, the organisms might become

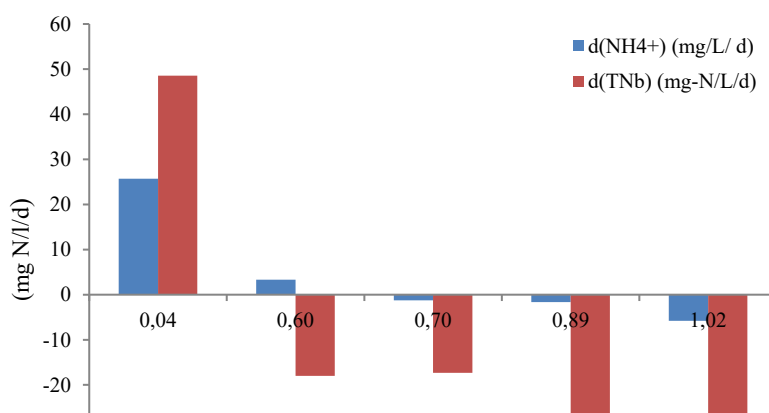


Figure 19: Turn-over rate of ammonium and TNb in experiment Batch 3. The negative rate bars indicated period of inactivity of inoculum leading to release of intracellular substrate at high DO concentrations.

temporarily inactive, release some intracellular pool of substrates in the cells and sometimes, some of the organisms in the biomass might die as the case may be.

These activities may influence the results presented if the organisms in the biomass were forced to release their pool of substrates stored intracellularly back to the solution of the medium, therefore, leads to an increase in concentrations of the substrates above initial concentrations which later become problematic in the calculation of the activities of the biomass as demonstrated in Figure.19.

In approach two which involves the tracer studies, ^{15}N labelled ammonium substrate was used to calculate the turnover rate of $^{29}\text{N}_2$ which directly isolate Anammox activities and therefore we can calculate the sensitivity of the Anammox process to different concentrations of DO in this approach. The method used was highly sensitive and the handling problem might have led to results, which were a little problematic to reconcile all the measurements in the different batch experiments. But the trend as observed in the charts depicts the influence of DO on the Anammox activities in all the batch experiments as shown in the results section. Although there is no linear relationship between the results of the batch experiments but when normalized with results from the control a clear trend is established. The general trend in the graphs indicated decreasing activities of Anammox bacteria as the concentration of DO increase in the vials as expected.

On a case by case basis, the trend of Anammox activities and the influence of different concentrations of DO used on test vials in the Batch experiments, Anammox activities were found in all the test vials in batch experiments as the oxygen concentration increases particularly in Batch 1 and 2 experiments. Thus, coming to a definite conclusion of what concentration of the oxygen Anammox activity was completely inhibited became difficult. In the first two batch experiments, the trend of influence of oxygen in the Anammox process was systematically examined Batch experiments 1 and 2 did not show complete inhibition of Anammox activities. But for the results obtained in Batch 3 which deals with higher concentrations of oxygen, revealed that at oxygen concentrations $> 0.6 \text{ mg-O}_2 \text{ l}^{-1}$, ammonium degradation was no longer visible as depicted in Figure 9, based on ammonium degraded and $^{29}\text{N}_2$ produced in the activity-inhibitor bar graph.

Kalvelage *et al.* (2011) reported on their investigation of the influence of oxygen on the activities of the Anammox process at the OMZ of Namibia and Peru. They found that dissolved oxygen is a significant factor that could affect the activities of Anammox bacteria in the OMZ. In their work, they demonstrated and found the upper limit of DO concentration above which Anammox activities cease was approximately $20 \mu\text{M O}_2$ ($\sim 0.64 \text{ mgO}_2/\text{l}$). On the evaluation of results of Anammox bacteria sensitivity to oxygen obtained in this work, our observations lie between the findings of Jensen *et al.* (2008) and Kalvelage *et al.*, (2011) based on the results of both approaches demonstrated in this work. Other literature has reported different results based on their findings of the response of Anammox organisms to different DO concentrations. Table 6 summarized some findings of inhibition concentrations of oxygen to Anammox activities. The results found are subject to different research assumptions and the degree of enrichment of Anammox organisms in the biomass used were not considered as well.

Table 6: Some inhibition DO concentration found in some literature

DO Concentration [mg O ₂ /l]	Source or ecosystem of Anammox	Literature
<0.04	Laboratory culture (man-made)	Strous <i>et al.</i> , (1997)
~0.43	Marine (natural)	Jensen <i>et al.</i> , (2008)
0.19-0.42	WWTP (man-made)	Szatkowska, <i>et al.</i> , (2003) *
0.64	Marine (natural)	Kalvelage, <i>et al.</i> , (2011)
0.4-0.64	Laboratory culture (man-made)	Current work

(*) Values taken and converted modified to the defined units from literature sources

These facts make the comparison of the results difficult. Tao and Gao, (2012) reported on their work the influence of oxygen on Anammox biomass containing AeAOB and NOB. They found oxygen to have no inhibitory effect on the Anammox organisms. This is because the nitrifying bacteria can metabolize oxygen and as such can offer a shield on the activities of Anammox organisms. More so, even when nitrifying activities in the biomass are inhibited, if the degree of inhibition of nitrifying organisms in the biomass is not complete, then nitrifying activities are still possible. This situation might influence the quantity of oxygen used in the experiments, as part of the oxygen might be metabolized leading to variable conclusions from different investigators. In this sense, reports on the sensitivity of Anammox bacteria to oxygen can be treated on a case by case basis. The amount of oxygen, which inhibit Anammox activities might be dependent on the source and degree of enrichment of the Anammox organisms in the microbial community. The results obtained in this work clearly showed an increase in oxygen concentrations will lead to the loss in Anammox activity.

5.7 Kinetics of Inhibition

The binding affinity constant K_i and the half-maximal inhibition concentrations IC_{50} of oxygen on the biomass were estimated from the results of inhibition kinetics in the batch experiments as shown in Table 2. The results obtained gave a low K_i value which implies that oxygen has a strong affinity for the enzyme regulatory site controlling Anammox activities in the biomass. The interaction of the oxygen with the Anammox enzyme is easily reversible as found in the recovery test. This observation was also reported in Jetten, (2001). Because the biomass was not pure, thus there was some variation in the K_i measured in the batch experiments. But generally, the low value shows that oxygen is a good inhibitor of Anammox activity. The IC_{50} is not a thermodynamic parameter like the Michaelis constant K_m or K_i which are constants. It depends on parameters like K_i , K_M , the substrate concentration [S] and the concentrations of the target organism in the biomass. In the Batch experiments, we used different dry mass concentrations of the biomass in the experiments. Batch 2 experiments; have almost twice the dry mass concentration of the biomass relative to batch 1. The IC_{50} value computed in the Batch 2 experiment may not have conformed to the theoretically expected. This may be as a result of using the mixed biomass. But in general, IC_{50} is a variable and responds to changing parameters as already mentioned. Considering the findings in the combined batch experiments for the sensitivity of Anammox bacteria to oxygen, doubling the concentration of IC_{50} , we approximately get the observed amount of

oxygen that inhibited the biomass used in approach one and this amount is sufficient to cause 100% inhibition in the batch experiments following the Approach 2.

5.8 Comparison of the methodological approaches

Two methodological approaches were described in this work to investigate the sensitivity of Anammox organisms to oxygen. On the one hand, Approach one helped in the biochemical characterization of some of the microbial activities taking place in the biomass. More so we observed in this approach, constant decrease in oxygen concentration in the test vial. The decrease in oxygen concentration can be linked to the metabolization of the oxygen by nitrifying bacteria present in the biomass, particularly the NOB or partially uninhibited AeAOB in the biomass. Also, we observed that oxygen treatment often leads to the loss of activity of Anammox organisms. Therefore, the continual treatment of the same biomass with different concentrations of oxygen might be an efficient way of investigating the influence of oxygen on the activities of Anammox organisms since the previous oxygen treatment might lead to some degree of loss of activity by the organisms.

Secondly, this approach also uses degradation of NH_4^+ to monitor and isolate the activities of Anammox activities in the biomass. As already mentioned, the case of partial inhibition of the AeAOB in the biomass might introduce some errors in the analysis of the result. The test for the accuracy of this approach was entirely checked by the measurement of TNb in the medium. But this check did not take into consideration other microbial activities that can influence the total inorganic-N in the medium like denitrifying activities that can also contribute to loss of inorganic nitrogen to get full reliable results for comparison.

On the other hand, approach two try to isolate the activities of Anammox bacteria through trace studies. With this approach, the influence of activities from other microbial groups can easily be isolated. It is worthy of note that since Anammox bacteria were usually found in mixed culture, the use of appropriate inhibiting compounds for the other microbial groups, which utilizes similar substrates as the Anammox organisms is recommended to stop their activities. Another option will be performing the experiments at Anammox optimum conditions as this can help suppress the activities of the other microbial groups in the biomass. Another important advantage of this approach was based on the fact that $^{29}\text{N}_2$ produced can be used as an estimate of Anammox activity separate from other microbial activity taking place in the biomass. The release of intracellular substrates from the biomass into the suspension solution does not influence the results of the measurement and hence the activity of the Anammox can be calculated.

5.9 Ecological implication of influence of dissolved oxygen in Anammox process

Oxygen plays important role in the way the marine ecosystem is structured defined by different strata, based on oxygen distribution in these layers (Gilly, et al., 2013). It controls the distribution of organisms starting from the microbes to very large organisms in this environment alongside other parameters such as temperature, light, salinity and dissolved nutrients. Anammox bacteria are widely found in different

natural ecosystems with very low oxygen conditions and contribute to the loss of nitrogen in these environments.

Hu *et al.*, (2011) and Tao and Gao, (2012) studied some environmental factors that could influence the Anammox process in different natural environments and how these factors contribute to nitrogen loss in the environments. Dissolve oxygen is one of such environmental factors that influence nitrogen loss via the Anammox process. Some investigations have suggested that Anammox organisms are always present in natural environments containing fixed nitrogen (Kartal *et al.*, 2013), leading to estimated nitrogen and the Anammox process account for different environments. This summarizes the importance of the Anammox process in the biogeochemical N-cycle according to Hu *et al.*, (2011).

This influence of loss of fixed nitrogen affects primary production and can lead to global warming. (Kartal, *et al.*, 2013). The direct consequence of low fixed nitrogen in the marine environment is a low primary production which also contributes to the influx of atmospheric CO₂ in the ocean since CO₂ is fixed to the cell biomass during the anabolic process of primary production. This process is one of the many mechanisms through which the ocean uses to act as a sink for atmospheric CO₂. The increasing human influence on the nitrogen content of the marine environment and Anammox activities in this environment can lead to an offset in the biogeochemical cycle. If these analyses and results are true, this could be linked to the Anammox process as one of such phenomena that affect global climate change. This phenomenon can also lead to loss of biodiversity in the marine environment if natural balance is not restored.

However, from the ecological viewpoint, DO have been seen as a very important factor influencing most microbial related activities in different elemental biogeochemical transformation. Stratification of DO in the marine environment as already defined by Murray, *et al.*, (2005), different activities of microbial organisms are determined by the concentration of oxygen present in the different strata in the environment. With support from Kalvelage, *et al.*, (2011) and Galán *et al.*, (2009) in their work, Anammox activities are found most in the suboxic strata in the ocean where an appreciable quantity of Anammox substrates is found due to the influence of activities of AeAOB and microbial nitrogen-fixing organisms in this region. Hence our results of the sensitivity of Anammox bacteria to oxygen, when applied to the ecosystem, Anammox activities have been reported in many OMZ in the marine environment. The region in the marine ecosystem has been defined based on DO concentration. Gilly, *et al.*, (2013) reported oxygen concentrations in the different marine OMZ in the range > 20-45 $\mu\text{M O}_2$ (i.e. 0.5 ml O₂ /l or 0.7 mg /l). In this work, we observed that Anammox activities are inhibited above 0.4 - 0.6 mg O₂ /l. These concentrations coincide with the oxygen concentrations found in different OMZ where Anammox activities have been reported. This result might be useful in determining possible environments containing fixed nitrogen, where Anammox activities could be found in different natural ecosystems.

Chapter 6

6 Conclusion

We investigated and determined specific metabolic activities of Anammox bacteria and their sensitivity to oxygen in a biomass culture. Two laboratory-scale Bioreactors were maintained and characterized as a source of Anammox biomass used in this investigation for two species of Anammox bacteria; *Ca. Brocadia Fulgida* and *Ca. Kuenenia Stuttgariensis* respectively. The results of the effluent recirculation analyses from the Bioreactors showed almost the same nitrogen substrate removal capacity and the Anammox process was the predominant microbial activity taking place. Although several other microbial processes were observed in the Bioreactors. The FISH characterization of the biomass revealed that the biomass in Bioreactor 1 contained a mix of two Anammox species: *Ca. Brocadia Fulgida* and *Ca. Kuenenia Stuttgariensis*; with the latter constituting > 78 % of the Anammox population.

Two methodological approaches were adopted and the outcome showed that results of **approach 2** were considered more reliable since the approach traces activities of only the Anammox organisms in the biomass and also try to explore other possible microbial activities taking place during the investigations. Combined microbiological-biochemical analyses of the biomass suggested that the predominant microbial activity in the biomass was dominated by Anammox analysis; with partial denitrification, DRNA and nitrification processes taking place in the biomass.

Batch experiments were used to calculate the SAA and investigate the influence of oxygen on the Anammox culture. The results of SAA in the biomass supported some results of the specific turnover rate of Anammox bacteria found in some literature and the discrepancies in the contemporary results was due to differences in study working approaches and difference in the degree of enrichment of Anammox organisms in the biomass by different investigators. Further inhibition kinetic calculations of the experimental results showed that oxygen is a good Anammox inhibitor based on the low K_i values. The results also showed that the mechanism of inhibition was non-competitive and is reversible

The sensitivity of Anammox organisms to oxygen showed that increasing concentrations of oxygen lead to the loss in Anammox activity in the biomass. At oxygen concentrations > 0.4-0.64 mgO₂/l the biomass was completely inhibited but recovers after purging with inert gases. Incorporating these results into ecological studies, establishing inhibition concentration of oxygen can be used for determining the spatial distribution of Anammox organisms in ecosystems where fixed nitrogen is present.

6.1 Limitations and some recommendations for future studies

There are hardly any scientific works free from limitations. In this work, we encountered some irregularities that needed to be considered and improved upon in further investigations related to this work. In this subsection, important limitations in this work will be highlighted and some suggestions and recommendations proffered going forward.

The batch experiments were carried under different experimental conditions ranging from varying biomass concentrations, DO concentrations and sometimes operational conditions like prevailing room temperature; all these parameters can influence the activities of the Anammox organisms. One of the major objectives of this work was to develop a methodological approach to investigate the influence of DO on the Anammox process. To reproduce and compare some of the results presented in this work, it is recommended that the batch experiments should be carried out in replicates under the same experimental conditions and handlings. This will make the results more reliable and easier to normalize.

Since this investigation is based on a mixed biomass culture, but the Anammox process is being investigated, it is important to completely isolate other microbial activities in the biomass. This will entail the prior complete characterization of the microbial constituents in the biomass and the use of appropriate inhibitor(s) to eliminate the influence of the unwanted organisms, which might affect the results investigated. Having just Anammox activities taking place in the biomass, the process can be easily monitored using the change in pressure in the system to be linked to Anammox activities as a result of N₂ gas produced as described in Dapena-Mora *et al.* (2007) and some other literature.

The results of turnover activities of Anammox organisms in the biomass can be done after eliminating other microbial activities in the biomass through the use of specific antibiotic inhibitors which does not affect the activity of Anammox bacteria. During the microscopic examination, characterization and analysis of the biomass to determine the degree of enrichment and the concentration of the Anammox organisms, one of the major challenges to overcome is the visualization and the counting of the Anammox cells which are usually found in clusters and this cluster formation is an inherent quality of Anammox bacteria which reflects their growth characteristics. The use of a membrane reactor for Anammox bacteria cultivation was suggested by Kartal *et al.* (2013).

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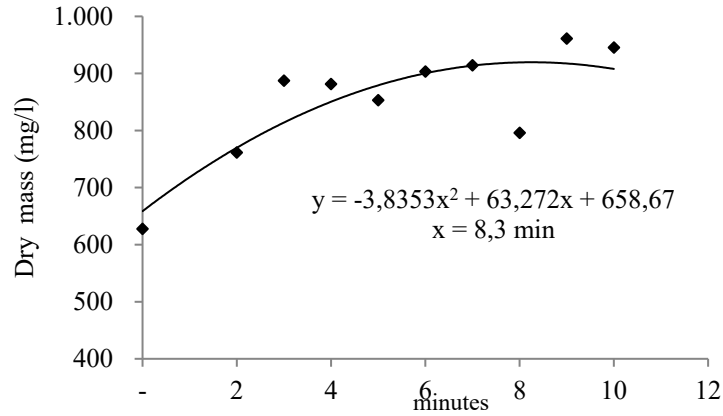
Appendices

Appendix 1: Determination of sonication time for dry mass measurements

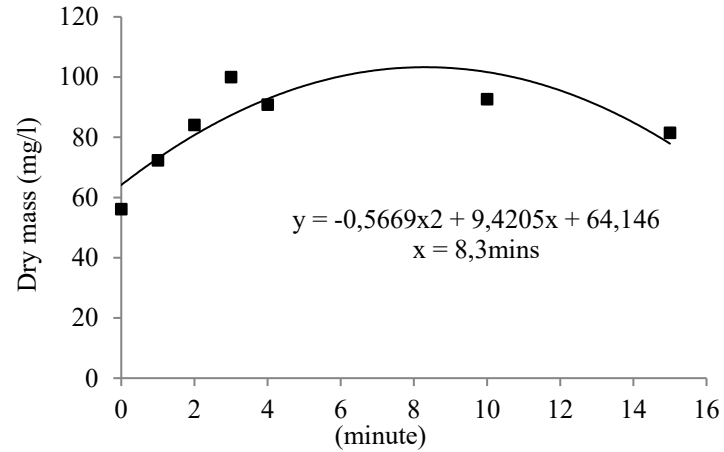
Date	Time (Sec)	Time (min)	Dry mass	%
17.11.2012	0,00	0,0	627,50	79
	30,00	0,5		
	60,00	1,0		
	120,00	2,0	761,4	90
	180,00	3,0	887,2	86
	240,00	4,0	881,4	88
	300,00	5,0	853,1	92
	360,00	6,0	903,4	95
	420,00	7,0	914,1	97
	480,00	8,0	796,0	91
	540,00	9,0	961,1	96
600,00	10,0	945,4	100	
09.01.2013	0.00		479,7	56,1
	30.00	0,5		
	60.00	1,0	618,3	72,3
	120.00	2,0	719,1	84,1
	180.00	3,0	855,0	100
	240.00	4,0	776,7	90,8
	600.00	10,0	792,0	92,6
	900.00	15,0	697,0	81,5
10.01.2013	0.00		224,2	61,1
	30.00		321,0	87,5
	60.00		366,8	100
	120.00		340,0	92,7
	180.00		344,0	93,8
	240.00		333,6	91,0

Table 7: Determination of Batch experiment

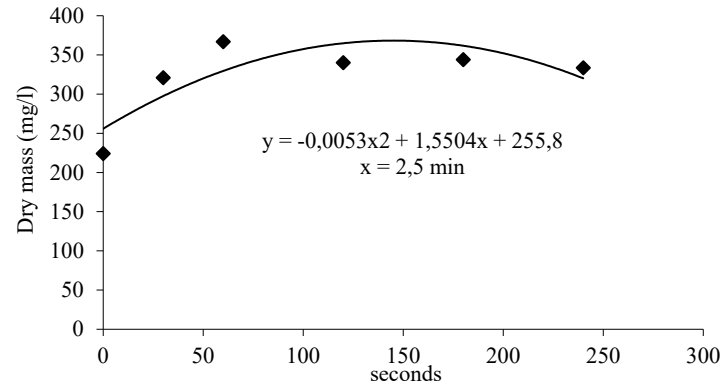
Experiment 1 Date: 7.11.12



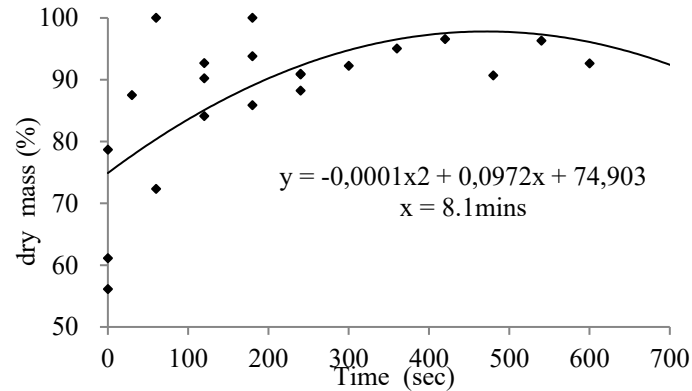
Experiment 2 Date: 9.01.13



Experiment 3 Date: 10.1.13



Combined results of the experiments



Appendix 2: RECIPE for reagents used in Fluorescent In situ Hybridization (FISH) analysis*Table 8-A: 4% Paraformaldehyde (PFA)*

PFA	0.8 g	1.0 g	2.0 g	4.0 g
Total Volume in 1xPBS (mL)	20.0	25.0	50.0	100.0

Table 9-A: 3x Phosphonate Buffered Saline (PBS)

NaCl (195 mM)	2.28 g	5.70 g	11.4 g	22.8 g
Na ₂ HPO ₄ (10.5 mM)	0.3 g	0.75 g	1.5 g	3.0 g
NaH ₂ PO ₄ x H ₂ O (4.3 mM)	0.12 g	0.30 g	0.6 g	1.2 g
Total Volume (ml)	100.0	250.0	500.0	1000.0
Adjust pH to 7.2				
Sterile filtration 0.2 µm syringe filter				

Table 10-A: Dehydrating ethanol solutions

Percentage/ Prozent	50%	80%	100%
Absolute Ethanol (ml)	25.0	40.0	50.0
Total Volume (ml)	50.0	50.0	50.0

Table 11-A: NaCl Solution

NaCl (5 M)	14.61 g	29.22	73.05
Total Volume (ml)	50.0	100.0	250.0

Table 12-A: 10% SDS (Sodium dodecyl sulfate)

SDS (v/v)	5.0 g	10.0 g	25.0 g
Total volume (ml)	50.0	100.0	250.0

Table 13-A: Tris/HCl pH 7.2

Tris (1 M)	6.05 g	12.12 g	30.29 g	60.58 g
Total volume (mL)	50.0	100.0	250.0	500.0
Adjust pH to 7.4 with HCl (25%)				
All solutions must be sterile filtered through 0.2 µm!				

Table 14-A: Preparation of lyophilized oligonucleotides

Probe buffer pH 7.4			
Tris/HCl (10 mM)	30.29 mg	60.57 mg	121.14 mg
EDTA (1 mM)	7.31 mg	14.61 mg	29.22 mg
Total volume (mL)	25.0	50.0	100.0

Adjust pH to 7.4 and sterile filtration through 0.2 µm syringe filter! Store at 4 °C.

Table 15-A: Wash solution

Strigency %	0	5	10	15	20	25	30	35	40	45	50
5M NaCl (µl)	9000	6300	4500	3180	2150	1490	1020	700	460	300	180
1 M Tris/HCl pH7.4 (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Deionized water (ml)	39.9	42.7	44.5	45.8	46.8	47.5	47.9	48.3	48.5	48.7	48.8
10% SDS (µl)	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Total volume (ml)	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0

Add NaCl, Tris/HCl in a 50 ml tube and fill with MilliQ to 50 ml. Mix gentle and finally add SDS

Recipe for the synthetic Anammox medium

Table 16-A: Mineral composition of Anammox feed. Source: (Krause, 2011)

Components	Chemical Formula	Concentration [mg/l]	Concentration [mmol/l]
Ferrous sulphate heptahydrate	FeSO ₄ ·7H ₂ O	12	0.08
Potassium dihydrogen phosphate	KH ₂ PO ₄	14	0.10
Potassium hydrogen carbonate	KHCO ₃	2500	24.97
Calcium chloride dihydrate	CaCl ₂ ·2H ₂ O	300	2.04
Magnesium chloride hexahydrate	MgCl ₂ ·6H ₂ O	114	0.68

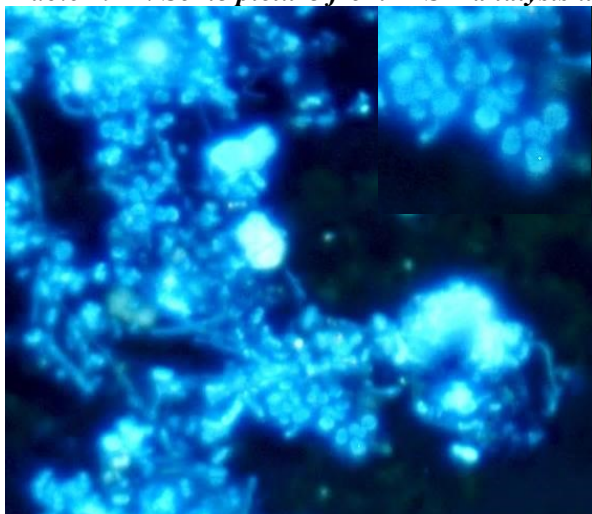
Table 17-A: Composition of trace element solution Source: (Krause, 2011)

Composition	Concentration [mg/l]
EDTA	21 825
ZnSO ₄ ·7H ₂ O	626
CaCl ₂ ·6H ₂ O	349
MnCl ₂ ·4H ₂ O	1183
CuSO ₄ ·5H ₂ O	364
NaMoO ₄ ·2H ₂ O	320
NiCl ₂ ·6H ₂ O	276
NaSeO ₄ ·10H ₂ O	153
FeSO ₄ ·7H ₂ O	14
H ₃ BO ₄	102

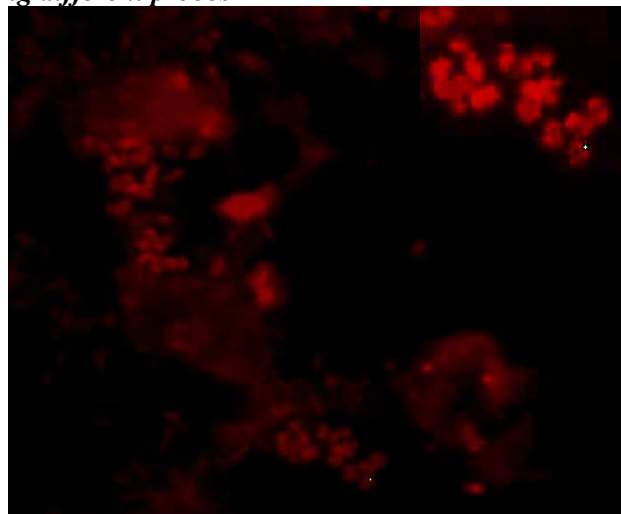
Appendix 3: Results of FISH analysisTable 18-A: **Properties of oligonucleotides probes used in the FISH analysis**

Oligonucleotide probe	Target	Florphor	Sequence	Position	Formamide (%)	Length	Anammox taxonomy / species
Ban162	16S rRNA	Texas Red	5'- CGG TAG CCC CAA TTG CTT -3'	162 - 179	40	18	Candidatus Brocadia Fulgidas
KST157	16S rRNA	Texas Red	5'- GTT CCG ATT GCT CGA AAC -3'	157 - 174	25	18	Candidatus Kuenenia stuttgartiensis
PLA46	16S rRNA	Texas Red	5'- GAC TTG CAT GCC TAA TCC -3'	46 - 63	30	18	Planctomycetales
EUB 338(I)	16S rRNA	Cy3	5'-GCT GCC TCC CGT SGG AGT -3'	338 - 355	20	18	Most bacteria

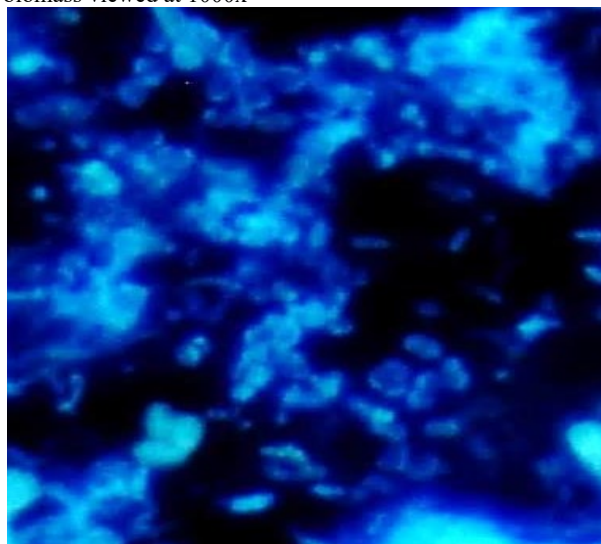
Table 19-A: Some picture from FISH analysis using different probes



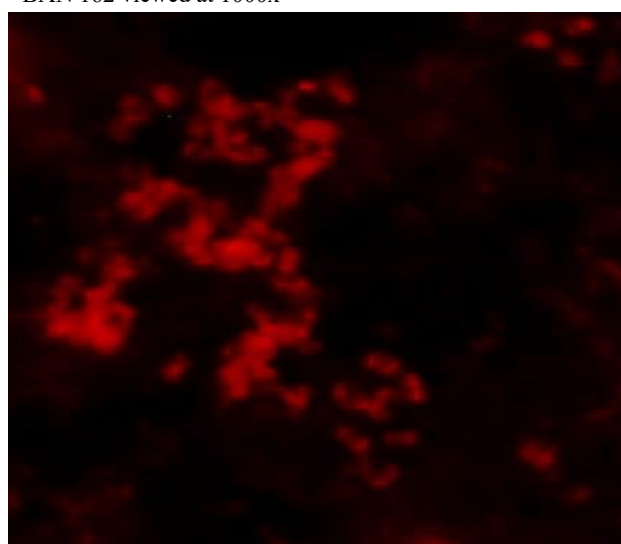
Picture of hybridized biomass counter-stained with DAPI biomass viewed at 1000x



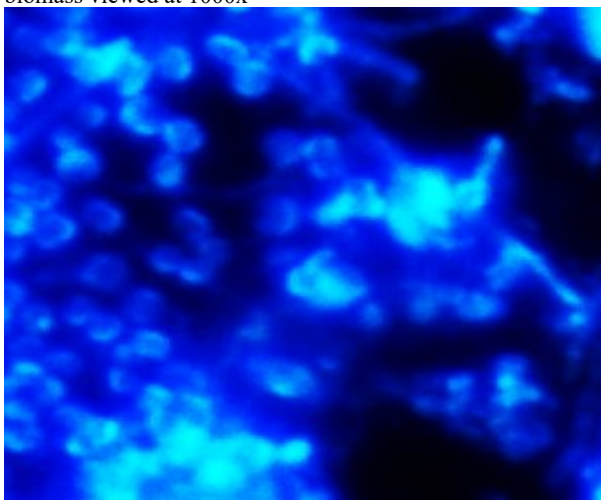
Picture of hybridized biomass stained with *Brocadia* probe BAN 162 viewed at 1000x



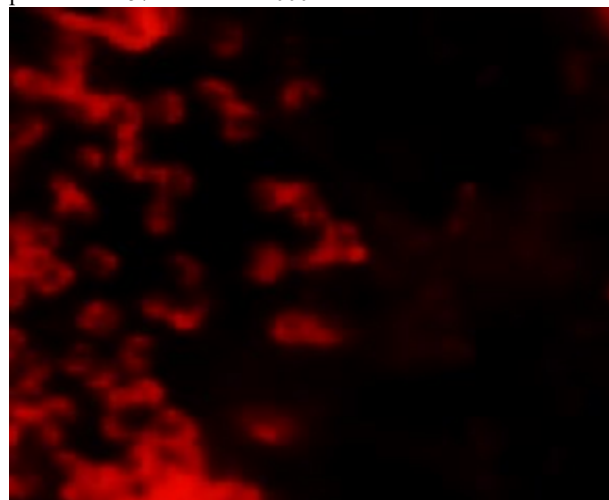
Picture of hybridized biomass counter-stained with DAPI biomass viewed at 1000x



Picture of hybridized biomass stained with *Kuenenia* specific probe KST157 viewed at 1000x



Picture of hybridized biomass counter-stained with DAPI biomass viewed at 1000x



Picture of hybridized biomass stained Planctomycete specific probe PLA 46 viewed at 1000x

Appendix 4: Biochemical characterization of biomass

Table 20: Results of test for microbial activities in the bioreactor 1
Source of biomass: Bioreactor 1

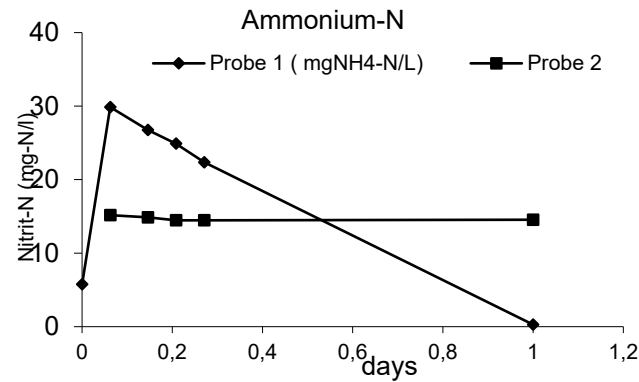
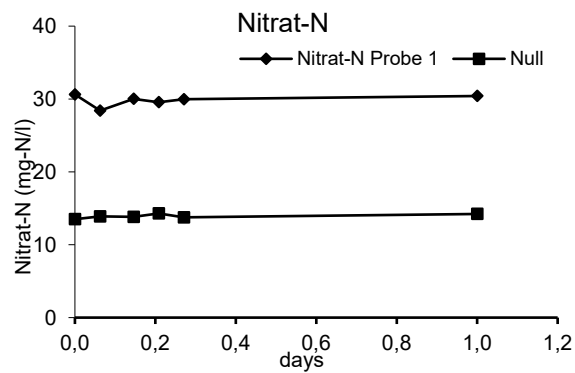
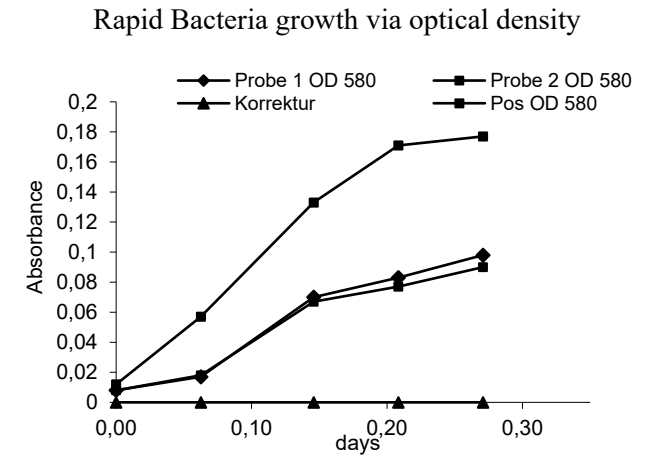
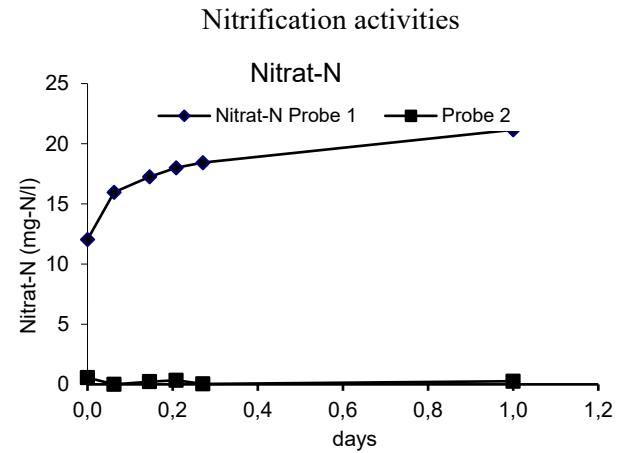
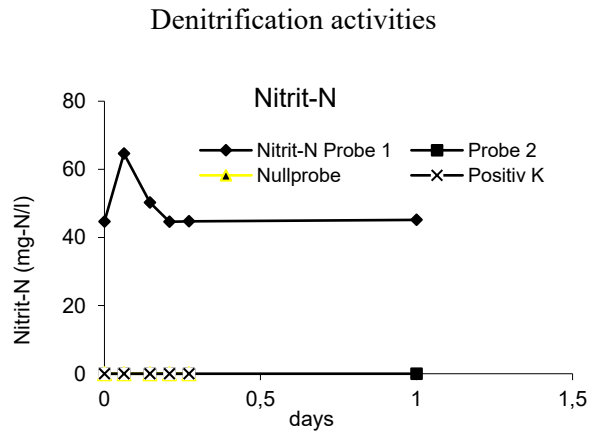
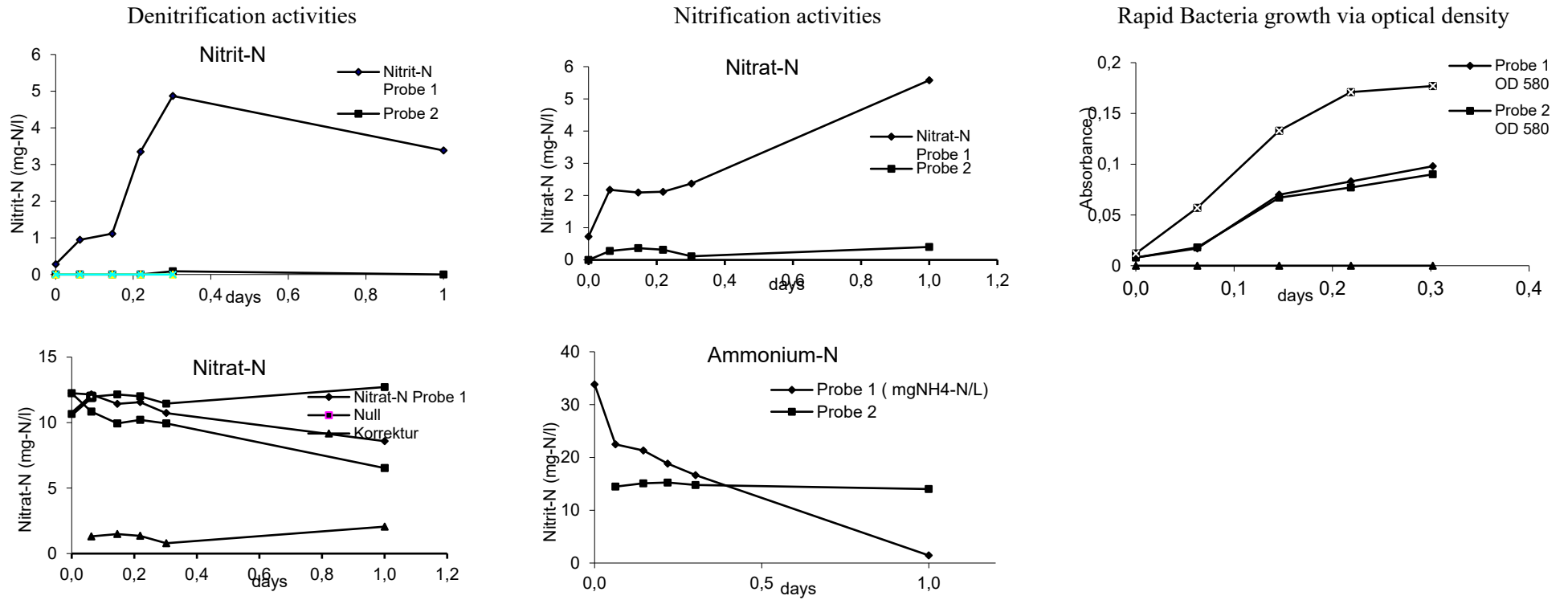


Table 21: Results for test for microbial activities in biomass from terminated biomass

Source of biomass: Terminated experiment from test vial in Batch 1



Appendix 5: Approach 1

Set-up without oxygen treatments + ATU

Biomass: Brocadia Fulgida

Date: 6.03.13

Volume of sample: 25 ml,

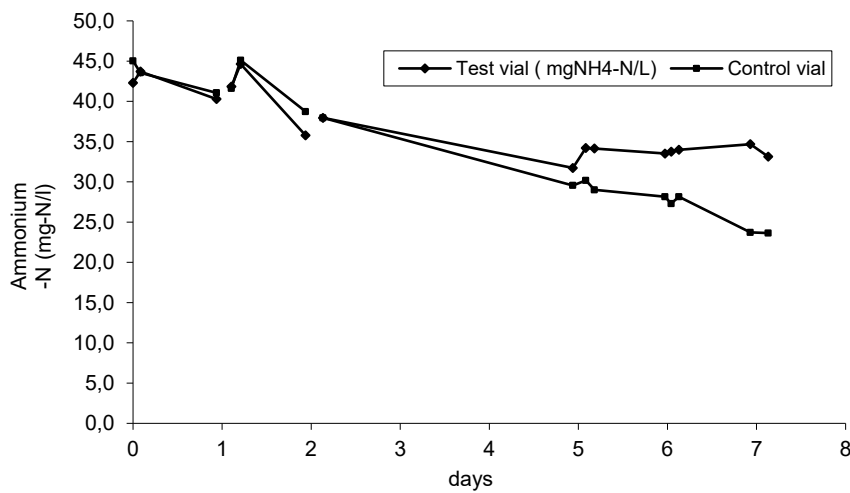
NH₄ -N: 48 mg-N /l

Volume of ATU: 25µl

NO₂ -N: 48 mg-N /l

Experiment 1

Time (Days)	Air (ml)	O ₂ (mg /l)	Test vial (mg-NH ₄ /l)	Control vial (mg-NH ₄ /l)
0,0	0.0	0.0	42,3	45,0
0,1	0.5	0,2	43,7	43,6
0,9	0.5	0,2	40,3	41,1
1,1	0.0	0,00	41,8	41,6
1,2	0.0	0,00	44,6	45,1
1,9	0.4	0,13	35,8	38,7
2,0	0.4	0,13		
2,1	0.4	0,13	38,0	38,0
4,9	0.4	0,13	31,7	29,6
5,1	0.4	0,13	34,2	30,2
5,2	0.0	0.0	34,1	29,0
6,0	0.5	0,2	33,5	28,2
6,0	0.5	0,2	33,8	27,3
6,1	0.5	0,2	34,0	28,2
6,9	0.0	0,00	34,7	23,7
7,1	0.0	0,00	33,1	23,6



Set-up + oxygen + without ATU

Biomass: Brocadia Fulgida

Date: 12.03.13

Volume of sample: 28 mL,

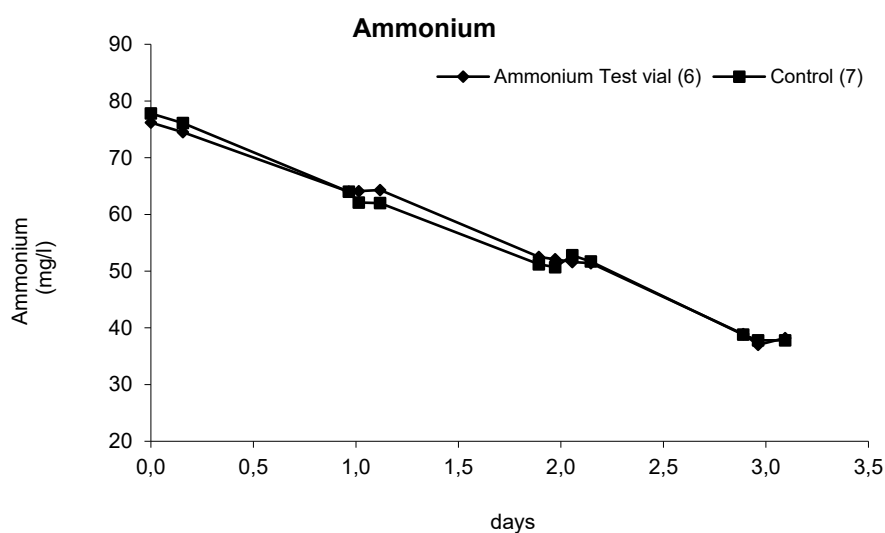
NH₄ -N: 80 mg-N /l

Volume of ATU: -

NO₂ -N: 80 mg-N /l

Experiment 2

Time (Days)	Air (ml)	O ₂ (mg/l)	Test vial (mgNH ₄ -N/l)	Control vial (mgNH ₄ -N/l)
0,0	0.0	0	76,20	77,80
0,16	0.0	0	74,50	76,10
0,97	0.5	0.13	64,00	64,00
1,01	0.5	0.13	64,10	62,10
1,12	0.5	0.13	64,30	62,00
1,89	0.0	0.00	52,50	51,20
1,97	0.0	0.00	52,1	50,70
2,06	0.0	0.00	51,60	52,80
2,15	0.0	0.00	51,40	51,7
2,89	0.0	0.00	38,90	38,80
2,96	0.0	0.00	37,00	37,80
3,09	0.0	0.00	38,20	37,80



Set-up + oxygen + ATU

Biomass: Brocadia Fulgida

Date: 13.03.13

Volume of sample: 28 mL,

NH₄ –N: 77.6 mg-N /l

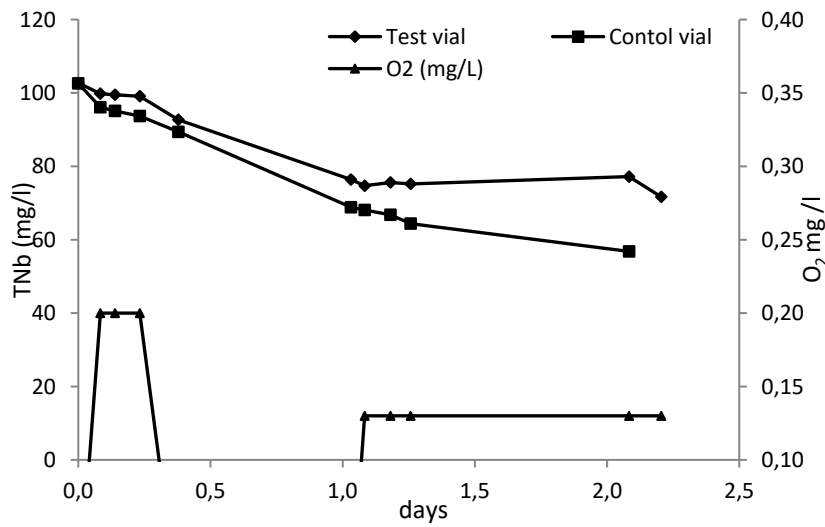
Volume of ATU used: -

NO₂ –N: 62.7 mg-N /l

TOC: 41.1 mg-C/l

Experiment 3

Time (Days)	Air (ml)	O ₂ (mg/l)	NH ₄ (mg-N/l)	TNb (mg-N/l)	NH ₄ -K (mg-N/l)	TNb (mg-N/l)
0,0	0,0	0,0		102,7		102,6
0,08	0,5	0,2	64,0	99,8	64	96,1
0,14	0,5	0,2	64,1	99,5	62,1	95,1
0,23	0,5	0,2	64,3	99,1	62	93,7
0,38	0,0	0,00		92,7		89,4
1,03	0,0	0,00	52,5	76,4	51,2	68,9
1,08	0,4	0,13	52,1	74,7	52,7	68,1
1,18	0,4	0,13	51,6	75,6	52,8	66,8
1,26	0,4	0,13	51,4	75,2	51,2	64,4
2,08	0,4	0,13	37,0	77,2	38,8	56,8
2,20	0,4	0,13	38,2	71,7	37,8	63,7



Set-up + oxygen + ATU

Biomass: Brocadia Fulgida

Date: 18.03.13

Volume of sample: 30 mL,

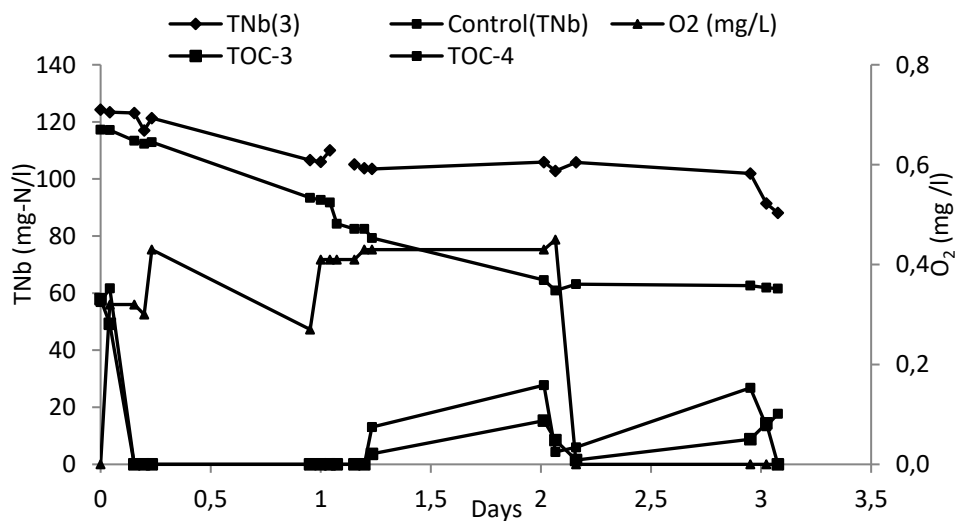
NH4 -N: 80 mg-N /l

Amount of ATU: 25µL

NO2 -N: 80 mg-N /l

Experiment 4

Time (d)	Test vial (T)					Control vial (K)		
	air (mL)	O ₂ (mg/l)	NH ₄ (mg -N/l)	TNb (mgN/l)	TOC (mg-C/l)	NH ₄ (mg -N/l)	TNb (mgN/l)	TOC (mg-C/l)
0,00	0	0.0	83,9	124,3	41,1	82,4	117,2	38,1
0,04	0.7	0,3	83,3	123,4	34,7	83,0	117,1	41,3
0,15	0.7	0,3	83,9	123,1	0,0	83,5	113,4	0,0
0,20		0,3		117,0	0,0		112,3	0,0
0,23	1	0,4	82,7	121,3	0,0	80,5	112,9	0,0
0,95	1	0,27	68	106,6	0,0	65,3	93,4	0,0
1,00	1	0,41	71,2	106	0,0	69,3	92,6	0,0
1,04	1	0,41	66,4	110	0,0	63,3	91,8	0,0
1,07	1	0,41	70,1		0,0	62,3	84,3	0,0
1,15	1	0,41	71,6	105,1	0,0	62,9	82,5	0,0
1,20	1+0.6	0,43	71	103,7	0,0	61,0	82,5	0,0
1,23	1+0.6	0,43		103,5	2,2		79,3	5,9
2,01	1+0.6	0,4	70,1	105,9	51,3	50,1	64,6	10,2
2,07	1+0.6	0,5	70,8	102,8	5,0	49,4	60,9	1,5
2,16	0	0,0	70,9	105,8	0,9	48,8	63,2	2,2
2,95	0	0,0	64,6	101,9	5,1	37,5	62,6	9,6
3,02	0	0,0	65	91,4	7,5	38,6	61,9	4,7
3,08	0	0,0		88,1	0,0		61,6	6,2
5,95	0	0,0		56,88			32,49	



Appendix 6: Approach 2 (¹⁵N- label experiments)**Batch 1 experiment**

BATCH 1 Experiments					
Anammox species	Brocadia Fulgida				
Date	26.03.13				
Substrate Conc.	¹⁴ NO ₂ -N			Volume	10 mL
	¹⁴ NH ₄	40.1 mg/l		TNb	82.2
After 1day of O ₂ treatment	¹⁵ NH ₄ -N	2.8 mg/l			
27.3.13 10:00	Vial 1	Vial 2	Vial 3	Vial 5	Vial 4
ATU	5µl	5µl	5µl	5µl	5µl
Vol of O ₂ (µL)	0	50	75	100	125
O ₂ (mg /l)	0.08	0.11	0.24	0.40	0.63
TNb (mg-N/l/d)	71.0	70.7	74.0	77.3	76.8
NH ₄ ⁺ (mg/l/d)	35.9	35.9	37.4	38.9	38.6
TOC (mg-N/l)	0.0	0.0	0.0	0.0	0.0
N29 (integral values)	176318.0	273916.0	138729.0	126912.0	165397.0
28.3.13 9:00	Vials purged with air/ CO ₂				
O ₂ (mg /l)	0.00	0.00	0.00	0.00	0.00
TNb (mg-N/l/d)	58.6	54.1	58.3	59.9	62.4
NH ₄ ⁺ (mg/l/d)	26.3	23.7	26.6	26.1	26.8
TOC (mg-N/l/d)	5.4	13.5	0.0	12.2	0.6
²⁹ N production (integral values)	244184.0	142537.0	127489.0	126881.0	264045.0
p ^H	7.3	7.3	7.3	7.3	7.3

Results of Activity calculations

Influence of DO anammox activity					
	Vial 1	Vial 2	Vial 3	Vial 5	Vial 4
O ₂ (mg /l)	0.08	0.11	0.24	0.4	0.63
TNb (mg-N/l/d)	11.7	12.0	8.6	5.1	5.7
NH ₄ ⁺ (mg/l/d)	4.3	4.3	2.8	1.3	1.6
²⁹ N ₂ (mg/l/d)	10.4	16.9	8.0	9.7	7.2
test of accuracy of assumption	-0.2	-0.9	-0.4	-2.8	-1.2

Recovery of biomass after purging with Ar/CO₂

	Vial 1	Vial 2	Vial 3	Vial 5	Vial 4
O ₂ (mg /l)	0	0	0	0	0
TNb (mg-N/l)	12.9	17.3	16.4	18.2	15.0
NH ₄ ⁺ (mg/l)	10.1	12.7	11.3	13.4	12.3

Calculations of SAA in the biomass

	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5
Kuenedia					
	3.06E-11	2.72E-11	1.28E-11	1.57E-11	1.16E-11
N ₂	0.026	0.042	0.020	0.024	0.018
NH ₄	8.74E-12	2.90E-11	1.87E-11	8.43E-12	1.06E-11
	0.004	0.004	0.003	0.001	0.002
Brocadia					
	4.99E-12	8.07E-12	3.81E-12	4.65E-12	3.43E-12
N ₂	0.0023	0.0038	0.0018	0.0022	0.0016
NH ₄	2.60E-12	2.60E-12	1.68E-12	7.55E-13	9.48E-13
	0.00122	0.00122	0.00079	0.00036	0.00045

Calculation of SAA

Turnover N₂-N						
O ₂ (mg/l)		0.08	0.11	0.24	0.40	0.63
Turn over ²⁹ N ₂ (mg/l/d)		8.4	13.5	6.4	7.8	5.8
SAA (mgN ₂ -N(cell ⁻¹)d ⁻¹)		5.07E-11	8.20E-11	3.87E-11	4.72E-11	3.49E-11
(mg N(g DM) ⁻¹ d ⁻¹)		0.024	0.039	0.018	0.022	0.016
Turnover NH₄						
O ₂ (mg/l)		0.08	0.11	0.24	0.40	0.63
Rate NH ₄ deg (mg/l.d)		4.3	4.3	2.8	1.3	1.6
SAA (mg- N(cell ⁻¹)d ⁻¹)		2.64E-11	9.62E-12	6.21E-12	2.80E-12	3.51E-12
(mg N(g DM) ⁻¹ d ⁻¹)		0.012	0.012	0.008	0.004	0.005

Percentage Activity (IC₅₀ raw data)					
O₂ (mg/l)	0.08	0.11	0.24	0.40	0.63
Log O₂	-1.10	-0.96	-0.62	-0.40	-0.20
% N₂ produced	100	162	76	93	69
% NH₄ degraded	100	100	65	29	37

IC₅₀ calculation for Batch 1 experiment

Nonlinear Regression

Sonntag, August 11, 2013. 09:32:52

Data Source: Data 1 in standard curve 1_group

Equation: Standard Curves; Four Parameter Logistic Curve

$$f1 = \min + (\max - \min) / (1 + (x / IC50)^{-Hillslope})$$

$$f = \text{if}(x \leq 0; \text{if}(Hillslope > 0; \min; \max); f1)$$

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9969	0.9939	0.9755	5.797

	Coefficient	Std. Error	t	P
min	32.80	5.2708	6.2230	0.1014
Max	100.00	3.7333	26.7857	0.0238
IC50	0.2394	9619.0484	2.4887E-005	1.0000
Hillslope	-43.3563	679100725.1014	-6.3844E-008	1.0000

Analysis of Variance:

	DF	SS	MS
Regression	4	26316.2311	6579.0578
Residual	1	27.8756	27.8756
Total	5	26344.1067	5268.8213

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	3	4518.6276	1506.2092	54.0333	0.0996
Residual	1	27.8756	27.8756		
Total	4	4546.5031	1136.6258		

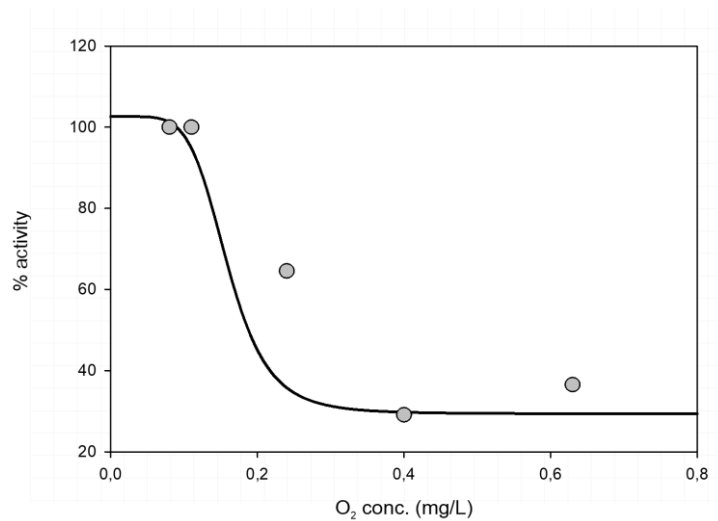


Figure 20-A: Curve of IC₅₀ estimation for Batch 1 experiment.

Batch 2 experimentRaw data from the ¹⁵N-label experiments

BATCH 2 Experiments					
Anammox species	Brocadia Fulgida				
Date	03.4.13 10:30 AM				
				OD dry mass	924.mg/l
				Gravimetric	1033.0
Substrate conc.	¹⁴ Nitrite-N	50.3mg/l		No cells /mL	4.35E+08
	¹⁴ NH4-N	49.2mg/l		volume	10 mL
	¹⁵ NH4-N				
	Vial K	Vial 2	Vial 3	Vial 4	Vial 5
ATU	5μL	5μL	5μL	5μL	5μL
Vol pure O ₂ (μL)	0	50	100	150	200
O ₂ (mg /l)	0.04	0.21	0.40	0.52	0.73
TNb (mg-N/l)	124.2				
NH ₄ ⁺ (mg/l)	49.2				
NO ₂ ⁻ (mg-N/l)	50.3				
TOC (mg-N/l)	25.2				
NO ₃ ⁻ (mg-N/l)	24.7				
3.4.13 17:00	vials purged with Ar/ CO ₂ +O ₂				
O ₂ (mg /l)	0.04	0.21	0.40	0.52	0.73
TNb (mg-N/l)	116.6	121.3	122.1	123.0	122.7
NH ₄ ⁺ (mg-N/l)	45.4	48.4	48.1	48.3	48.5
NO ₂ ⁻ (mg-N/l)	42.5	44.6	45.0	45.3	44.7
NO ₃ ⁻ (mg-N/l)	28.7	28.4	28.9	29.4	29.4
²⁹ N ₂ (mg/l.d)	103745.0	21882.0	22091.0	20659.0	24713.0
4.4.13 9:40	vials purged with Ar/ CO ₂				
O ₂ (mg /l)	0.00	0.00	0.00	0.00	0.00
TNb (mg-N/l)	91.2	98.7	98.4	99.2	99.4
NH ₄ ⁺ (mg-N/l)	36.6	39.5	39.9	40.4	40.9
NO ₂ ⁻ (mg-N/l)	36.2	38.6	38.1	38.8	37.9
TOC (mg-N/l)	25.7	26.7	24.5	29.5	33.2
NO ₃ ⁻ (mg-N/l)	18.5	20.5	20.5	20.0	20.6
²⁹ N ₂ (integral)	198125.0	205854.0	231594.0	218956.0	210922.0

Influence of O₂ on N-degradation

	Vial K	Vial 2	Vial 3	Vial 4	Vial 5
O ₂ (mg /l)	0.04	0.21	0.4	0.52	0.73
NH ₄ ⁺ (mg/l/ d)	14.1	3.2	4.0	3.4	2.6
N ₂ production (mg/l/d)	24.6	5.2	5.2	4.9	5.9
TNb (mg-N/l/d)	28.1	10.6	7.8	4.3	5.5
% accuracy of hypothesis	29.0	39.6	73.6	61.7	-23.0

Recovery / Reversibility test

	Vial K	Vial 2	Vial 3	Vial 4	Vial 5
O ₂ (mg /l)	0	0	0	0	0
d(TNb) (mg-N/l)	36.5	32.6	34.1	34.3	33.6
NH ₄ ⁺ (mg/l/ d)	12.8	12.8	11.9	11.4	11.0
N ₂ production (mg/l/d)	19.6	20.5	23.3	21.9	21.0
NO ₃ ⁻ (mg-N/l/d)	14.7	11.3	12.2	13.6	12.8
Excess d(TNb) (mg-N/d)	10.5	6.6	9.8	11.0	11.2
contribution of denit	28.4		28.5	31.7	33.1

Calculations of SAA (Turnover rate N₂)

	Vial K	Vial 2	Vial 3	Vial 4	Vial 5
O ₂ (mg/l)	0.04	0.21	0.4	0.52	0.73
Rate ²⁹ N ₂ production (mg/l.d)	24.6	5.2	5.2	4.9	5.9
SAA (mgN ₂ -N(cell ⁻¹)d ⁻¹)	2.00E-12	4.21E-13	4.25E-13	3.97E-13	4.75E-13
(mgN ₂ - N(g TSS) ⁻¹ d ⁻¹)	9.39E-04	1.98E-04	2.00E-04	1.87E-04	2.24E-04

Turnover rate NH₄

	Vial K	Vial 2	Vial 3	Vial 4	Vial 5
O ₂ (mg/l)	0.04	0.21	0.40	0.52	0.73
Rate NH ₄ deg (mg/l.d)	14.07	3.16	4.02	3.45	2.58
SAA (mg N (cell ⁻¹) d ⁻¹)	1.14E-12	2.56E-13	3.26E-13	2.79E-13	2.09E-13
(mg N (gTSS ⁻¹ d ⁻¹)	5.37E-04	1.20E-04	1.53E-04	1.31E-04	9.86E-05

Activity in terms v/v_o

	Vial K	Vial 2	Vial 3	Vial 4	Vial 5
O₂ (mg/l)	0.04	0.21	0.40	0.52	0.73
N₂	100	21	21	20	24
NH₄	100	22	29	24	18

	Vial K	Vial 2	Vial 3	Vial 4	Vial 5	
	O₂ (mg /l)	0.04	0.21	0.4	0.52	0.73
Date	Time (days)	NH₄ (mg-N/l)				
3.4.13 10:30	0	49.2	49.2	49.2	49.2	49.2
3.4.13 17:00	0.3	42.8	48.4	48.1	48.3	48.5
4.4.13 9:40	1.0	36.6	39.5	39.9	40.4	40.9
5.4.13 16:30	2.3	24.9	39.8	38.2	39.1	39.2
8.4.13 9:30	5.0	4.3	18.9	17.3	21.9	22.0
Degr rate (mg-N/l/d)		-13.1	-10.1	-9.7	-9.2	-8.6
recovery rate%		100	77	74	70	66
Degr rate (mg-N/l.d)		-7.6	-7.7	-7.7	-6.3	-6.3

Table showing relationship activity of denitrification activity in batch 3 experiments

Date	Time (days)	NO3 (mg N/l)	TOC (mg C/l)	C/N
3.4.13 10:30	0.0	24.7	25.5	0.2
3.4.13 17:00	0.3	28.7		
4.4.13 9:40	1.0	18.5	25.7	0.3
5.4.13 9:50	2.0	21.7	7.7	0.1
5.4.13 16:30	2.3	22.2		
8.4.13 9:30	5.0	21.9		

IC₅₀ calculation for Batch 2 experiment

Nonlinear Regression

Sonntag, August 11, 2013. 09:57:48

Data Source: Data 1 in standard curve 1_group

Equation: Standard Curves; Four Parameter Logistic Curve

$$f1 = \min + (\max - \min) / (1 + (x / IC50)^{-Hillslope})$$

$$f = \text{if}(x \leq 0; \text{if}(Hillslope > 0; \min; \max); f1)$$

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9943	0.9886	0.9543	7.3583

	Coefficient	Std. Error	t	P
min	23.4694	4.2635	5.5047	0.1144
max	112.5344	6020063120.6780	1.8693E-008	1.0000
IC50	0.04621	641739.3508	2.8149E-008	1.0000
Hillslope	-12.5292	243826453.1235	-5.1386E-008	1.0000

Analysis of Variance:

	DF	SS	MS
Regression	4	12203.2486	3050.8122
Residual	1	54.1441	54.1441
Total	5	12257.3928	2451.4786

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	3	4685.5477	1561.8492	28.8462	0.1358
Residual	1	54.1441	54.1441		
Total	4	4739.6918	1184.9229		

Statistical Tests:

Normality Test (Shapiro-Wilk) Passed (P = 0.9202) W Statistic = 0.9774

Significance Level = <0.0001

Constant Variance Test Passed (P = 0.0500)

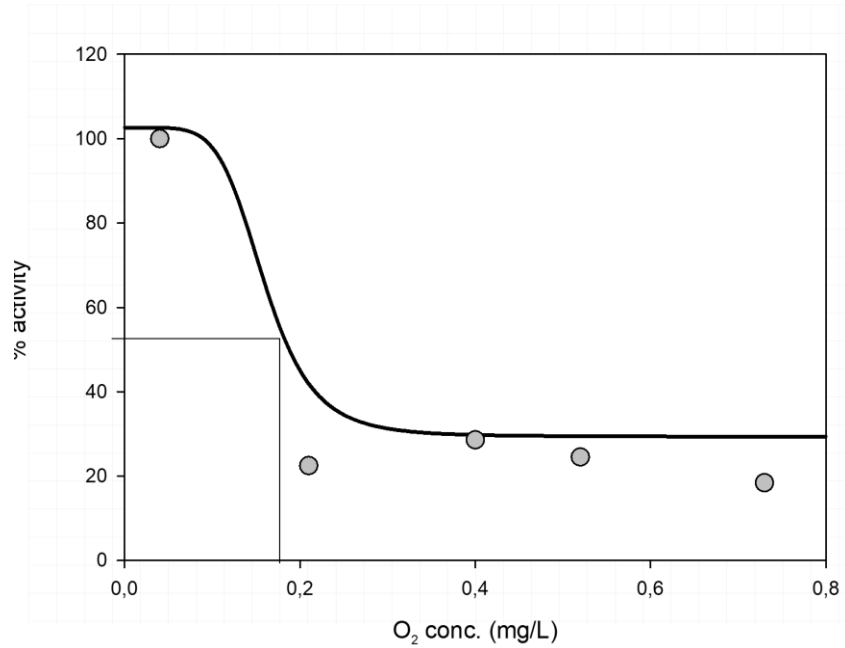


Figure 21-A: Curve of IC₅₀ estimation for Batch 2 experiment

IC₅₀ Combined Batch 1 and 2 experiments

Nonlinear Regression

Sonntag, August 11, 2013. 08:43:26

Data Source: Data 1 in Notebook1

Equation: Standard Curves; Four Parameter Logistic Curve

$$f1 = \min + (\max - \min) / (1 + (x / IC50)^{-Hillslope})$$

$$f = \text{if } (x \leq 0; \text{if } (Hillslope > 0; \min; \max); f1)$$

R	Rsqr	Adj Rsqr	Standard Error of Estimate	
0.9332	0.8709	0.8064	15.4840	
Coefficient Std. Error		t	P	
min	29.3671	7.1387	4.1138	0.0063
Max	02.6369	12.6693	8.1012	0.0002
IC50	0.1593	0.0443	3.5977	0.0114
Hillslope	-5.7255	4.6316	-1.2362	0.2626

Analysis of Variance:

	DF	SS	MS
Regression	4	37162.9777	9290.7444
Residual	6	1438.5217	239.7536
Total	10	38601.4994	3860.1499

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	3	9704.2370	3234.7457	13.4920	0.0045
Residual	6	1438.5217	239.7536		
Total	9	11142.7587	1238.0843		

Statistical Tests:

Normality Test (Shapiro-Wilk)

Passed (P = 0.2342) W

Statistic= 0.9026

Significance Level = <0.0001

Constant Variance Test

Passed (P = 0.6313)

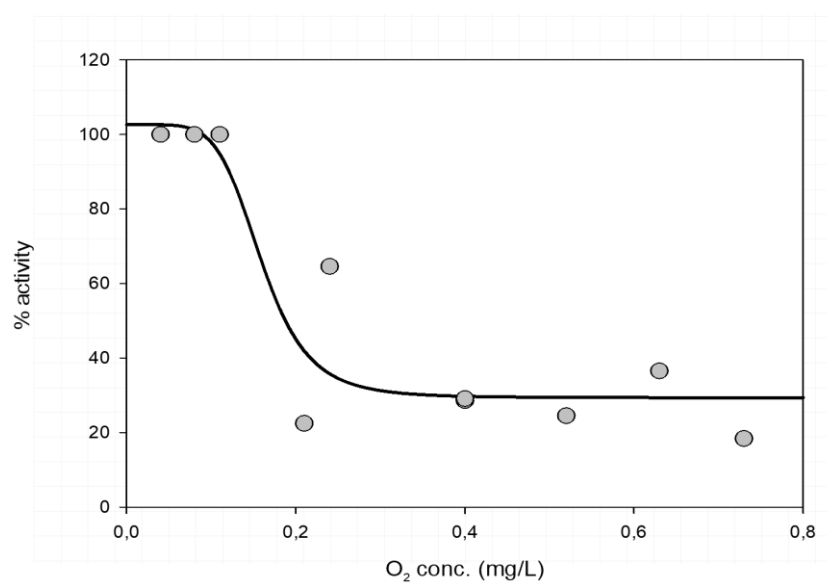


Figure showing a graphical presentation of a sigmoid plot for the calculation of IC_{50} for the combined batch experiments.

Batch 3 experiment

Anammox species	Brocadia Fulgida				
Date	09.04.13				
				Dry weight gravimetric	580.0
				No cells/ml	680.0
Substrate conc.	¹⁴ Nitrite-N	53.2 mg/l			
	¹⁴ NH ₄ -N	45.9 mg /l			
				volume	
After 1day of O ₂ treatment 9.4.13 @ 12:30	¹⁵ NH ₄ -N				
	Vial K	Vial 2	Vial 3	Vial 4	Vial 5
ATU	5µL	5µL	5µL	5µL	5µL
Vol. pure air (µL)	0	150	200	250	300
O ₂ (mg /l)	0.04	0.60	0.70	0.89	1.02
TNb (mg-N/l)	115.2				
NH ₄ ⁺ (mg-N/l)	44.8				
NO ₂ ⁻ (mg-N/l)	53.2				
NO ₃ ⁻ (mg-N/l)	17.2				
TOC (mg-C/l)	39.2				
10.4.13 9:30	vials purged with Ar/ CO ₂ +O ₂				
O ₂ (mg /l)	0.04	0.60	0.70	0.89	1.02
TNb (mg-N/l)	106.1	118.6	118.5	120.2	120.5
NH ₄ ⁺ (mg-N/l)	40.0	44.2	45.0	45.1	45.9
NO ₂ ⁻ (mg-N/l)	47.9	52.3	52.3	52.3	51.6
NO ₃ ⁻ (mg-N/l)	20.3	19.7	21.1	19.8	20.1
²⁹ N ₂ (integral values)	151283.0	176288.0	108290.0	131280.0	116929.0
TOC (mg-C/l)	28.9	43.3	35.6	40.0	44.8
10.4.13 16:45	vials purged with Ar/ CO ₂ @ 10.04.2013 09:30:00				
O ₂ (mg /l)	0.00	0.00	0.00	0.00	0.00
TNb (mg-N/l)	100.0	116.2	118.4	120.1	120.5
NH ₄ ⁺ (mg-N/l)	36.4	41.6	41.8	41.1	41.2
NO ₂ ⁻ (mg-N/l)	41.4	49.6	48.9	50.1	50.5
NO ₃ ⁻ (mg-N/l)	22.0	24.1	26.8	27.6	27.8
TOC (mg-C/l)	8.5	3.9	6.7	18.2	14.9
Influence of O₂ on N-degradation					
	Vial K	Vial 2	Vial 3	Vial 4	Vial 5
O ₂ (mg /l)	0.04	0.60	0.70	0.89	1.02
NH ₄ ⁺ (mg-N/l/d)	5.51	0.71	-0.27	-0.36	-1.24
TNb (mg-N/l/d)	10.41	-17.99	-17.33	-26.60	-28.34
²⁹ N ₂ (production) (mg-N/l/d)	7.87	8.28	-	-	-
% accuracy test					

Recovery test after purging with inert Ar/ CO₂ gas

	Vial K	Vial 2	Vial 4	Vial 5
O ₂ (mg /l)	0.00	0.00	0.00	0.00
d(TNb) (mgN/l/d)	-6.73	-5.45	-7.75	-8.76
NH ₄ ⁺ (mg-N/l)	-3.62	-2.59	-4.09	-4.72
Excess (TNb) (mgN/l/d)	0.65	-0.16	0.59	0.86
N ₂ production (mg/l)	9.63		7.55	9.88

		Vial K	Vial 2	Vial 4	Vial 5
O₂ (mgL⁻¹)		0.04	0.6	0.89	1.02
Date	Time (days)	NH ₄ (mg-N /l)			
9.4.13 12:30	0.0	44.8	44.8	44.8	44.8
9.4.13 17:00	0.2	40.0	44.2	44.7	45.9
10.4.13 16:40	1.2	36.4	41.6	41.1	41.2
12.4.13 9:40	2.9	33.0	38.4	39.4	38.2
15.4.13 10:30	5.7	13.0	16.7	21.1	18.5
Degr rate (mg-N/l.d)		-4.1	-2.1	-2.0	-2.5
recovery rate%		100.0	52.1	48.6	59.8

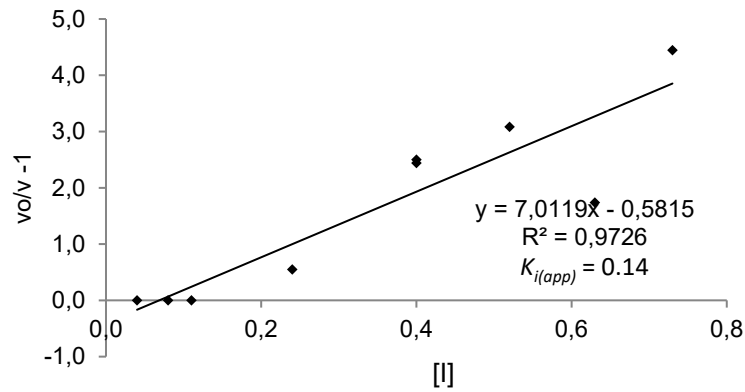
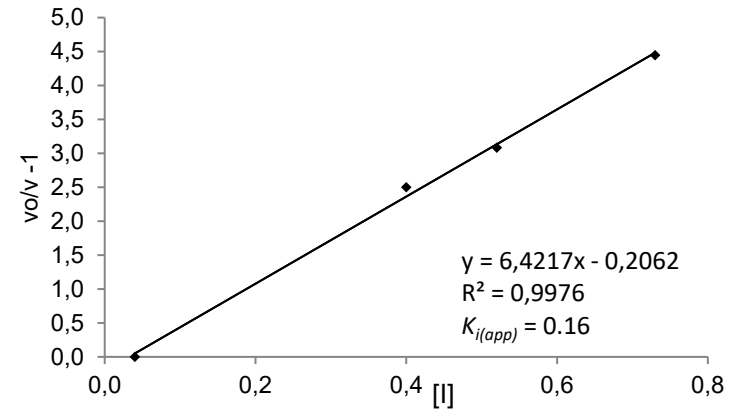
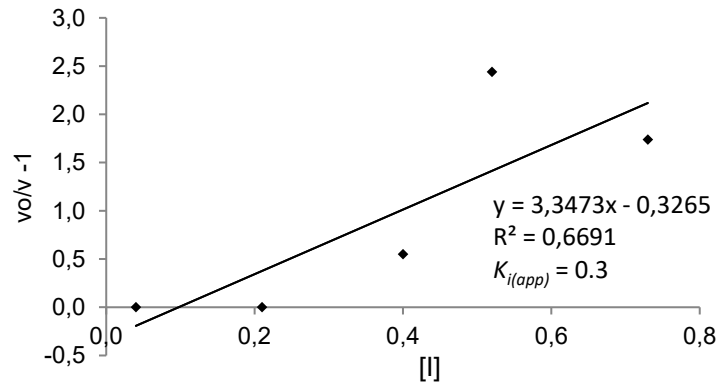
Plots of C:N and NO₃

Date	Time days	TOC (mgC/l)	C:N	NO ₃ (mgN/l)
9.4.13 12:30	0.00	39.2	0.34	17.21
9.4.13 17:00	0.19	28.9	0.27	20.28
10.4.13 16:45	0.99	8.5	0.09	22.04
12.4.13 9:40	1.70	8.1	0.09	19.49
15.4.13 10:30	3.03	0.0	0.00	13.68

Appendix 7: Kinetics of inhibition

Determination of $K_{i(app)}$					
	[O ₂]	activity %	Inhibition %	v/v ₀	(v ₀ /v) - 1
Batch 1	0.08	100	0.00	1.00	0.0
	0.11	100	0.00	1.00	0.0
	0.24	65	35.47	0.65	0.5
	0.40	29	70.93	0.29	2.4
	0.63	37	63.47	0.37	1.7
Batch 2	0.04	100	0.00	1.00	0.0
	0.21	22	77.55	0.22	
	0.40	29	71.43	0.29	2.5
	0.52	24	75.51	0.24	3.1
	0.73	18	81.63	0.18	4.4

Determination of K_i (app)



K_M obtained from Ijioma (2012)