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BACHELORARBEIT

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Biological Nitrate-dependent Ammonium Oxidation In An Anammox Reactor

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Biologische nitrat-abhängige Ammoniumoxidation in einem Anammox-Reaktor

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Biological Nitrate-dependent Ammonium Oxidation In An Anammox Reactor

Kurzbeschreibung:

Die biologische Ammoniumoxidation ist ein zentraler Bestandteil des globalen Stickstoffkreislaufs. Angesichts der extremen Massen Stickstoff anthropogenen Ursprungs in der Umwelt, liegt die Entfernung reaktiven Stickstoffs im Interesse der Umwelt und der öffentlichen Gesundheit. In der folgenden Arbeit werden Bedingungen zur anaeroben Ammoniumoxidation mit Nitrat in einem Anammox-Reaktor untersucht. Dabei wurden 2 Laborreaktoren für eine Zeit von insgesamt 116 Tagen betrieben und beobachtet, die ausschließlich als Elektronendonatoren und Akzeptoren Ammonium und Nitrat enthielten. Zusätzlich wurden Batchkulturen mit Zellen eines Reaktors angezüchtet und auf ihre Gaszusammensetzung abhängig unterschiedlicher Eigenschaften untersucht. Hierbei wurde eine Reihe unterschiedlicher analytischer Quantifizierungsmethoden genutzt und es konnte gezeigt werden, dass ein Abbau unter den Bedingungen stattfindet. Die aktuelle Forschung zu dieser Reaktion ist spärlich und verleiht der Bachelorarbeit dadurch Relevanz.

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Gia-Khoa Vu

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List of abbreviations

Amxb	-	anammox bacteria
Anammox	-	anaerobic ammonium oxidation
AOA	-	ammonia-oxidizing archaea
AOB	-	ammonia-oxidizing bacteria
Commamox	-	complete ammonia oxidation to nitrate
DRNA	-	dissimilatory nitrate reduction to ammonia
GC-MS	-	gas chromatography – mass spectrometry
HPLC	-	high performance liquid chromatography
IC-MS	-	ion chromatography – mass spectrometry (IC-MS)
N	-	nitrogen
N_2	-	dinitrogen gas
N_2H_4	-	hydrazine
N_2O	-	nitrous oxide
NO	-	nitric oxide
NOB	-	nitrite-oxidizing bacteria
NO_2^-	-	nitrite
NO_3^-	-	nitrate
NH_4^+	-	ammonium
WWTP	-	wastewater treatment plant

1. Introduction

As the fourth most abundant cellular bound element in form of biomass and biopolymers, nitrogen (N) and its biogeochemical cycle subject for interdisciplinary research for centuries. The Haber Bosch process allowed humanity to obtain incomparable amounts of ammonia from the exceptionally stable nitrogen molecules in out of air. This especially revolutionized agricultural industry through availability of ammonium based (NH_4^+) fertilizers and allowed the explosion of human population (Smil, 2011). Satisfaction of this increasing world population hunger of the 20th century came with the unaccountable price of high influxes of reactive nitrogen into environment. Excessive use of nitrogen fertilizers and mass livestock farming are major contributors to anthropogenic nitrogen fixation in the environment. This imbalance in the biosphere causes disastrous consequences for environment and public health e.g. eutrophication and infant methemoglobinemia. Without doubt, nitrogen harbors significant meaning to biosphere and its cellular life. Still, our understanding of the relevant microorganisms their cellular mechanisms and involved enzymes in the transformation of reactive nitrogen raises new questions with each discovery. Novel discoveries can approve thermodynamics-based predictions proposed several decades before like the finding of anaerobic ammonium oxidation (anammox) (Strous *et al*. 1999). Or they create paradigm shifts like the discovery of complete ammonium oxidation to nitrate (comammox) a reaction believed exclusively conducted in two separate reactions by different bacteria genera (Daims *et al*. 2015). In face of a further growing world population and societal shifts in so-called emerging countries in terms of wealth, food demands will not cease. Efforts in transformation of the way agriculture is managed are noticeable but need to be accompanied with scientific innovation in ammonia removal. This thesis investigates conditions for ammonia oxidation with nitrate (NO_3^-) as sole electron acceptor. Observations were made in lab-scale reactors of anammox reactor effluent where continuous ammonia and NO_3^- consumption occurred with simultaneous gas production. These findings are unusual, as key substrates for the dominant anammox strain are absence. Scientific literature to this subject is sparsely available and it is therefore hoped to elucidate another missing puzzle piece in the grand scheme of ammonia oxidation and the nitrogen cycle with these studies. Through an array of analytical methods applied on

different ways of cultivations evidence can be found whether these observations are coincidental or based on a new reactions or reaction yet unknown to other microorganisms.

2. Scientific background

In its elemental form, dinitrogen gas (N_2) accounts for 78% of the air composition of the Earth's atmosphere. While most organisms cannot metabolize it directly, all living creatures are dependent on it and must absorb it in a form that they can metabolize. Few examples of essential nitrogen containing biomolecules are amino acids, proteins, but also nucleic acids, which define the chemistry of life. Thus, inert nitrogen molecules with their triple covalent binding must be fixed before it is transformed into a variety of oxidative states used by different groups of microorganisms. This process termed nitrogen fixation transforms the inert nitrogen into a more reactive species, NH_4^+ or ammonia, and is exclusively conducted by prokaryotes aerobically or anaerobically. Bacterial and fungi induce ammonification, i.e., transforming organic-bound nitrogen into NH_4^+. A variety of chemolithoautotrophic bacteria or archaea metabolize NH_4^+ for their energy conservation and growth. These microorganisms are generally termed ammonium-oxidizing bacteria (AOB) and ammonium-oxidizing archaea (AOA).

2.1 Ammonium oxidation

2.2.1 Aerobic ammonium oxidation

Nitrification conducted by genera like *Nitrosomonas,* oxidizes ammonia to nitrite (Eq. 1) and releases energy simultaneously. In similar fashion nitration known by genera such as *Nitrobacter* oxidize nitrite to NO_3^- (Eq. 2) earning the related bacteria the term nitrite-oxidizing bacteria (NOB). This two-step dogma reaction remained untouched for over a century before another chemolithoautotrophy of the genus *Nitrospira* demonstrated comammox (Daims *et al*. 2015). Predictions on the existence of such bacteria were postulated almost a decade before discovery based advantageous growth yields with longer pathways and under biofilm like conditions (Costa *et al*. 2016). However, there was another discovery that galvanized research in nitrogen transforming microorganisms and innovations in wastewater treatment.

(1) $2\ NH_4^+ + 3\ O_2 \rightarrow 2\ NO_2^- + 2\ H_2O + 2\ H^+$

(2) $2\ NO_2^- + O_2 \rightarrow 2\ NO_3^- + e^-$

2.2.2 Anaerobic ammonium oxidation

In 1977, Austrian physiochemist Broda postulated the missing discovery of two missing lithotrophs on evolutionary and thermodynamic grounds (*Zeitschrift Für Allgemeine Mikrobiologie*, *17*(6), 491–493). One of them, a bacterium that oxidizes NH_4^+ to N_2 anaerobically, was found 1999 in a pilot plant in Delft (Strous *et al*. 1999). Due to the reaction occurring in absence of oxygen (O_2) it is termed anaerobic ammonia oxidation (anammox). Besides not relying on oxygen to activate the rather inert NH_4^+, they use the intermediate hydrazine (N_2H_4) in their final reduction step, a highly toxic compound used among other things as rocket fuel. Still, their intricacies are not limited to the used metabolites. Attention to their existence showed their omnipresence in anoxic environments. Especially their contribution in oxygen minimum zones, the major maritime sources of dinitrogen gas allowed them an allocation as central player in the nitrogen cycle as denitrification was believed to be the only N_2 producing biological reaction. Denitrification is the anaerobic reduction of NO_3^- or nitrite to N_2 via (nitrite,) NO and nitrous oxide (N_2O).

(3) $1\ NH_4^+ + 1\ NO_2^- \rightarrow N_2 + 2\ H_2O$

2.2 Unusual findings in an anammox reactor

In October 2021, effluent of an anammox enrichment reactor with *Candidatus Kuenenia stuttgartiensis* as dominant strain, was intended to be setup as a new anoxic reactor. After removing the majority of the cells by centrifugation and flushing the reactor with N_2, NH_4^+ and NO_3^- concentrations dropped. At this point no nitrite, which is a crucial substrate for the bacteria, was supplied. Cell density increased to a magnitude of 10^8 cells/ml and continuous pH decrease was noticed. In January 2022, 200 ml culture in the above-mentioned set-up was transferred into a new setup that contained 300 ml supernatant of a running anammox CSTR. Continuous NH_4^+ and NO_3^- consumption was observable again with increasing cell density. Air tightness was ensured and based on pressure response from the gas sensor and stoichiometry, 0.36 mM N loss/ day was calculated. When gas production fell below average it was restored through NO_3^- addition. 16S rRNA-targeted denaturing gradient gel electrophoresis (DGGE) and proteomics analysis allowed to investigate microbial community of the culture and revealed that besides anammox bacteria unknown phylotypes were found and no aerobic ammonium oxidizers were present. Thus, the question arises what causes this nitrate-

dependent ammonia oxidation and is it possible to recreate these observations with precise monitoring of key parameters in another reactor and batch tests. For these reasons a similar approach is chosen of introducing supernatant of an anammox reactor into an anoxic reactor with NO_3^- as sole electron acceptor and NH_4^+ as sole electron acceptor and bicarbonate as inorganic carbon source.

2.3. State of research and positioning of this work

Ammonia oxidation is an intensely researched field and has had exciting discoveries in recent years while genomic approaches allowed hypothesis about their enzyme-induced mechanisms, physiology, and ecology. However, the use of NO_3^- as the sole electron acceptor in this process without additional electron donors such as organic acids was not observed. Research on this specific subject is sparsely available and only one publication has been found in that regard. Wang *et al*. (2023) investigated exactly this reaction in anammox culture through using its internal storage of organics in form of glycogen. These reasons emphasize the significance of the following work. NO_3^- accumulation is an indicator anammox growth, as nitrite is used in two partial reactions: One is the oxidation to NO which is coupled to hydrazine synthesis in the N_2 producing reaction (Eq. 4). And in carbon fixation nitrite is reduced to NO_3^- regenerating electrons to the cyclic flow, the base of their energy metabolism. The latter reaction is suggestively coupled to the nitrite reduction to NO (Kartal *et al*. 2012). Requirement is an nitrite:nitrate-oxidoreductase complex (Nxr), an enzyme complex also found in NOB and commamox. Under anaerobic conditions Nxr demonstrated NO_3^- reduction activity to nitrite (Meincke *et al*. 1992). Indeed, anammox bacteria can adopt various alternative lifestyles e.g. as “disguised denitrifier” and reduce NO_3^- to NH_4^+ before conducting the regular anammox reaction, a process termed dissimilatory nitrate reduction to ammonium (DRNA). Even so, this is only observable in absence of NH_4^+ and therefore requires external organic or inorganic electron donor. Interestingly, Broda predicted one of the missing lithotrophs to oxidate NH_4^+ with NO_3^- as acceptor. This idea was hypothesized about in context of anammox metabolism but does not concur with what we know so far. If existent, it probably is conducted by another organism (Kartal *et al*. 2012)

(4) $NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$
(5) $NO + NH_4 + 2\ H^+ + 3e^- \rightarrow N_2H_4 + H_2O$
(6) $N_2H_4 \rightarrow N_2 + 4\ H^+ + 4\ e^-$

3. Materials

All chemicals were supplied by Sigma-Aldrich (Seelze, Germany) if not stated otherwise. For synthetic media or sample dilution Milli-Q water was used.

For setup 1 and setup 2 no synthetic media was prepared as culture media is reactor effluent from anammox CSTR. The components of the solutions fed into the reactor are listed in the appendix. For the first batch cultivations the labeled ammonia containing media is listed below (Table 1) Stock solutions components and the remaining batch media are also listed in the appendix.

Table 1: Batch media composition first and third trial

Component	Stock concentration	Final concentration
Salt solution	100 x	1 x
NaCl	Powder	60 mM
Sodium sulfate	Powder	0.1 mM
Trace elements with Copper	20000 x	1 x
Phosphate buffer (pH 7.4)	350 mM	3.5 mM
Selen, Tungsten, Molybdenum solution	20000 x	1 x
NaHCO3	Powder	10 mM
Vitamin 10 solution	1000 x	1 x
^{15}N-labeled NH_4Cl	1 mM	50 uM

4. Methods

The goal was to recreate the reaction of simultaneous NH_4^+ and NO_3^- depletion in different systems and find hints to the responsible mechanism. The first step was to establish a new reactor setup with the liquid of an anammox reactor as it was done before prior to this bachelor project on 5th July 2022 (referred as "setup 1"). By doing this, frequent measurements can be taken to analyze parameters verifying that the reaction is occurring. In the second step batch trial with labeled NH_4^+ media were set up to investigate the influence of abiotic reactions and gas production and gas composition. With such set-up, hints could be found to estimate whether the reaction is related to known reactions e.g. anammox, denitrification or a novel reaction. Finally, metabolomics investigations around the reactor setups were performed to recognize substrates, intermediates, relevant cellular mechanisms, and physiological state of the culture.

4.1 Reactor set-ups

The base for is the effluent of a lab-scale anammox enrichment CSTR, which has been stored in 4°C. This CSTR had a moderate growth rate and was mainly used for biochemical investigation on physiological properties, growth optimization and base for inoculations. The new system followed the setup as for the reactor from 2022/07. In February 2023, reactor liquid was poured out slowly to prevent disturbance in the precipitated cell mass on the bottom of the vessel. The remaining cells were concentrated and stored away. The liquid was centrifuged and filtered a second time through a filter paper with 7 µm pore size to further remove particles. The reactor vessel, a "Widdelkolben" (inverted Erlenmeyer flask) was washed to reuse in this method. Finally, the reduced cell liquid was poured back into the reactor vessel and reestablished as a new setup and will be referenced as "setup 2" (named as 2023/02). Setup 2 contained approximately 1.8 l liquid with 500 ml headspace with estimated NH_4^+ and NO_3^- concentration 20 mM and 10 mM respectively. Anoxic conditions were established through flushing the liquid phase and headspace with nitrogen for two hours. To prevent any gas leakages the setup, with bottle neck and inlets, was inverted and wrapped up in a heating blanket to keep temperature constant and block direct light. In the later stage the setup 2 was stored in 30°C incubation room.

4.2 Batch trials setup

For three batch trials synthetic mineral media was prepared with a final concentration of 50 µM of ^{15}N-labeled NH_4^+ (Table 1, Appendix). In an anaerobic glovebox 50 ml medium was aliquoted into 210 ml serum bottles with 160 ml headspace, closed with rubber septa and aluminum crimp caps. As the gas mixture of the anaerobic box contains N_2, the headspace in the serum bottles was flushed with helium for 10 minutes for individual bottles in the first batch trial and 15 minutes in the later trials in sequences of 4 bottles that were serially connected. By minimizing N_2 in the headspace, detection sensitivity for labeled dinitrogen gas ($^{29}N_2$ and $^{30}N_2$) is increased. In the first trial differences in autoclaved and untreated media were tested. Cells containing conditions were inoculated 10% v/v with liquid of reactor setup 1. All bottles were inverted to prevent possible gas leakage and incubated static at 30°C in the dark. In the second trial final ^{15}N-labeled NH_4^+ concentration was 1 mM and 800 µM sodium sulfite was added to bottles as a condition before inoculation for oxygen control. Bottles were inoculated with the same concentration. Sulfite at this concentration was proven to be toxic to anammox cells. Finally, the third batch tested 20 µM sodium sulfite instead and inoculated 0.1% v/v instead of 10% v/v so as to reduce the amount of unlabeled NH_4^+ brought in by the inoculum. Final ^{15}N-labeled NH_4^+ concentration was reduced to 50 µM in each bottle. Furthermore, at a later point unlabeled nitrate was added for a final concentration of 50 µM.

4.3 Analytical methods

4.3.1 Reactor sampling

For quantification of NH_4^+, NO_3^-, pH and cell density, samples were taken on a regular base from the reactor setups with sterile disposable syringes or a Hamilton syringe pump. For cell counting, untreated homogenous reactor liquid was stained with SYBR Green in triplicates and loaded onto agarose-coated slides. Pictures were taken randomly through a digital camera mounted on a fluorescence microscope. The remaining sample liquid was centrifuged (20 minutes at 14.000 g) and the resulting supernatant was diluted. NH_4^+ and NO_3^- quantification were done as duplicates and triplicates with high performance liquid chromatography (HPLC, Ultimate3000, ThermoScientific, LiChrospher® 100 RP-

18 endcapped (5 µm)) and ion exchange chromatography (IC, ICS-5000, Dionex IonPac^{TM} A18 column 2x250 mm), respectively. For HPLC analysis of NH_4^+, 200 µL of supernatant were taken, and mixed with 1 mL of borate-methanol solution (7:3 v/v, borate buffer is 50 mM, pH 9.0) and 6 µL of diethyl ethoxymethylenemalonate (DEEMM).

4.3.2 GC-MS analysis

For investigations on gas composition in the batch bottles, 500 µL headspace were taken with a Hamilton syringe directly through the rubber septa and injected and measured with gas chromatography – mass spectrometry (GC/MS, 7890A GC System, Agilent Technologies) on a weekly base. Since there was no separation in the GC column, all gases came out at the same retention time (~2.4 min). In between injections the syringe was purged with helium gas. Besides control conditions, each condition had 4 to 5 replicates depending on the batch trial. The following ion masses were analyzed for each bottle for their respective compounds are listed in Table 2. For evaluation the ratio of $^{28}N_2$ to $^{29}N_2$ was compared and plotted. Biotic $^{29}N_2$ production was calculated based on the natural average ratio of $^{28}N_2$ to $^{29}N_2$ in the atmosphere and subtracted from the measurement of $^{29}N_2$.

Table 2: Ion masses and their suspected compounds analyzed in GC/MS

Ion mass	**28**	**29**	**30**	**31**	**32**	**40**	**44**	**46**
suspected compound	$^{14\text{-}14}N_2$	$^{14\text{-}15}N_2$	$^{15\text{-}15}N_2$ or ^{14}NO	^{15}NO	O_2	Ar	CO_2 or $^{14}N_2O$	$^{15}N_2O$

4.3.3 Metabolomics

Metabolomics of both the anammox reactor and the setups in this study was investigated. Samples were taken from reactors, filtered immediately through 0.2µm syringe filter, and chilled before measurement with ion chromatography – mass spectrometry (IC-MS). The IC-MS system consists of an ion-chromatograph (ICS-6000, organic acid column, Dionex IonPac^{TM} CS19-4 µm, RFIC, 2 x 250 mm) and an Orbitrap system (Orbitrap Exploris 240 Mass Spectrometer, ThermoFisher). For the detection, a makeup pump with 0.15 ml/min flow rate (methanol), a regenerant pump with 0.5 ml/min flow rate (MilliQ water), and a ICS-6000 pump with 0.38 ml/min flow rate was applied. Column temperature and

compartment temperature was 30 and 20 °C, respectively. EGC 500 KOH was employed to generate the concentration gradient as described in **Error! Reference source not found.**:

Table 3: eluent gradient of ion chromatograph (ICS-6000)

Time (min)	Concentration (mM)
0	1
5	1
14	10
23	20
38	60
48	80
55	80
55.1	1
60	1

A detection MS range of 40-900 was set with a detection resolution of 120,000. The H-ESI ion source was applied with static spray voltage, 3500 V (positive ions) and 2500 V (negative ion). Temperatures were set to 325 °C for the Ion transfer Tube Temperature and 300 °C at the vaporizer. A full scan method with dd MS^2 detection (secondary MS) was applied.

The obtained data were normalized against the signal intensity of phosphate (1e11), based on the assumption that phosphate was constant in all cultures (3.5 mM) in the beginning, and was consumed only negligibly.

4.3 Methods summary

To sum up the methodology we used, two anoxic lab-scale reactors were operated and investigated in their liquid phase on the fate of the NH_4^+ as electron donor and NO_3^- as electron acceptor mainly through analytical methods. In addition, three batch trials containing ^{15}N-labled NH_4^+ and inoculum of setup 1 have been performed with different inoculum volumes, NH_4^+ concentrations and defined additions. These batches were investigated and compared on their nitrogen gas composition. Finally, the metabolome of setup 1 and setup 2 were analyzed to give hints about the occurring reaction and relevant cell metabolism inducing the reaction.

5. Results

Results are structured in two parts separated by the two different kinds of setups that were used for cultivation as well as set of quantification methods. The first part will include setup 1 (2022/07) and setup 2 (2023/02) and their monitoring of the key parameters and finally metabolomics. In the second part insights are shown to the GC/MS analysis of the batch bottles.

5.1 Reactor setups

Two anoxic lab-scale reactors with NH_4^+ as sole electron acceptor and NO_3^- as sole electron acceptor were monitored for a total of 116 days operation. The main difference between both setups is the 7 months lead time setup 1 was operated for compared to setup 2 and will be respected. Setups are presented individually before difference and similarities are drawn in direct comparison. Overviews of measured parameters on given timepoints are shown in Table 4 and Table 5.

Table 4: Overview table of key parameters for setup 2

Date	Sampling time	Time(d)	pH	Cell density (cells/ml)	NH_4^+ (mM)	NO_3^- (mM)
03.02.23	14:39	0	7,4	2,05E+07		11,73
08.02.23	10:27	5		3,40E+07		11,69
20.02.23	10:17	17		2,70E+07		11,64
06.03.23	09:24	31		2,70E+07		11,22
14.03.23	11:04	39		4,60E+07	18,57	11,12
27.03.23	10:15	52	7,72	2,80E+07	20,28	11,08
05.04.23	10:00	61		5,50E+07		11,03
20.04.23	10:55	76		4,60E+07	18,51	11,31
26.04.23	10:15	82		4,30E+07	18,59	
03.05.23	13:15	89		3,50E+07	18,72	
05.11.23	10:50	97	7,8	3,00E+07	18,32	11,29
16.05.23	13:15	108		4,10E+07	18,28	11,27
24.05.23	14:30	116		4,70E+07	17,76	11,27
25.05.23	13:00	117	Take filtered samples for metabolomics			

Table 5: Overview table of key parameters for setup 1

Date	Sampling time	Time(d)	pH	Cell density (cells/ml)	NH_4^+ (mM)	NO_3^- (mM)
03.02.23	14:39	213				
08.02.23	10:27	218				6,99
20.02.23	10:17	230		5,67E+07		6,73
06.03.23	09:24	244				6,45
14.03.23	11:04	252		6,90E+07	8,52	6,35
27.03.23	10:15	265	7,71	9,40E+07	9,01	6,22
05.04.23	10:00	274		8,80E+07		6,14
20.04.23	10:55	289		7,80E+07	8,92	6,20
26.04.23	10:15	295		4,70E+07	7,36	
03.05.23	13:15	302		5,40E+07	8,67	
11.05.23	10:50	310	7,67	7,50E+07	8,52	6,06
16.05.23	13:15	321		1,00E+08	8,42	6,01
24.05.23	14:29	329		1,20E+08	8,02	5,96
25.05.23	13:00	330	Take filtered samples for metabolomics			

5.1.1 Setup-2

Setup 2 had due to the filtering a reduced cell density of 2.05×10^7 cells/ml and started out with a pH of 7.4 and NO_3^- concentration of 11.73 mM (Table 4). Reactor liquid therefore looks transparent at this point. During the first weeks, cell density did not increase steadily and showed a high degree of fluctuation between cell counts and between replicates. Figure 1 shows an increase on day 39 at which point a yellow tint was noticeable and pellets were visibly observable after centrifugation. In a series of alternating between increases and decreases, cell density finally reached a concentration of 4.70×10^7 cells/ml on day 116. NH_4^+ levels were not quantified before day 52 and were 18.57 mM as starting point. In a total of 9 weeks from then the most significant changes were the unexpected increase on read on day the steep decrease on day 116. Otherwise, the trend is a very slow decrease averaging in a weekly change of -0.029 mM NH_4^+ per week. For NO_3^- concentration a similar trend is observable. Overall, a flat curve with one

significant time at day 61 when it increased. From that point it started lowering in a slow manner again. On day 31 a significant NO_3^- decrease was observed. This most likely relates to a 50 ml addition of culture liquid of setup 1 to setup 2 in assumption of a boosting effect. Nevertheless, the average weekly change for NO_3^- is -0.033 mM. pH was measured sparsely but increased from 7.4 to 7.8 in 116 days.

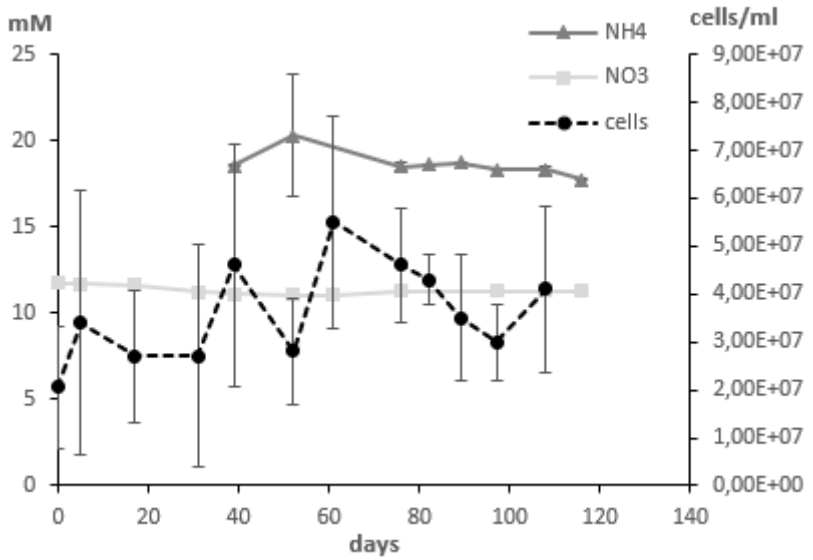

Figure 1: Performance of setup 2. NH4 and NO3 degradation and cell growth over time

5.1.2 Setup 1

In direct comparison, setup 1 had significantly more cells from the beginning. Although not quantified, biofilm and flocs were visible around pH sensor and needles that reached into the liquid. Growth in setup 1 was more consistent for the first 50 days before cell density slowly lowered and steeply reduced until day 293. Worth mentioning is the inoculation of the first batch trial liquid of this culture on day 289 of operation. After day 293 cell density steadily increased to 1.2×10^8 cells/ml on the final day of measurement. NH_4^+ concentrations fluctuate for the most time of measurements between 8 and 9 mM except for day 265 when concentration was 9.01 mM and day 295 when concentration was 7.36 mM. However, it is worth pointing out that the first case occurred on the same day NH_4^+ concentration also peeked for setup 2 and for the latter case deviation is significantly high compared to the other measurements. Overall, albeit very slow with an average of -0.12 mM per week NH_4^+ reduction occurred at a higher pace compared to setup 2. In contrast to the NH_4^+ curve, NO_3^- reduction was stable and steadily decreased

with an average of -0.08 mM per week. Besides one measurement, nitrite, a crucial substrate for anammox bacteria was never detected. It is suspected that nitrite originated from a previous sample of the sequence unrelated to this experiment.

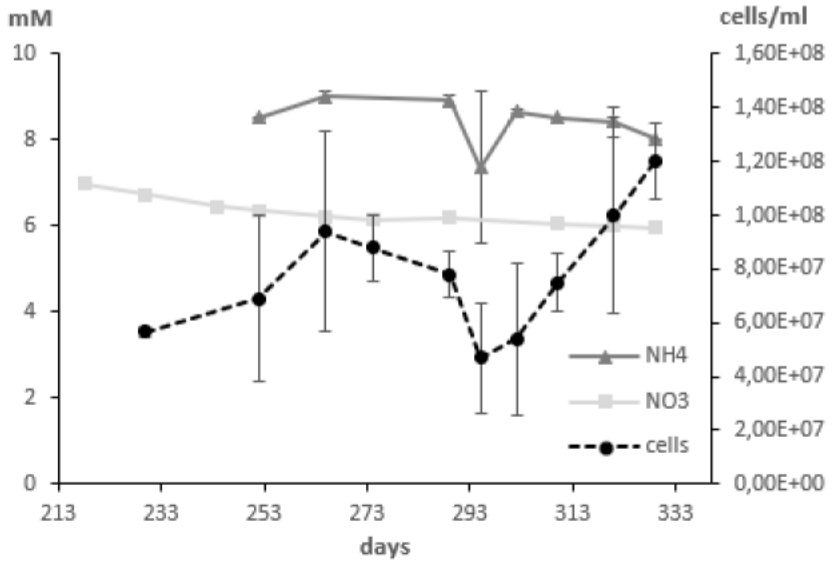

Figure 2: Performance of setup 1. NO_3^- and NO_3^- degradation and cell growth over time

5.1.3 Metabolomics

Metabolomic analysis was conducted on both setups at the end of the monitoring period (05/2023). Table 6 lists the organics with the highest abundances detected in the analysis during the thesis and the analysis done in 10/2022 of setup 1 and an operating anammox CSTR. The latter was conducted as single replicate while the current reading represents the setups as average of triplicates. Superficially, a massive reduction in total organic acids occurred.

Table 6: Organics detected with highest abundance in the supernatant of samples analyzed (color coded for the abundance). Numbers shown are intensity of the respective peaks in IC-MS.

Organic compound	Formula	Anammox on 20221025	Setup1 on 20221025	Setup1 on 20230526 averaged	Setup2 on 20230526 averaged
Sum of signals of all organic acids detected		8.1E+10	5.1E+10	1.3E+10	1.6E+10
4-Sulfobenzoic acid	C7 H6 O5 S	2.6E+10	1.5E+10	9.0E+08	1.0E+09
Ethanol	C2 H6 O	1.0E+10	7.9E+09	3.1E+09	3.6E+09

1-{[(Ethylsulfonyl) methyl]sulfonyl}ethane	C5 H12 O4 S2	1.5E+10	7.3E+09	9.7E+07	1.2E+08
Isobutyraldehyde	C4 H8 O	3.3E+09	4.3E+09	1.6E+09	1.7E+09
4-Methoxychalcone	C16 H14 O2	3.0E+09	2.4E+09	9.1E+08	1.8E+09
4-Phenolsulfonic acid	C6 H6 O4 S	3.3E+09	1.6E+09	7.0E+07	8.5E+07
Benzyl cinnamate	C16 H14 O2	1.8E+09	1.3E+09	7.7E+08	1.2E+09
12-Oxo phytodienoic acid	C18 H28 O3	1.8E+09	2.0E+09	1.9E+08	2.3E+08
Glycolic acid	C2 H4 O3	4.6E+08	5.3E+07	1.6E+09	1.9E+09
butadiene	C4 H6	1.7E+09	1.3E+09	3.8E+08	4.0E+08
4-Formylbenzenesulfonic acid	C7 H6 O4 S	2.1E+09	1.3E+09	7.3E+07	7.5E+07
Acetone	C3 H6 O	7.5E+08	6.0E+08	5.4E+08	6.9E+08
2-Acetyl-5-hydroxyphenyl hydrogen sulfate	C8 H8 O6 S	1.7E+09	7.0E+08	1.7E+07	2.0E+07
Trimethylamine	C3 H9 N	1.8E+09	2.4E+08	5.4E+06	6.4E+06
(S)-2-methylbutanal	C5 H10 O	8.0E+08	7.2E+08	1.2E+08	1.5E+08
Oxalic acid	C2 H2 O4	8.4E+08	4.5E+08	1.1E+08	1.6E+08
D-Sulfophenylacetic acid	C8 H8 O5 S	7.8E+08	3.8E+08	1.0E+07	1.1E+07
N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-5-methyl-3-isoxazolecarboxamide	C13 H12 N2 O4	1.4E+08	3.0E+08	2.3E+08	3.6E+08
Methanesulfonic acid	C H4 O3 S	1.0E+08	7.1E+07	3.8E+08	3.2E+08
13(S)-HpOTrE	C18 H30 O4	3.6E+08	2.9E+08	8.1E+07	7.4E+07
b-Sulfinyl pyruvate	C3 H4 O5 S	4.4E+08	1.5E+08	2.3E+05	2.8E+05
Fumaric acid	C4 H4 O4	3.4E+08	1.4E+08	2.9E+06	2.7E+06
L-(+)-Lactic acid	C3 H6 O3	2.7E+08	5.7E+07	4.6E+07	6.0E+07
(1S,6S)-6-Aminooctahydro-1-indolizinyl acetate	C10 H18 N2 O2	1.4E+08	1.1E+08	4.5E+07	5.9E+07
5-Amino-2-hydroxybenzoate	C7 H6 N O3	9.0E+06	5.7E+06	1.4E+08	1.7E+08
Acrylic acid	C3 H4 O2	2.3E+08	7.5E+07	2.4E+06	2.2E+06
trans-Piperylene	C5 H8	1.1E+08	6.2E+07	4.0E+07	5.6E+07
Pyruvic acid	C3 H4 O3	1.5E+08	5.5E+07	2.7E+07	3.0E+07
HEPES	C8 H18 N2 O4 S	7.6E+07	4.1E+07	7.1E+07	6.9E+07
chavicol hydrogen sulfate	C9 H10 O4 S	1.7E+08	6.3E+07	1.8E+06	1.3E+06
Miglitol	C8 H17 N O5	3.3E+06	2.4E+06	1.0E+08	1.2E+08
3-Acetylpyridine	C7 H7 N O	7.7E+07	6.5E+07	3.7E+07	4.0E+07

5-Sulfosalicylic acid	C7 H6 O6 S	1.1E+08	8.8E+07	3.5E+05	7.5E+05
Crotonic acid	C4 H6 O2	5.9E+07	6.2E+07	3.1E+07	4.2E+07
D-(+)-Malic acid	C4 H6 O5	1.1E+08	5.1E+07	1.6E+07	1.7E+07
MFCD20642996	C3 H4 O6 S	1.4E+08	5.1E+07	3.2E+05	3.7E+05
uredepa	C7 H14 N3 O3 P	4.6E+06	2.5E+06	8.5E+07	9.3E+07
Methyl mesylate	C2 H6 O3 S	1.3E+06	3.0E+05	9.0E+07	9.0E+07
L-(+)-Tartaric acid	C4 H6 O6	1.2E+08	5.5E+07	3.6E+05	3.8E+05
N-(1,3-Benzothiazol-2-yl)-5-methoxy-1H-indole-2-carboxamide	C17 H13 N3 O2 S	3.8E+07	6.8E+06	4.7E+07	7.6E+07
4-nitrophenolate anion	C6 H4 N O3	1.0E+07	7.1E+06	6.9E+07	8.1E+07
Terephthalic acid	C8 H6 O4	7.3E+07	4.8E+07	1.8E+07	2.1E+07
Calcium oxide	Ca O	5.0E+06	1.2E+07	6.8E+07	7.2E+07
5-Chloro-4-fluoro-2-nitroaniline	C6 H4 Cl F N2 O2	1.1E+08	4.2E+07	2.7E+05	3.3E+05
sulfoacetaldehyde	C2 H4 O4 S	9.2E+07	4.6E+07	3.4E+06	3.8E+06
3-sulfolactic acid	C3 H6 O6 S	9.5E+07	4.5E+07	2.1E+06	1.8E+06
Oxepane	C6 H12 O	3.3E+07	2.5E+07	3.4E+07	4.9E+07
Glyoxylic acid	C2 H2 O3	9.4E+07	4.1E+07	2.0E+06	3.0E+06
Phthalic anhydride	C8 H4 O3	5.5E+07	4.3E+07	1.2E+07	1.2E+07
AJ4900000	C2 H4 O5 S	8.0E+07	3.7E+07	2.4E+06	1.8E+06

5.2 Batch trials and GC/MS analysis

Based on the performance, setup 1 was chosen to be the inoculation culture for the batch tests. We started the batch trials on different days to scope any usable results before continuing testing different conditions, therefore the three batch trials differ in the period they were observed and in direct consequence the amount of data points. The first batch contains 50 ml media with 50 µM ^{15}N-labeled NH_4^+ and was inoculated 10% v/v and received based on quantifications for day 76, 60 µmol NO_3^- and 93 µmol unlabeled NH_4^+. This trial tested the effects of autoclaving media before inoculation. Clearly observable is the steady decrease of the $^{28/29}N_2$ ratio by inoculated cultures with untreated media. This occurred in correspondence with increasing amount of the microbially produced $^{29}N_2$

that was calculated. Interestingly, the autoclaved culture controls performed poorly in direct comparison and behaved like the controls of this batch. However, unlike the autoclaved media cultures, an increasing amount of $^{29}N_2$ was detected in the later stage of the trial reaching almost the same level as the regular inoculated cultures on day 40. All inoculated cultures showed colorless precipitation by day 26 indicating microbial growth.

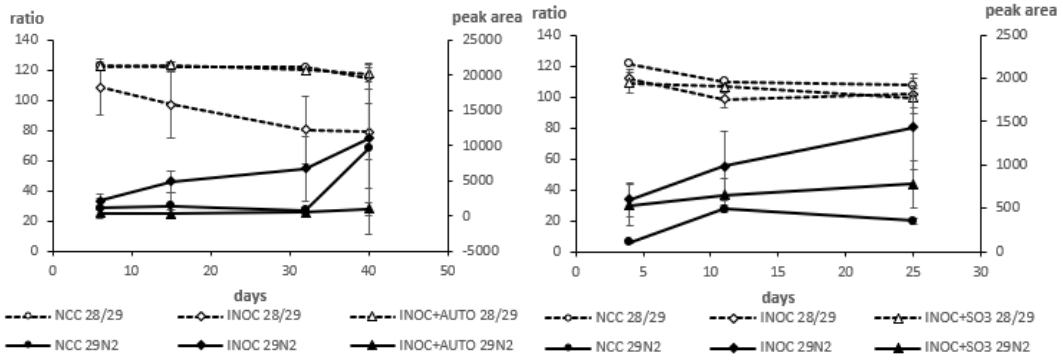

Figure 3: Effects of autoclaving media (left) and 800 µM sodium sulfite (right). ratio of $^{28}N_2$ and $^{29}N_2$ over time and peak area for biotic $^{29}N_2$ over time. NCC = No cells control, INOC = inoculated, AUTO = autoclaved

In the second batch we tested the effects of sulfite on the cultures. Due to a calculation error a final concentration of 800 µM Na_2SO_3 was introduced into the sulfite condition bottles. This concentration was proven to be toxic for anammox bacteria. Still, even though in the starting stage differences were not evident after 25 days the regular cultures without any additions slightly outperform the sulfite containing cultures. Performance overall was comparably slower than the untreated cultures of the first batch. Another difference worth mentioning is the unstable $^{28/29}N_2$ ratio in the controls.

Finally, the last batch contained the proper concentration of 20 µM Na_2SO_3 but was inoculated 0.1% v/v and therefore received 0.91 µmol unlabeled NH_4 and 0.56 µmol. Furthermore, 50 µM $NaNO_3$ were added to all inoculated bottles at a later stage. The sulfite cultures are barely producing more $^{29}N_2$ than the cultures without any additions and slightly outperform the untreated conditions in terms of $^{28/29}N_2$ ratio. Furthermore, for the last measurement all samples were analyzed on additional molar masses of relevant intermediate compounds of anammox and denitrification: ions 30, 31, 45 and 46. The corresponding compounds (Table 2) are intermediates of NO_2^- and NO reduction in anammox and denitrification pathway. Ion 31 was not detected in any of the samples

besides two cases barely in detection range. Ions 45 and 46 on the other hand were detected in trace amounts in every sample, including the controls, of the first and second batch trial. In addition, a similar ratio of 3:1 was observed on average for every sample. In the last trial only ion 45 was barely detectable in the non-control conditions.

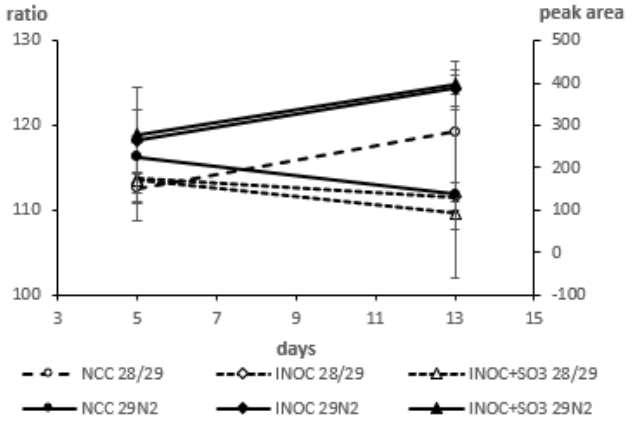

Figure 4: Effects of 20 µM sodium sulfite (right) compared to untreated (left). ratio of $^{28}N_2$ and $^{29}N_2$ over time and peak area for biotic $^{29}N_2$ over time. NCC = No cells control, INOC = inoculated

Table 7: GC/MS readings on batch trials for additional ions and final ratio.

Batch condition		**peak area**			
		30	**45**	**46**	**45/46 ratio**
1st trial	No cells	4012	1281	391	3,3
	Inoculated	814	2708	927	2,9
	Autoclaved + Inoculated	90	3221	1129	2,9
2nd trial	No cells	33	2036	694	2,9
	Inoculated	63	2700	919	2,9
	Sulfite + Inoculated	107	2393	823	2,9
3rd trial	No cells	111	0	0	-
	Inoculated	7	26	0	-
	Sulfite + Inoculated	26	26	0	-

5.2 Results summary

In this project two lab-scale reactors that originated from anammox enrichment cultures were operated and monitored for a total time of 116 days with NH_4^+ as sole electron donor and NO_3^- as electron acceptor. In addition, metabolomic data of both setups was extracted, creating a snapshot of the current state of the cells in the culture. Out of the two setup 1,

which was already operating before this project, showed greater consumption rates of these two mentioned substrates and stable growth over time. Thus, three batch trials were conducted with inoculations of that reactor and analyzed on their headspace composition. The first batch demonstrated constant $^{29}N_2$ gas production over time when media was not autoclaved. In the second batch trial additions of 800 µM and higher substrate concentrations showed reduced activity. The last batch trial showed slight superiority of 20 µM sulfite additions over untreated cultures but was tested for a comparably short period. Overall activity in the first two weeks was also in direct comparison to the previous trials.

6. Discussion

6.1 Performance evaluation of the reactors

Two lab-scale reactors with solely NH_4^+ and NO_3^- as substrate and mixed culture were operated and monitored for a total of 116 days. Setup 2 which was built at the start of this project exhibited unstable growth expressed through alternating between increases and decreases in cell density. This could be due to the slow growing nature of anammox bacteria. While studies showed their versatility in lifestyles and electron acceptors, the lack of nitrite might have an inhibiting effect. Zhong *et al.* (2013) successfully showed simultaneous NO_3^- and NH_4^+ consumption in batch tests only when glucose was added as organic energy source for the denitrification. Their batch results also report that in presence of NO_3^- as sole electron acceptor NH_4^+ concentrations never decreased without glucose or nitrite addition. However, we intentionally only included sodium bicarbonate as alternative electron acceptor and carbon source as it would be thermodynamically disadvantageous. In contrast, NH_4^+ concentration was decreasing with NO_3^- as sole electron acceptor during the experiments albeit very slow.

NH_4^+ and NO_3^- degradation in this reactor were calculated as weekly average of 0.029 and 0.033 mM respectively. Bacterial growth is clearly observably but alternates between increases and decreases and substrate consumption speed is slower than expected. Setup 1 was already in operation before monitoring and therefore cannot be fully compared to the other setup in all regards. While the growth curves show differences, more observation time would be necessary to verify that the frequency for increases and decreases is not simply much slower while following the same trend. In terms of weekly substrate consumption setup 1 outperforms setup 2 for NH_4^+ 4-fold and NO_3^- 2.3-fold. However, these results pale compared to the consumption rates that were observed (Chang Ding, personal communication) before in setup 1 before this bachelor project. NH_4^+ and NO_3^- consumption rates up to 30 mg/l per week were observed. Therefore, here during the bachelor project consumption rates for NH_4^+ in presence of NO_3^- could not been successfully recreated. Even so, it might be possible by further operation under these conditions, that cultivation of a microorganism responsible for the reaction is feasible.

6.2 Metabolomics

The extracted metabolomic data allows comparisons between each individual setup and in addition between setup 1 in 10/2022 and 05/2023. Starting with the most abundant compound, after 5 months of operation setup 1 showed only slightly above half the abundance of 4-sulfobenzoic acid. During the next 8 month it was reduced by another 1.41×10^{10} down by 2 orders of magnitudes. Reduction is therefore apparently occurring in correspondence with time, but this raises the question why in setup 2 abundance is only slightly more. Due to the lead in operation time of setup 1 compared to setup 2 it would be reasonable to see higher abundances in case of reduction with setup 2 e.g. 4-methoxychalcone. However, the same observation is also seen with other compounds such as ethanol and acetone. In contrast, compounds such as glycolic acid and calcium oxide increased in abundance after operating the setups in this manner. Unexpectedly, some compounds in setup 2 are even less densely expressed than in setup 1 such as fumaric acid and acrylic acid. Overall, with few exceptions, both setups are very similar in organic composition and abundance. These low abundances in their composition of organic metabolites could have major impact and explain their currently slow activity. Nevertheless, the values for the measurement of 10/2022 are derived from single samples. Furthermore, small molecule metabolites are continuously absorbed, synthesized, or interact with other molecules. Our metabolomic analysis therefore can only offer a snapshot of a highly dynamic system.

6.3 Effects of batch additions

In the batch trials microbial activity and effects of different additions were shown successfully. $^{29}N_2$ gas production was increasing and therefore shifting the ratio of $^{28/29}N_2$. Autoclaving media beforehand appeared to be counterproductive as the ratio barely decreased due to minimal changes in $^{29}N_2$. These results are almost identical with the control condition of the second trial and are outperformed by the controls of this batch. Therefore, it could be assumed that autoclaving eliminated all biotic traces of the used water for media preparation or water storage units. Therefore, increasing gas production was observed in the control conditions as they were not autoclaved either. These findings agree with those of Tao *et al*. (2011) where the contribution of side communities like AOB, NOB, DNB and DRNA is stressed. In their batch tests, inoculated from anammox

SBR and MRB reactors, these organisms survived in the same magnitude as anammox and did not have any negative impact. In fact, they might be beneficial in reducing residual DO and organic matter. In the second trial the added sulfite barely showed negative impact compared to the untreated conditions. Interestingly, while identically prepared the untreated conditions performed very poorly compared with higher substrate concentrations compared to the untreated cultures of the first batch trial. This raises the question what benefitted the cells during the first trial. Unfortunately, the last batch trial is limited through the period that the experiment was conducted. Given that, in terms of ratio and overall gas production performance is poor in direct comparison to untreated conditions from the previous batch trials. In the same timeframe of the first 2 weeks only the autoclaved conditions apparently had less activity. Nevertheless, it should be emphasized that in the last batch only one hundredth of the cells were inoculated. Therefore, a higher inoculation concentration is recommended for higher activity. To prevent any disturbance to the batch cultures other analytical methods that were conducted with setup 1 and setup 2 were renounced.

6.4 Hints in the gas composition

$^{29}N_2$ production suggests anammox activity as one nitrogen atom originates from the labeled ^{15}N- NH_4^+ and the other stems from unlabeled NO_3^- resulting into total molecular weight of 29.

Nitric oxide (NO), an important intermediate in anammox and denitrification derived from nitrite reduction was only detected in its labeled molar weight of 30 and thus shares the same weight with $^{15\text{-}15}N_2$. However, $^{30}N_2$ would be unexpected as this would require ^{15}N-labled NO_3^- or nitrite as base for denitrification while NO on the other hand is a very reactive radical. Jetten *et al*. (1999) labeling experiments with $^{15}NH_4^+$ and $^{14}NO_3^-$ agrees with these findings as almost exclusively $^{29}N2$ was produced in an almost pure anammox culture. Furthermore, in their enrichment reactor no $^{15}NO_3^-$ and no other anammox intermediate was ever observed in the presence of $^{15}NH_4^+$ without nitrite additions. Anammox versatility in their metabolic pathways enables the bacteria also to DRNA, a reaction with striking similarities to canonical denitrification resulting in ammonia accumulation with N_2O as intermediate (Kartal *et al*. (2007). This alternative pathway could contribute to the slow ammonia decrease that was observed in the reactor setups.

Even so, this reaction requires organic acids as electron donor and would not produce any $^{30}N_2$, unless $^{15}NO_3^-$ was used. Against the expectations, consistent traces of 45 and 46 suspectedly $^{14\text{-}15}N_2O$ and $^{15\text{-}15}N_2O$ respectively were detected in an average ratio of 3:1. This indicates denitrifying activity contributing to $^{29}N_2$ production as anammox produce no to very little N_2O (Krampscheur *et al*. 2008 in Kartal *et al*. 2012). In denitrification NO_3^- or nitrite is used as terminal electron acceptor therefore it was expected that the reaction produces $^{28}N_2$. This raises the question whether $^{15}NO_3^-$ or $^{15}NO_2^-$ originates from nitrifiers / aerobic ammonia oxidizers or anammox produced.

6.5 Technical and experimental limitations

A variety of factors may have limited the investigations during this bachelor project. First, with the new setup compromises were made to assure anoxic conditions. Because air tightness of setup 2 was only given through inverting the vessel (Widdelkolben) sensors that reached into the liquid or were attached to the inlets of the flask needed to be removed. Thus, parameters like pressure, redox potential, oxygen concentration and pH were not precisely monitored in real-time. Furthermore, the inverting meant there was no mechanical stirring system installed to keep the cells in suspension and no homogenization as consequence. In contrast, setup 1 had a magnet stirrer homogenizing full-time, if not taken out for sampling. Oxygen concentration was regularly checked with via tracer spots on the inside of the reactor vessels. Still, leaking could have potentially occurred on sampling events. Especially in the case of setup 1 which was also used for inoculations between day 76 and 111 this could facilitate more opportunities for oxygen into the system even though efforts were made to minimize disturbance. This may contribute to the steep increase in growth observed in the later stage as it may benefit growth of aerobic nitrifiers dormant in the anammox culture (Tao *et al*. 2012).

6.6. Prospects for future research

Broda (1977) predicted two “impossible” autotrophic ammonia oxidizing groups based on thermodynamics. Anammox found in a denitrification pilot plant several decades later was the missing link that could oxidized ammonia with nitrite as oxidant. This novelty caused a reassessment in the N cycle related microorganisms and galvanized research

them and enhancement in their use in wastewater treatment plant. Few published research is currently conducted whether biological nitrate-dependent oxidation is possible. In the limited time of 5 months this thesis and the conducted experiments may not illuminate the mentioned decade old question which organisms may perform this reaction or whether our observations are microbial novelty. However, it might be a steppingstone as the applied methods under these conditions are rarely found in public literature. This underlines the complexity of research on mixed cultures with their microorganisms fitting into different ecological roles in terms of nitrogen cycling. Noticeable, current efforts are made to figure out if this reaction lies within the abilities of anammox bacteria (Wang *et al*. 2023). Here they demonstrated energy conservation based on NO_3^- as sole substrate while gaining NH_4^+ through DRNA and their internal glycogen storage. For future research it is recommended to combine the methods conducted in this thesis e.g. gas analysis of the lab-scale setups and substrate quantifications on the batch cultures for stoichiometry-based estimates. Long-term operation of the reactor setups might favor microorganisms responsible for nitrate-dependent ammonia oxidation.

7. Summary

In this bachelor thesis investigations were conducted whether biological nitrate-dependent ammonium oxidation could be established in a reactor setup and consequently in batch cultures for activity tests. To explain the unusual findings in our lab-scale anammox reactors an array of analytical methods has been used like chromatography-based quantification methods, fluorescence microscopy and isotope labeling experiments. Along an already examined setup, a new one was built with effluent of anammox enrichment CSTR. Through 116 days of monitoring, we confirmed continuous NO_3^- degradation and NH_4^+ consumption in absence of nitrite. In 9 weeks, 0.81 mM and 0.5 mM NH_4^+ were reduced in setup 2 and setup 1, respectively and therefore did not reach the rates observed previously. Out of the two, setup 1 was picked for batch cultivations due to more stable cell growth and weekly substrate decrease. Batch cultivations with ^{15}N-labeled ammonia and NO_3^- from inoculum demonstrated continuous $^{29}N_2$ production and is most likely caused by anammox activity. However, traces from intermediates of other nitrogen transforming bacteria were also consistently detectable. Considering all these results we could not demonstrate nitrate-dependent ammonia-oxidation in the magnitude that was hoped for. Therefore, identifying the responsible reaction or microorganism remains elusive. Even so, this thesis shows that the reaction is occurring, albeit very slowly. Without doubt, more efforts must be made in method optimization and will show if these results will hold any relevance. Eventually then, a new discovery in this exciting field of research may be witnessed.

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Appendix

Supportive Methods & Materials

Supportive Material 1: Stock solution composition of Trace solution (20000 x)

Chemical	Amount	Stock concentration
HCl (20% solution, w/w)	125 ml	5%
MilliQ water	To ~400 ml	-
$FeCl_2 \cdot 4H_2O$	15 g	30 g/l
$CoCl_2 \cdot 6H_2O$	1.9 g	3.8 g/l
$MnCl_2 \cdot 2H_2O$	0.82 g	1.64 g/l
$ZnCl_2$	0.7 g	1.4 g/l
H_3BO_3	0.06 g	0.12 g/l
$NiCl_2 \cdot 6H_2O$	0.24 g	0.48 g/l
$CuCl_2 \cdot 2H_2O$	0.2 g	0.4 g/l
MilliQ water	To 500 ml	-

Supportive Material 2: Stock solution composition Selen/Tungsten/Molybdenum solution (20000 x)

Chemical	Amount	Stock concentration
Na_2SeO_3	0.04 g	0.08 g/l
$Na_2WO_4 \cdot 2H_2O$	0.08 g	0.16 g/l
$Na_2MoO_4 \cdot 2H_2O$	0.36 g	0.72 g/l
NaOH	0.5 g	1 g/l
MilliQ water	To 500 ml	-

Supportive Material 3: Stock solution composition salt solution (100 x)

Chemical	Amount	Stock concentration
$MgCl_2 \cdot 6H_2O$	10 g	10 g/l
KCl	30 g	30 g/l
$CaCl_2 \cdot 2H_2O$ / $CaCl_2$	1.5g / 1.13g	1.5 g/l / 1.13g/l
MilliQ water	To 1000 ml	-

Supportive Material 4: Stock solution composition phosphate buffer (pH 7.2)

Chemical	**MW (g/mol)**	**Amount**	**Stock concentration**
KH_2PO_4	136.09	16.0 g	16 g/l
K_2HPO_4	174.2	40.5 g	40.5 g/l
MilliQ water		To 1000 ml	-

Supportive Material 5: Stock solution composition Vitamin10 solution (1000 x)

Chemical	**MW (g/mol)**	**Amount**	**Stock concentration**
D(+)Biotin	244.31	20 mg	20 mg/l
Folic acid	441.41	20 mg	20 mg/l
Pyridoxamine dihydrochloride monohydrate	259.13	252 mg	252 mg/l
(-)-Riboflavin	376.37	50 mg	50 mg/l
Thiamine chloride hydrochloride	337.27	50 mg	50 mg/l
Nicotinic acid	123.11	50 mg	50 mg/l
Calcium-D(+)-pantothenate	476.55	50 mg	50 mg/l
p-aminobenzoic acid	137.1	50 mg	50 mg/l
DL-α-Lipoic acid	206.33	50 mg	50 mg/l
Vitamin B12	1355.37	50 mg	50 mg/l
MilliQ water		To 1000 ml	-

Supportive Material 6: Batch medium composition second trial

This medium is higher concentrated with labeled $^{15}N\text{-}NH_4^+$ compared to trial 1 and 3. The final concentrations are shown in the following table.

Component	**Stock concentration**	**Final concentration**
Salt solution	100 x	1 x
NaCl	Powder	60 mM
Sodium sulfate	Powder	0.1 mM
Trace elements with Copper	20000 x	1 x
Phosphate buffer (pH 7.4)	350 mM	3.5 mM
Selen, Tungsten, Molybdenum solution	20000 x	1 x
$NaHCO_3$	Powder	10 mM

Vitamin 10	1000 x	1 x
^{15}N-labeled NH_4Cl	1 mM	1 mM

Statement of authorship / Selbstständigkeitserklärung

I hereby declare that I have prepared this thesis independently and without outside assistance. Text passages, which are based literally or in the sense on publications or lectures of other authors, are marked as such. The work has not yet been submitted to any other examination authority and has not yet been published.

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Mittweida, 21.06.2023

Vu Gia-Khoa