In search of Comammox in oxygen limiting conditions

Master Thesis

by

Mu-En Lu

Student no.: 4903803

Master of Science
in Life Science & Technology

at the Delft University of Technology,
to be evaluated on Monday June 15, 2020 at 14:00.

Supervisor:	Prof. Robbert Kleerebezem
	PhD Candidate Gerben Stouten
Thesis committee:	Prof. David Weissbrodt
	Prof. Jules van Lier

Abstract

Comammox bacteria are capable of catalysing the full nitrification pathway – oxidation of ammonium to nitrate – and have been encountered in many ecosystems recently (Lawson &Lücker, 2018). What the ecological role of comammox bacteria is in hypoxic enrichment cultures remains unclear. Based on the thermodynamics and biochemistry of known nitrogen cycle conversion, we propose that comammox is oxidizing ammonium to nitrite with both oxygen and nitrate as electron acceptor in hypoxic condition. Our hypothesis suggests that when comammox cooperates with anammox, they can harvest most energy per unit of oxygen supplied. We tried to cultivate bacteria toward a community of anammox and comammox consortium by limiting the oxygen and supplying ammonium and nitrate. Although the predicted optimal state has not been achieved during this work, we did observe that the community indeed developed towards higher consumption of ammonium under limited oxygen supply.

Acknowledgements

Robbert, thank you for guiding me to become a master. Whenever I look back, I will always remember you spending time discussing with me with a big smile!

Gerben, thank you for helping, challenging, encouraging me all the way. You stimulate me to use my brain. You are my joy!

Professor Jules and David, thank you for giving your time and advice. You made the ending of my thesis an invaluable lesson.

Ben, thank you for your time and eager explaining to me FISH, NGS and qPCR.

Zita, thank you for organizing the lab to give us a comfortable working environment.

Dmitrii , thank you for insights of what bacteria might be doing

Roel, thank you for teaching me FISH explicitly and maintaining my bioreactor during lockdown.

Zejia, thank you for maintaining my reactors during Christmas

Chris, thank you for helping me get in the lab to fetch my lab journal and prepare my reactors for the lockdown.

Viktor, thank you for teaching me DA, and bringing me the activated sludge

Michele, thank you for the AMX inoculum and knowledge

Maxim, thank you for commenting on my writing, helping me explain things more clear.

Shengle & Chuan, thank you teaching me the basic lab routine
Maxim for giving me feedback on writing

Jure, thank you for connecting me to EBT and this thesis, and advice to
organize notes of paper

Apilena, Astrid, Jeannie, Marcel, thank you for facilitating all the lab work.

My office mates, Vincent, Eva, Melati, Reneta, Shannara, Lemin, Mythili, Sergio, Jeffrey, thank you for sharing news, life in general, and nice cakes. You add spices and colors to my thesis life.

To all EBTers, thank you all for your birthday, I am so happy to be around with wonderfully diverse you.

Contents

1. Introduction

Nitrogen compounds removal from wastewater is important to prevent the discharged water from causing eutrophication in aquatic ecosystems. The process involves two steps: nitrification and denitrification. Nitrification is the oxidation of ammonium to nitrite or nitrate. Ammonium oxidation to nitrite is done by Ammonium Oxidizing Bacteria (AOB), which have the required enzymes: ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). Nitrite can be further oxidized to nitrate by Nitrite Oxidizing Bacteria (NOB), which has the required enzyme: nitrite oxidoreductase (NXR). AOB and NOB are commonly found to coexist in wastewater treatment plant. Denitrification is the reduction of nitrite or nitrate to dinitrogen gas usually by heterotrophs, coupled with oxidation of organic matter.

Another route for N removal from wastewater is through anaerobic ammonium oxidation. The bacteria that perform this reaction are called anammox (AMX). Anammox oxidize ammonium and reduce nitrite at the same time to produce dinitrogen gas. This reaction can be seen as a variation of denitrification.

AOB: $NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$

NOB: $NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$

AMX: $NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$

Understanding how different conditions affect the N conversion community is crucial for wastewater treatment plants to maximize the N removal efficiency and lower the cost of operation. One way to maximize the N removal is to lower the aeration, limiting the oxygen supply so that ammonium is oxidized to nitrite by AOB, and then nitrite is denitrified with ammonium by AMX. This approach saves oxygen from oxidizing nitrite to nitrate, and excludes the need for organic for denitrification by heterotrophs

However, the recent discovery of comammox complicates the control of N conversion community. Comammox (complete ammonium oxidation, CMX) is a type of bacteria that can oxidize ammonium to nitrate. Comammox was first discovered in 2015 (Daims et al., 2015; vanKessel et al., 2015) Van Kessel and Daims provided genomic and FISH evidence that the comammx belong to genus *Nitrospira* and have AMO, HAO, and NXR, the whole set of enzymes to oxidize ammonium all the way to nitrate. They also showed the bacteria were indeed oxidizing ammonium to nitrate under aerobic condition.

$$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2\ H^+$$

Surprisingly, Van Kessel enriched the comammox under hypoxic condition. Whether comammox was growing on complete ammonium oxidation to nitrate or on another conversion under severe oxygen limitation remains unclear.

Based on the enzymes CMX have and their coexistence with AMX in van Kessel's experiment, we hypothesize that in severe oxygen limiting conditions, CMX is oxidizing one ammonium by one nitrate and one oxygen, producing two nitrite, by using the enzymes AMO, HAO, and reversing NXR:

$$NH_4^+ + NO_3^- + O_2 \rightarrow 2\ NO_2^- + H_2O + 2\ H^+$$

The nitrite produced by CMX can then be used by AMX, forming a cross-feeding symbiosis.

This research tries to enrich a community of CMX + AMX and verify the hypothesis that CMX is oxidizing ammonium to nitrite with both oxygen and nitrate as electron acceptor in hypoxic condition. Cultivation conditions of Daims and van Kessel were analyzed regarding their degree of oxygen limitation. A thermodynamic and biochemical model was used to analyse the N conversions stoichiometries of different nitrification and denitrification pathways. The stoichiometries were used to compare N removal and growth yield on oxygen requirements as described in the next section of this report. Based on this theoretical analysis, an experimental setup was defined to investigate if the CMX conversion proposed could be enriched in oxygen limiting conditions. Experiments were conducted and the development of the nitrogen conversions observed and the microbial community structure are described in the subsequent sections of this report.

2. Theory

In this section, the ecological niche of comammox's full nitrification pathway is discussed, based on the enrichment conditions described by Daims and van Kessel. The N conversions of the different consortia are compared. In short, this section explains why our hypothesized comammox reaction collaborating with anammox is the most competitive under oxygen limitation, and that comammox's full nitrification under oxygen limitation is unlikely.

2.1. CMX's full nitrification is predicted to outcompete AOB+NOB when NH_4^+ is limiting.

While CMX's full nitrification is the same as the sum of AOB's and NOB's conversions (Table 1), CMX has been predicted to have a lower maximum specific growth rate but a higher growth yield on ammonium than its cross-feeding competitors; CMX's longer pathway makes it grow slower, but harvest more energy (Costa, Pérez, &Kreft, 2006). Costa et al. postulated that comammox would out-compete incomplete ammonia oxidizers under conditions of ammonium limitation and low wash out that allow slow growth, in aggregates such as flocs, microcolonies or biofilms.

Table 1
AOB, NOB, CMX's CMX's N conversions and the energy released from the conversions.

Microbe	Conversion	Energy $\Delta G^{0'}$ (kJ/mol)
AOB	$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$	-274.7
NOB	$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$	-74.1
CMX	$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2H^+$	-348.9

A simplified explanation of CMX's higher growth yield:
CMX gets the whole 348.9 kJ/mol of energy from nitrification, whereas AOB and NOB share the energy. Since CMX gets more energy than the individual AOB and NOB in the consortium, CMX would have higher growth yield on ammonium, rendering a competitive advantage in case of ammonium limitation.

2.2. Daims' CMX adapts to low NH_4^+, and the O_2 in the culture was sufficient for full nitrification

Daims enriched comammox and revealed that the comammox adapts to low NH_4^+ conditions. Daims found that the comammox's specific growth rate μ_{max} is 0.0061 h^{-1} and ammonia affinity K_M (NH_3) is 0.049 (Dimitri Kits et al., 2017). The small maximum specific growth rate and high ammonia affinity align with Costa's prediction.

Although Daims did not mention how much oxygen they supplied in the system, estimation suggests that the oxygen availability allowed full oxidation of ammonia:

Microbes were grown in 25mL medium in a 100mL schott bottle. The NH_4^+ concentration was 0.01~1mM. The maximum amount of NH_4^+ in the bottle = 1 mM NH_4^+ * 25mL = 25 µmol.
Since Daims did not mention how they supplied gas nor purge N_2, we assume that they did not supply gas, and the oxygen came from the headspace of the bottle:
75mL /24.5 L/mol *0.2095 = 0.64 mmol O_2 = 640 µmol O_2.
More oxygen could be supplied to the system when they opened the lid to sample.

Comparing the 640 µmol O_2 and the 25 µmol NH_4^+, we could say that the oxygen in the system was sufficient for full nitrification of ammonium.

2.3. Van Kessel's case is O_2 limiting, NH_4^+ excessive

In contrast to Daims', van Kessel's enrichment condition was hypoxic (they claimed to have DO < 3.1 µM). They sparged the reactor and medium with 95% Ar/ 5% CO_2, trying to make an anaerobic condition. Although some oxygen might leak into the system during operation, the oxygen influx should be very low. They operated the reactor as SBR, but their methods are not described in full detail. During decanting, 600mL supernatant is removed from the reactor in 30 minutes, that means it is possible that the reactor drew back in 600mL of ambient air. (It is supposed that there is no gas supply during decanting because the sludge settled.) The air leaking into the system is a probable source of oxygen in the system, with dissolved oxygen in the medium as second possible contributing factor. Considering the fact that they kept bubbling N_2 and CO_2 through the 7 L bioreactor at 10 ml/min during the operational cycles of 12 and 24 hour, if oxygen was in the liquid, then it would be stripped out. It is difficult to calculate how much oxygen was transferred into their system, but it makes sense that the oxygen transfer rate was low.

The low oxygen transfer rate in the system led us to doubt that the comammox was doing complete oxidation of ammonium. We hypothesize that instead of using two O_2 to oxidize one NH_4^+, comammox might use NO_3^- together with O_2 to oxidize NH_4^+ to NO_2^-. Oxidation of NH_4^+ to NO_2^- can be performed by AMO and HAO, and reduction of NO_3^- can be done by reversing NXR. The hypothesized reaction is asterisked (CMX*) to distinguish it from the full nitrification CMX.

CMX*: $NH_4^+ + O_2 + NO_3^- \rightarrow 2\ NO_2^- + H_2O + 2\ H^+$

The oxidation of NH_4^+ to NO_2^- gives out six electrons, of which four electrons are accepted by O_2, and the remaining two electrons can reduce NO_3^- to NO_2^-.
The nitrite produced by CMX* can then feed AMX

AMX: $NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$

Since nitrite was below detection limit in van Kessel's experiment, we eliminate nitrite by adding up one unit of CMX* with two units of AMX reaction.
The resulting cross-feeding combination of CMX* + AMX is:

CMX* + AMX: $3\ NH_4^+ + O_2 + NO_3^- \rightarrow 2\ N_2 + 5\ H_2O + 2\ H^+$

If van Kessel could show that the nitrate concentration decreased, then this hypothesis would be supported more strongly. However, their medium was prepared from aquaculture water, which had varying NO_3^- concentration (300–1,848 μM), so they were unable to show whether nitrate was consumed. In this study we use synthetic medium to control NO_3^- concentration to verify our hypothesis

2.4. When oxygen is limiting, what is the thermodynamically favored conversion?

To explain which N conversion is more favorable when oxygen is limiting, we will compare the Gibbs free energy of metabolisms of different consortia, as well as microbes' growth yields on O_2 by using the blackbox model of metabolism. Based on this model, CMX* + AMX consortium's metabolism is the most thermodynamically favorable, and has the highest growth yield on O_2, and thus is expected to win the survival competition under O_2 limitation in presence of ammonium and nitrate.

What is a blackbox model?
The blackbox model sees metabolism as a chemical equation, where the substrates are the reactants and the product is the biomass. The metabolism (Met) equation can be dissected into anabolism (An) and catabolism (Cat). Anabolism is the synthesis of biomass; catabolism is the reaction that releases energy for anabolism. Both Cat and An are redox reactions, which means they each can be further dissected into electron donating reaction (ED) and electron accepting reaction (EA).
In short, we can express metabolism in equations as below (Kleerebezem &VanLoosdrecht, 2010)

$$Met = An + \lambda * Cat$$
$$An = An' + ED$$
$$Cat = ED + EA$$

We assume that for all microbes the biomass have the same chemical formula $CH_{1.8}O_{0.5}N_{0.2}$, and the Gibbs free energy of Met is -3000 kJ/mol, meaning it would dissipate 3000 kJ/mol to produce one mole of biomass from the carbon and nitrogen source which in these cases are CO_2 and NH_4^+ respectively. One unit of Met comprises one unit of An and several units of Cat to satisfy the energy requirement, therefore we multiply Cat with a coefficient λ. Since the microbes we are discussing are all autotrophs, their electron accepting reactions in anabolism are all CO_2 receiving electrons, which we denote as An'.

Blackbox model of AOB, NOB, AMX, and CMX are summarized as follows:

AOB

ED	$NH_4^+ + 2H_2O \rightarrow NO_2^- + 6e^- + 8H^+$
EA	$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$
An'	$CO_2 + 0.2NH_4^+ + 4.2e^- + 4H^+ \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 1.5H_2O$
Cat	$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+$
Met	$13.1\ NH_4^+ + CO_2 + 18.3\ O_2 \rightarrow 12.9\ NO_2^- + CH_{1.8}O_{0.5}N_{0.2} + 12.3\ H_2O + 26H^+$

NOB

D	$NO_2^- + H_2O \rightarrow NO_3^- + 2e^- + 2H^+$
A	$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$
An'	$CO_2 + 0.2\ NH_4^+ + 4.2e- + 4H^+ \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 1.5H_2O$
Cat	$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$
Met	$0.2\ NH_4^+ + 44\ NO_2^- + CO_2 + 20.9\ O_2 + 0.6H_2O \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 44\ NO_3^- + 0.2\ H^+$

AMX

ED	$NH_4^+ \rightarrow 0.5\ N_2 + 3e^- + 4H^+$	(Donor for catabolism)
ED	$NO_2^- + H_2O \rightarrow NO_3^- + 2e^- + 2H^+$	(Donor for anabolism)
EA	$NO_2^- + 3e^- + 4H^+ \rightarrow 0.5\ N_2 + 2\ H_2O$	
An'	$CO_2 + 0.2NH_4^+ + 4.2e^- + 4H^+ \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 1.5H_2O$	
Cat	$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$	
Met	$9.3\ NH_4^+ + 11.2\ NO_2^- + CO_2 \rightarrow$	
	$9.1\ N_2 + 2.1\ NO_3^- + CH_{1.8}O_{0.5}N_{0.2} + 17.7\ H_2O + 0.2\ H^+$	

CMX, full oxidation of ammonia to nitrate

D	$NH_4^+ + 3H_2O \rightarrow NO_3^- + 8e^- + 10H^+$
A	$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$
An*	$CO_2 + 0.2NH_4^+ + 4.2e^- + 4H^+ \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 1.5H_2O$
Cat	$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2H^+$
Met	$10.2\ NH_4^+ + 18.9\ O_2 + CO_2 \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 10.0\ NO_3^- + 9.4\ H_2O + 20.1\ H^+$

CMX*, alternative catabolism we propose in this study,
using one O_2 and NO_3^- to oxidize one NH_4^+

ED	$NH_4^+ + 2\ H_2O \rightarrow NO_2^- + 6e^- + 8H^+$
EA	$O_2 + NO_3^- + 6\ e^- + 6\ H^+ \rightarrow NO_2^- + 3H_2O$
An*	$CO_2 + 0.2\ NH_4^+ + 4.2e^- + 4H^+ \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 1.5H_2O$
Cat	$NH_4^+ + NO_3^- + O_2 \rightarrow 2NO_2^- + H_2O + 2H^+$
Met	$18.1\ NH_4^+ + 17.2\ NO_3^- + 17.2\ O_2 + CO_2 \rightarrow$
	$CH_{1.8}O_{0.5}N_{0.2} + 17.3\ H_2O + 36.1\ H^+ + 35.2\ NO_2^-$

After constructing the metabolism of these microbes, we want to combine them to see which consortium maximizes the use of resources. Because NO_2^- can be either oxidized to NO_3^- or reduced to N_2 to produce energy, we assume that NO_2^- produced by AOB or CMX* will be consumed by NOB or AMX. To achieve no NO_2^- accumulation, we consider four scenarios: AOB+NOB, AOB+AMX, CMX*+AMX, AOB+AMX+CMX*.

We derive the consortium's overall metabolism by finding a linear combination that satisfies NO_2^- production equals consumption. Then we normalize the metabolism with O_2 consumption, to compare the utilization of NH_4^+ (and nitrate) and the yields of biomass per O_2.

Example AOB + NOB:
AOB produces 12.9 NO_2^- and NOB consumes 44 NO_2^-, according to the Met.
AOB:

$$13.1\ NH_4^+ + CO_2 + 18.3\ O_2 \rightarrow\ 12.9\ NO_2^- + CH_{1.8}O_{0.5}N_{0.2} + 12.3\ H_2O + 26\ H^+$$

NOB:

$$0.2\ NH_4^+ + 44\ NO_2^- + CO_2 + 20.9\ O_2 + 0.6\ H_2O \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 44\ NO_3^- + 0.2\ H^+$$

By multiplying NOB by 12.9/44, and then adding with AOB, the NO_2^- is eliminated.
AOB + 12.9/44 * NOB =

$$13.1\ NH_4^+ + 1.3\ CO_2 + 24.4\ O_2 \rightarrow\ 12.9\ NO_3^- + 1.3\ CH_{1.8}O_{0.5}N_{0.2}\ + 12.1\ H_2O + 26\ H^+$$

Then, dividing this equation by 24.4 to normalize by O_2, we get

$$0.54\ NH_4^+ + 0.053\ CO_2 + O_2\ \rightarrow\ 0.53\ NO_3^-\ + 0.053\ CH_{1.8}O_{0.5}N_{0.2} + 0.50\ H_2O + 1.1\ H^+$$

Applying the same method, the metabolisms for the other consortia can be derived.
AOB + AMX:

$$1.3\ NH_4^+ + 0.12\ CO_2 + O_2\ \rightarrow\ 0.57\ N_2 + 0.13\ NO_3^-\ + 0.12\ CH_{1.8}O_{0.5}N_{0.2} + 1.8\ H_2O + 1.4\ H^+$$

AMX + CMX*:

$$2.7\ NH_4^+ + 0.6\ NO_3^- + 0.2\ CO_2 + O_2\ \rightarrow\ 1.7\ N_2 + 0.24\ CH_{1.8}O_{0.5}N_{0.2} + 4.2\ H_2O + 2.1\ H^+$$

AOB + AMX + CMX*:
(In this scenario, both NO_2^- and NO_3^- are set to have no accumulation)

$$1.6\ NH_4^+ + 0.14\ CO_2 + O_2\ \rightarrow\ 0.8\ N_2 + 0.14\ CH_{1.8}O_{0.5}N_{0.2} + 2.2\ H_2O + 1.6\ H^+$$

$\Delta G^{0\prime}$ can be calculated from the stoichiometry, and are arranged in the following table together with yields:

Table 2. Summary of N conversion communities' yields and $\Delta G^{0\prime}$

	AOB+NOB	AOB+AMX	AOB+AMX+CMX*	AMX+CMX*
Consumption of NH_4^+ per O_2	0.54	1.3	1.6	2.7
Yield of biomass per O_2	0.053	0.12	0.14	0.24
Production of NO_3^- per NH_4^+ consumed	0.98	0.10	0	-0.23
$\Delta G^{0\prime}$ kJ/mol O_2	-158.9	-352.5	-416.9	-719.2

From Table 2, we can see that AMX+CMX* is the most exergonic conversion, consumes the most NH_4^+, and has the highest biomass yield on oxygen. Based on this model, we propose that under oxygen limitation and supply of NH_4^+ and NO_3^-, AMX+CMX* will win the competition for oxygen. Furthermore, as the consumption of NH_4^+ per O_2 and yield of biomass per O_2 increase from left to right across the table, the production of NO_3^- per NH_4^+ consumed decrease. Hence, the experimental measurement of NH_4^+ and NO_3^- can be used to indicate the development of the conversion and community. It is expected that if CMX* is enriched, NO_3^- production will decrease and eventually turn to consumption.

2.5. Is it possible that NH_4^+ is oxidized by NO_3^- without O_2?

In the above discussion, oxidation of one NH_4^+ is either by AMX using one NO_2^-, or AOB using 1.5 O_2, or CMX using one NO_3^- and one O_2.

Some people might ask, why cannot AMX or CMX just use NO_3^- to oxidize NH_4^+?

$5\ NH_4^+ + 3\ NO_3^- \rightarrow 4\ N_2 + 9\ H_2O + 2\ H^+$ $\Delta G^{0'}$ = -1483.6 kJ/reaction

= -296.7 kJ/mol-NH_4^+

This reaction is thermodynamically favorable, but it has not been found in any organism. The reaction is impossible for CMX because the only enzyme that CMX can use to oxidize NH_4^+ is AMO, which requires O_2 to function.

AMX has the enzymes to oxidize ammonium by nitrite, and it also has enzyme to oxidize nitrite to nitrate, reversing of which can turn nitrate to nitrite. It seems that AMX's set of enzymes can perform the oxidation of ammonium by nitrate. However, such reaction has not been found in AMX. This puzzle could be explained as we look at the coordination of the enzymes.

Table 3. AMX's enzymes (Kartal, vanNiftrik, Keltjens, Op den Camp, &Jetten, 2012)

Enzyme	**abbrev.**	**Reaction**
Hydrazine synthase	HZS	$NH_4^+ + 2\ H^+ + NO + 3\ e^- \rightarrow N_2H_4 + H2O$
Hydrazine dehydrogenase	HDH	$N_2H_4 \rightarrow N_2 + 4\ H^+ + 4\ e^-$
Nitrite reductase	Nir	$NO_2^- + 2\ H^+ + e^- \rightarrow NO + H2O$
Nitrite::Nitrate oxidoreductase	Nar	$NO_2^- + H_2O \rightarrow NO_3^- + 2\ e^- + 2\ H^+$

The AMX catabolic reaction $NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$ is composed of the reactions of HZS, HDH, Nir. The conversion of nitrogen compounds and the electron flow can be visualized as Fig.T1

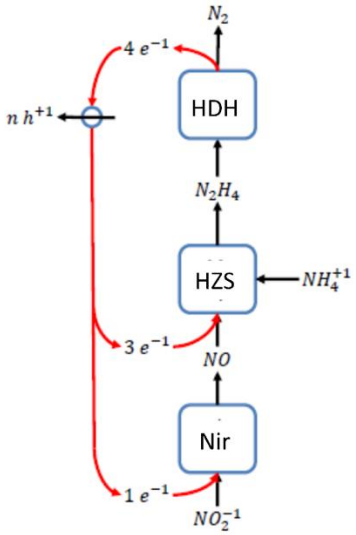


Fig. T1. The catabolism of Anammox. The electron flow is a closed cycle.
*The figure is adapted from Kleerebezem, R. (2019). Electron_puzzles_in_N-cycle_conversions [PowerPoint slides].
Retrieved from: https://brightspace.tudelft.nl/d2l/le/content/128351/Home

As can be seen from Fig. T1, the 4 electrons produced from HDH are exactly consumed by Nir and HZS. This closed loop of electron flow makes using NO_3^- to oxidize NH_4^+ impossible for AMX as it does not have an enzyme that catalyzes the oxidation of NH_4^+ to N_2: $2\ NH_4^+ \rightarrow N_2 + 6\ e^-$. Therefore this closed loop of electron flow makes using NO_3^- to oxidize NH_4^+ impossible for AMX.

For example. if we break the reaction $5\ NH_4^+ + 3\ NO_3^- \rightarrow 4\ N_2 + 9\ H_2O + 2\ H^+$ into steps carried out by the enzymes, we notice the problem. First, the only reaction that involves nitrate is catalyzed by Nar:

$$NO_2^- + H_2O \rightarrow NO_3^- + 2\ e^- + 2\ H^+$$

The reverse of this reaction is reduction of NO_3^- to NO_2^-.

$$NO_3^- + 2\ e^- + 2\ H^+ \rightarrow NO_2^- + H_2O$$

Suppose $3\ NO_3^-$ is reduced to $3\ NO_2^-$, the $3\ NO_2^-$ can then react with $3\ NH_4^+$, forming $3\ N_2$ and $6\ H_2O$, via the typical anammox pathway catalyzed by Nir, HDH, and HZS

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

Finally, $2\ NH_4^+$ are left to be oxidized to N_2 and produce $6\ e^-$ to reduce the $3\ NO_3^-$ to $3\ NO_2^-$ in the beginning, fulfilling the electron balance. However, such an enzyme is not identified in the world. Therefore, anammox's repertoire of enzymes does not allow them to use NO_3^- to oxidize NH_4^+.

3. Materials and Methods

3.1. Bioreactor setup

We tried to enrich a consortium of comammox and anammox by providing a microaerobic condition and long solid retention time in membrane bioreactors (MBR).

A 1.5 L reactor (Applikon, The Netherlands) was used for the cultivation. The liquid volume was 1.45 L. The temperature was controlled at 25 ℃ by thermostat bath (Lauda, Germany). The pH was controlled at 7.0 by computer, adding base (0.3 M NaOH) if pH was below 6.9. The stirring speed was 200 rpm (SC4, DASGIP, Germany). To avoid growth of phototrophic organisms, the reactor was covered by aluminium foil. The system maintained overpressure of 0.1 bar by a valve connected to the offgas tube. The medium contained 10mM NH_4^+, 2.3 mM NO_3^-, essential minerals and 3.5 mg/L yeast extract. (see Appendix 8.1 for the complete recipe)

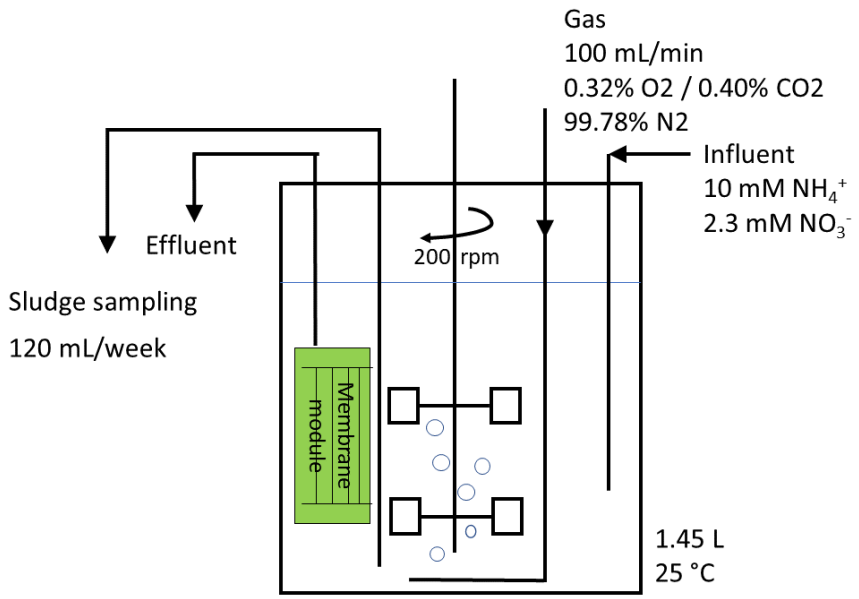


Fig M1. Scheme of the MBR, Reactor 1.

Two reactors were run to try different conditions. The only differences were oxygen transfer rate and medium feed rate. Reactor 1 (R1) oxygen supply was lower, keeping it more hypoxic, Reactor 2 (R2) oxygen transfer rate was higher, in order to accelerate the growth. R1 was operated similar to a sequential batch reactor (SBR) where the fill and decant took place once a week. Every Friday, more than half of the liquid was pumped out through a membrane ultrafiltration module which was placed within the reactor vessel. Then, new medium was fed until reaching the level sensor where the liquid volume would be 1.45L. R2 was run as a continuous stirred-tank reactor (CSTR), with HRT of two days and then later adjusted to one day.

To maintain a microaerobic condition and provide a carbon source, the reactors was sparged continuously at 100 mL/min: R1 with 8 mL/min 4%O_2 5%CO_2 91% N_2 mixed gas

and 92 mL/min N_2, resulting in 0.32% O_2,0.40% CO_2, 99.28% N_2 ingas compostition; R2 with 50 mL/min 4%O_2 5%CO_2 91%N_2 and 50 mL/min N_2 and later adjusted to 100 mL/min 4%O_2 5%CO_2 91% N_2. The flowrate was controlled by Brooks mass flow controller (MFC; Brooks Instrument, Hatfield, PA, USA)

3.2. The membrane module

The membrane module was modified from UF membrane modules ZeeBlok ZBLS 2.5 from SUEZ. The membrane material was PVDF (Polyvinylidene fluoride). The pore size of the membrane was 0.04 μm.

3.3. Inoculum

The inoculum for comammox/anammox enrichment culture was activated sludge (6 g TSS/L, 120mL) and anammox culture (0.26 gVSS/L, 1L). The activated sludge used as inoculum was sampled from the aeration tank of a nearby WWTP. The plant performs biological nutrient removal with an SRT of 25 days and has a PHOSIM-configuration (Van Nieuwenhuijzen et al., 2008). The anammox inoculum was sampled from a highly-enriched planktonic culture of *Ca. Kuenenia stuttgartiensis* (79±4 % as estimated by 16S rRNA gene-based amplicon sequencing analysis). The culture originated from a 10 L (working volum) membrane bioreactor (MBR; pH 7, 30^{o}C) operating at steady state with an average loading rate of ca. 380 mg-N-NO_2^-/L/d. The AMX reactor was maintained by Dr. Micheles Laureni, TU Delft (Soler-Jofra et al., 2020).

3.4. Sampling and storage

Every Monday, 100 mL of broth was taken from the reactor by a 60 mL syringe and transferred to two 50 mL falcon tubes and six 2 mL vials. After being added a few drops of 37% formaldehyde to deactivate the biomass, the falcon tubes were stored in 4 ℃. The six vials were centrifuged under 13000g for 5 minutes, and the supernatant was transferred to new vials, and the precipitates and the supernatant were stored in -20 ℃.

From Tuesday to Friday, 4 mL of broth was taken from the reactor, transferred into two 2mL vials, and centrifuged. The resulting supernatant was transferred into another two 2mL vials. The precipitates and the supernatant were stored in -20 ℃.

3.5. NH_4^+, NO_2^-, NO_3^- measurements

Supernatant samples were thawed and vortexed under room temperature. Concentrations of NH_4^+, NO_2^-, NO_3^- of the supernatant were measured on a Thermo Fisher Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, USA).

In the beginning, while we were trying different cultivation conditions, we also used HACH LANGE toolkit to have a quick look at the NH_4^+, NO_2^-, NO_3^- concentration.

3.6. TSS and VSS measurement

To determine the concentration of biomass, total suspended solids (TSS) and volatile suspended solids (VSS) were measured once per week in triplicates according to the Standard Methods (APHA, 1998).

3.7. Oxygen transfer rate measurements

The offgas composition was determined by a mass spectrometer (PRIMA BT Benchtop, Thermo Scientific, UK). The offgas was dewatered when it left the reactor through a reflux condenser operated at 4 ℃. The MS converted the raw signals of N_2, O_2, CO_2 and Ar into percentage. The oxygen transfer rate was determined by O_2 flow rate of ingas minus offgas. The O_2 flow rate was calculated from gas flow rate times the percentage of O_2. The amount of N_2 in ingas and offgas were assumed to be the same, neglecting the much smaller amount of N_2 generated by bacteria.

3.8. kLa determination

To estimate the required gas supply composition and flow rate, the mass transfer coefficient k_La of oxygen is needed. For k_La determination, the reactor with medium was sparged with N_2 to remove oxygen, and then sparged with air to see the DO increase. The DO was recorded every second (Mettler Toledo, USA).

k_La can then be calculated from the equation:

$$\frac{dC_O}{dt} = k_L a * (C_O{}^* - C_O)$$

C_O is the concentration of oxygen in the solution.
C_O* is the saturated concentration of oxygen in the solution.

After integration, we get

$$-ln(C_O{}^* - C_O) = k_L a * t + constant$$

Because $C_O = C_O{}^* * DO\%$, we can rewrite the equation as

$$-ln(C_O{}^* * 100\% - C_O{}^* * DO\%) = k_L a * t + constant$$

Then we can move $C_O{}^*$ and % to the right-hand side of the equation and get

$$-ln(100 - DO) = k_L a * t + constant$$

As we plot $-ln(100 - DO)$ against time, the slope of the regression line is the k_La.

3.9. Amplicon sequencing of 16S rRNA genes

The microbial diversity of the reactors was characterized by amplicon sequencing of the 16S rRNA genes. Samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V3-4 region of the 16S-rRNA gene (position 341-806) on an Illumina paired-end platform. After sequencing, the raw reads were quality filtered, chimeric sequences were removed and OTUs were generated on the base of ≥ 97% identity. Subsequently, microbial community analysis was performed by Novogene using Mothur & Qiime software (V1.7.0). For phylogenetic determination the most recent SSURef database from SILVA (http://www.arb-silva.de/) was used.

3.10. Fluorescence in situ hybridization (FISH)

To see the abundance of AOB, AMX, and NOB, and how they distribute within flocs, we used FISH. The FISH was performed according to TU Delft Environmental Biotechnology lab's protocol.

4. Results

The goal of this research is to investigate if oxygen limitation enables the enrichment of the AMX+CMX* conversion as proposed in Chapter 2, which is predicted to be thermodynamically most favorable. The hypothesized AMX+CMX* conversion entails the combined consumption of O_2 and NO_3^- as electron acceptor for oxidizing ammonium to dinitrogen gas, and AMX and CMX population increase.

To this end, a 1.45 L membrane bioreactor (MBR) seeded with planktonic anammox and activated sludge were run under O_2 limiting conditions in sequencing batch mode. The medium contained 10 mM NH_4^+, 2.3 mM NO_3^-, 3.5 mg/L yeast extract and minerals (See medium recipe). The main carbon source was CO_2 supplied by gas. The gas supply was fixed at 100mL/min 0.32% O_2 / 0.40% CO_2 / 99.28% N_2 from week 4 on.

The removal of effluent and refill of medium took place once a week (except for initial 10 weeks), making HRT 12 ± 3 days. To be able to enrich CMX which grows slowly, the SRT was uncoupled from the HRT by pumping out effluent through a membrane ultrafiltration module (pore size of 0.04 µm) immersed in the reactor. The SRT was ~85 days caused by sampling. The reactor is still running currently, but no offline data was measured after lockdown due to Covid-19.

To monitor the N compounds concentration and the microbial community structure, ~4mL of sludge samples were taken daily and centrifuged (except for weekends and special days). The NH_4^+, NO_2^-, NO_3^- concentrations of the supernatant were measured, and biomass pellets were stored in -20°C and later sent for 16S rRNA sequencing. Total Suspended Solid (TSS) and Volatile Suspended Solid (VSS) were determined once per week.

Because Reactor 1 has been run for longer period and had more data to analyze, this study is focused on results of Reactor 1. Results of Reactor 2 are put in the appendix.

4.1. N conversion performance

The nitrogen compounds concentrations in supernatant over time are plotted as Fig. 1.

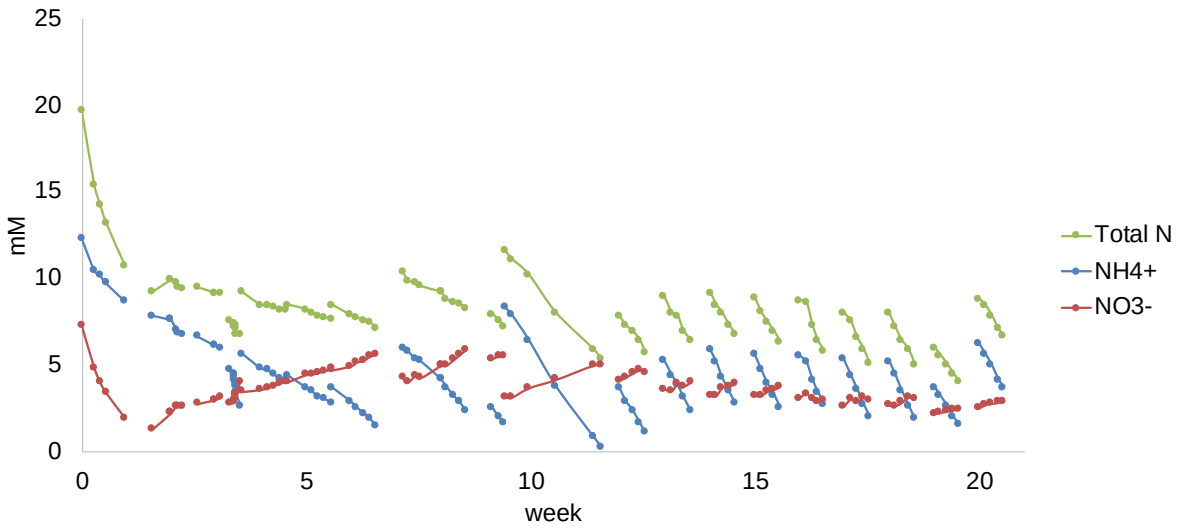


Fig. 1. N compounds concentrations over time in reactor 1. NO_2^- concentrations were below 1% of NO_3^- concentrations at all times.

Total N was calculated by summing up the concentrations of NH_4^+, NO_2^-, and NO_3^-. There was no accumulation of NO_2^- (not shown in the figure). The initial concentration of NH_4^+ was higher than the 10 mM of the medium, due to the high NH_4^+ from the anammox inoculum.

In every batch, concentrations of NH_4^+ and total N decreased. Concentration of NO_3^- increased in every batch, except for week 0. In week 0~11, the refill of medium took place occasionally when the remaining concentrations N compounds were estimated to be insufficient to run for another full week. Starting from week 12, new medium was refilled regularly once per week.

To derive the consumption and production rates of the N compounds, linear regression of concentration (mM) versus time (day) was performed for each batch. The rates over weeks are plotted in Fig.2. The uptake rate of NH_4^+ and total N removal rate increased over time, while NO_3^- production rate remained nearly unchanged.

NH_4^+ uptake rate increased steadily from 0.23 mM/d in week 4 to the highest 0.84 mM/d in week 18. The NH_4^+ uptake rate was always greater than the N removal rate, due to the production of nitrate in the process. The N removal rate increased from 0.14 mM/d in week 4 to 0.74 mM/d in week 17. The NO_3^- production rate fluctuated between 0.07 and 0.24 mM/d, with an average of 0.13 mM/d in week 4~20.

The rates in week 0 were not in the trend as data in other weeks and were not plotted in the figure. Week 0 has the highest N removal rate, 1.3 mM/d, among the weeks, and week 0 is the only week that NO_3^- was consumed instead of produced. This could be caused by heterotrophic denitrification with the large amount of organic in the inoculum.

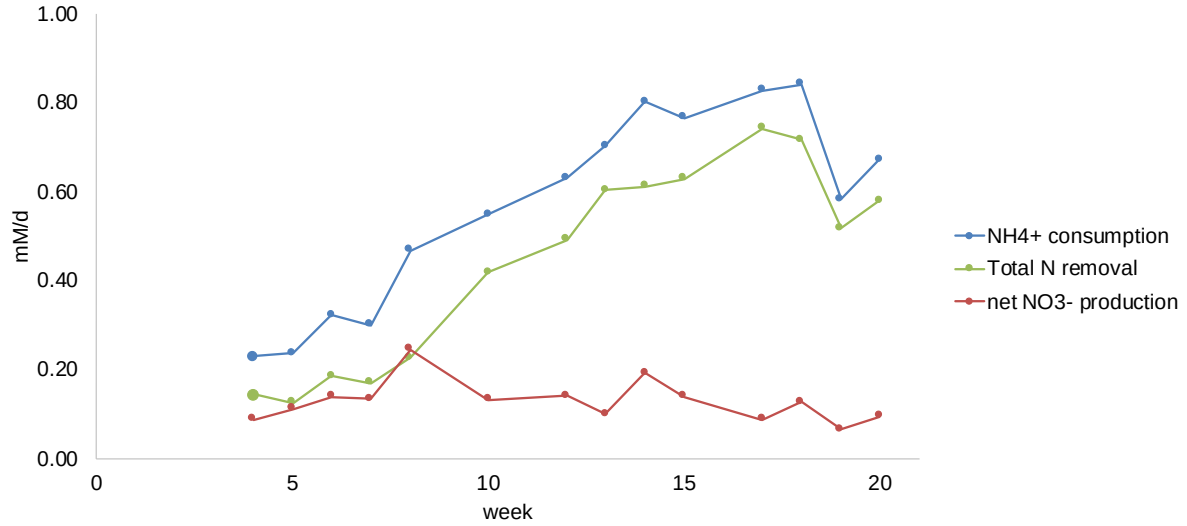


Fig.2. N conversion rates over weeks. The consumption of NH_4^+ and N removal were taken to be positive. The rates were obtained from the regression line slopes of N concentration over days each week. The unit of the rates is mM/day. The observed net NO_3^- rate represents the sum of production and consumption.

To compare the reactor's overall conversion to the theoretical stoichiometry, the ratio of NO_3^- production to NH_4^+ consumption was taken and plotted in Fig.3. The ratio started from 0.38 and increased to 0.52 in week 8, then decreased to 0.14 in week 20.

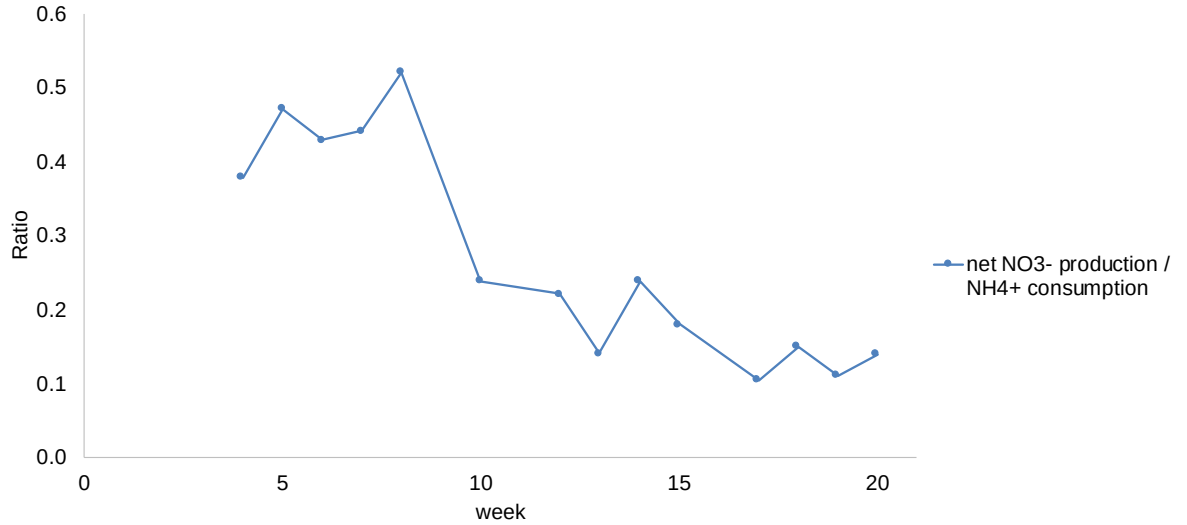


Fig.3. The ratio of net NO_3^- production rate / NH_4^+ uptake rate.

The cultivations are performed under oxygen limitation. Due to the low gas flows, and limited oxygen uptake, gas measurements are complicated. The oxygen transfer rate (OTR) was calculated in two ways: 1) off gas measurements and 2) stoichiometrically derived from N conversion, as plotted in Fig.4.

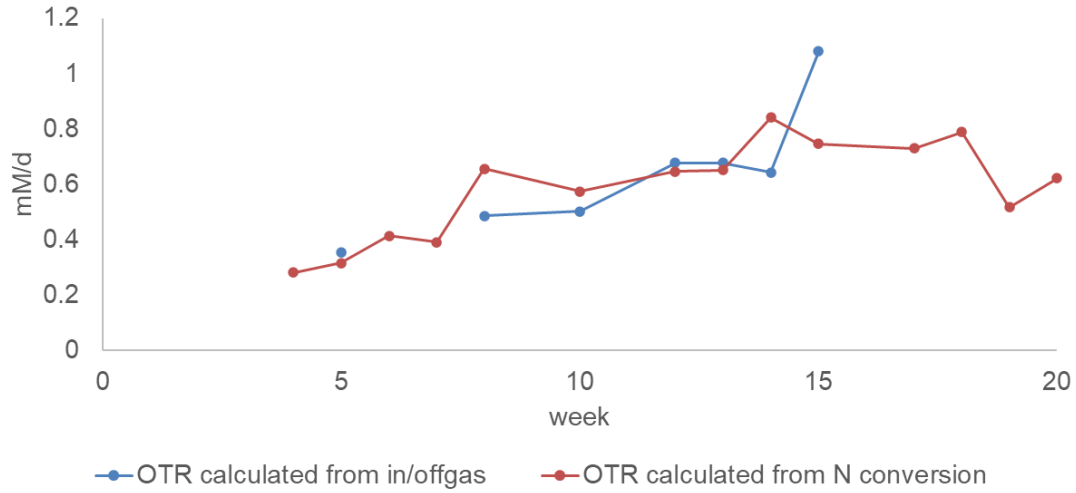


Fig.4. Oxygen Transfer Rate (OTR) over time. An OTR was calculated by in/offgas mass balance. The other OTR was calculated by assuming the ammonium was consumed by AOB+AMX and AOB+NOB's catabolic reactions. OTR = RNO_3^- * 2 + (-RNH_4^+ - RNO_3^-) *0.75.

OTR calculated from N conversion gradually increased from 0.3 mM/d to 0.6 mM/d. OTR calculated from in/offgas mass balance had the same order of magnitude and also showed an increasing trend.

4.2. Biomass concentration

The biomass concentration was monitored by volatile suspended solid (VSS). VSS was taken once per week in triplicate. The average value and the standard deviation were plotted in Fig.5. The biomass concentration of the sludge decreased over time from 1 g/L to 0.05 g/L.

VSS predicted was calculated by dividing VSS by the dilution factor due to sampling, assuming that there was no growth. Every week about 120 mL of sample was taken from the 1450 mL bioreactor, so the dilution factor was 1450/ (1450-120) = 1450/1330. For example, predicted VSS of week 1 = VSS of week 0 * (1330/1450) = 1 g/L * 1330/1450 = 0.92 g/L.

The measured VSS was always lower than the VSS predicted by dilution from week 0. The difference between predicted and measured VSS is considered to be biomass consumption by heterotrophs. The widest gap between measured VSS and predicted VSS was around 0.4 g/L, and the gap started to narrow down from around week 8 on.

The decrease of biomass concentration could also be observed by the opacity decreasing over time. Initially the sludge was thick dark, and gradually it became light and transparent. Membrane fouling was also observed, as biofilm covered the membrane and the reactor

surfaces. The reactor was cleaned at the beginning of week 6 and at the end of week 18 to remove the biofilm. Some of the detached biofilm was put back to the reactor in week 6, resulting in a spike of VSS in week 6.

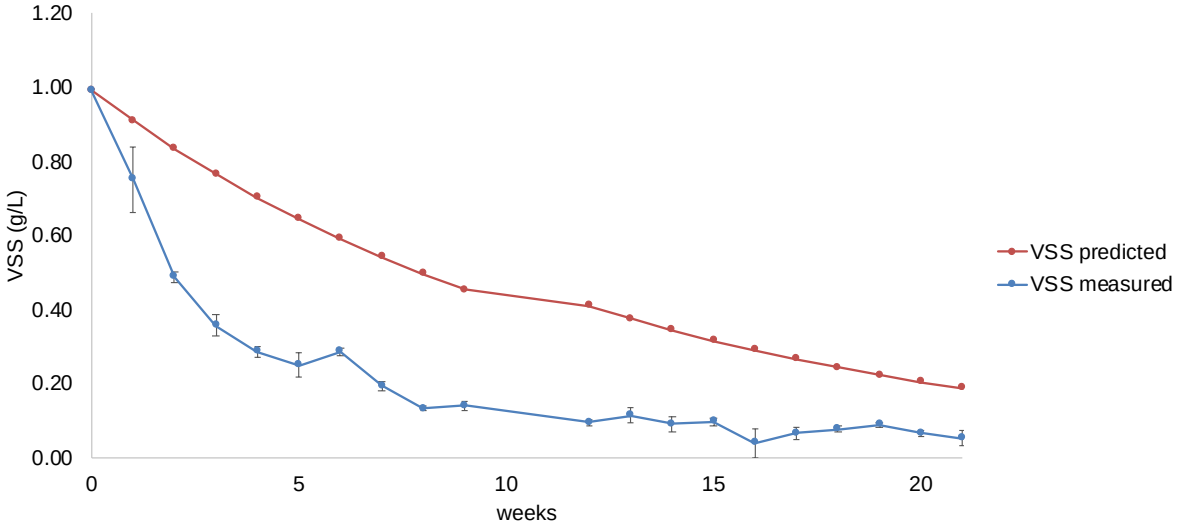


Fig.5 Biomass concentration over time represented by VSS. VSS predicted was calculated from VSS of week 0 divided by the dilution factor, assuming that there was no growth. Week 9~11 had smaller dilution factors because of not adding new medium to the working volume.

4.3. Abundance of AOB, *Nitrospiraceae*, AMX

To know the change of microbial community structure, relative abundance of the dominant OTU's involved in nitrogen conversion was plotted (Fig.6A). Data after week 13 were unavailable due to Covid-19 lockdown.

Interpretation of 16S rRNA data should be taken carefully, since different species may have different amounts of ribosomes in a cell. In addition, some species' ribosomes may not be detected by the primers. Therefore, the relative abundance detected by 16S rRNA does not represent the real abundance of the species. The 16S rRNA trend could somewhat qualitatively reflect the enrichment or elimination of species.

Total AOB (all OTUs affiliated with the family *Nitrosomonadaceae*; no ammonia oxidizing archaea or gammaproteobacterial AOB were detected) increased one order from 1% in week 1 to 13 % in week 13. Interestingly, *Nitrosomonas europaea* was enriched among the AOB population, from 7% to 97% during week 1~13. (Fig.6B)

The OTUs affiliated with the family *Nitrospiraceae* (which NOB and CMX belong) consisted of *Nitrospira defluvii* and two unidentified genera. The OTU of *Nitrospira defluvii,* a typical NOB (Lücker et al., 2010), accounted for >99.5% reads within the family *Nitrospiraceae* of every sample. The relative abundance of *Nitrospiraceae* stayed stable around 1~3% during week 1~13. *Nitrobacter,* another kind of NOB, was not detected.

AMX (all OTUs affiliated with the family *Brocadiaceae*) dropped 2.5 orders from 12% to 0.03% during week 1~8, then rose 1.5 orders to 0.8% during week 8~13. In every sample, *Candidatus Keunenia* accounts for >95% of AMX, and *Candidatus Brocadia* <5%, no other AMX genera were detected.

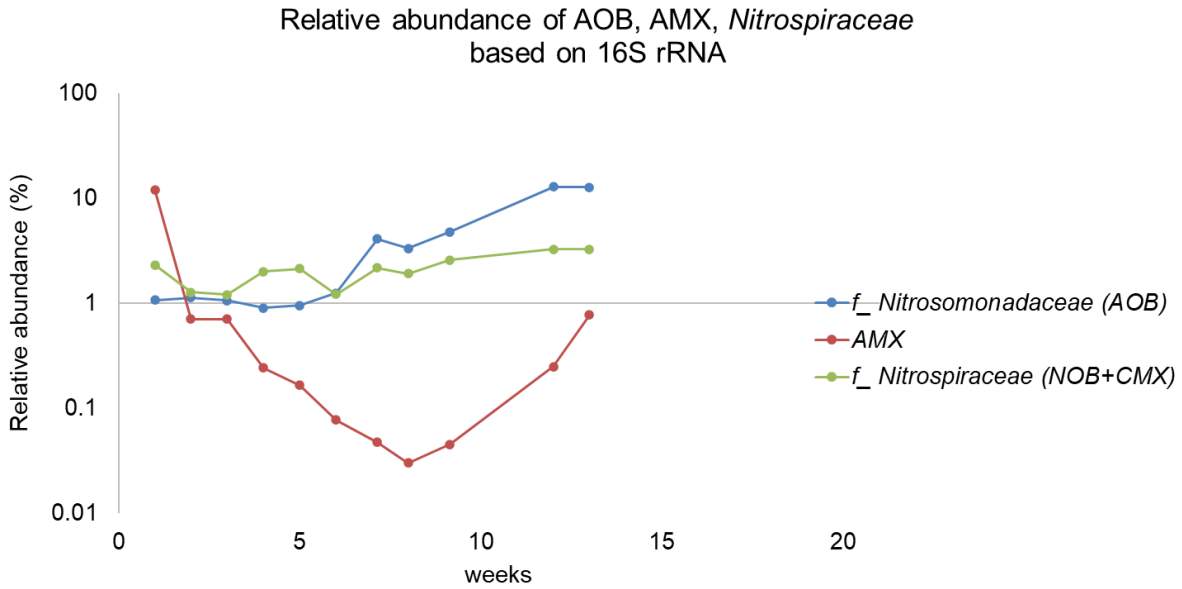


Fig 6A. Relative abundance of total AOB (*Nitrosomonadaceae*), AMX, *Nitrospiraceae* (NOB and CMX) based on 16S rRNA. The y-axis is on a logarithmic scale.

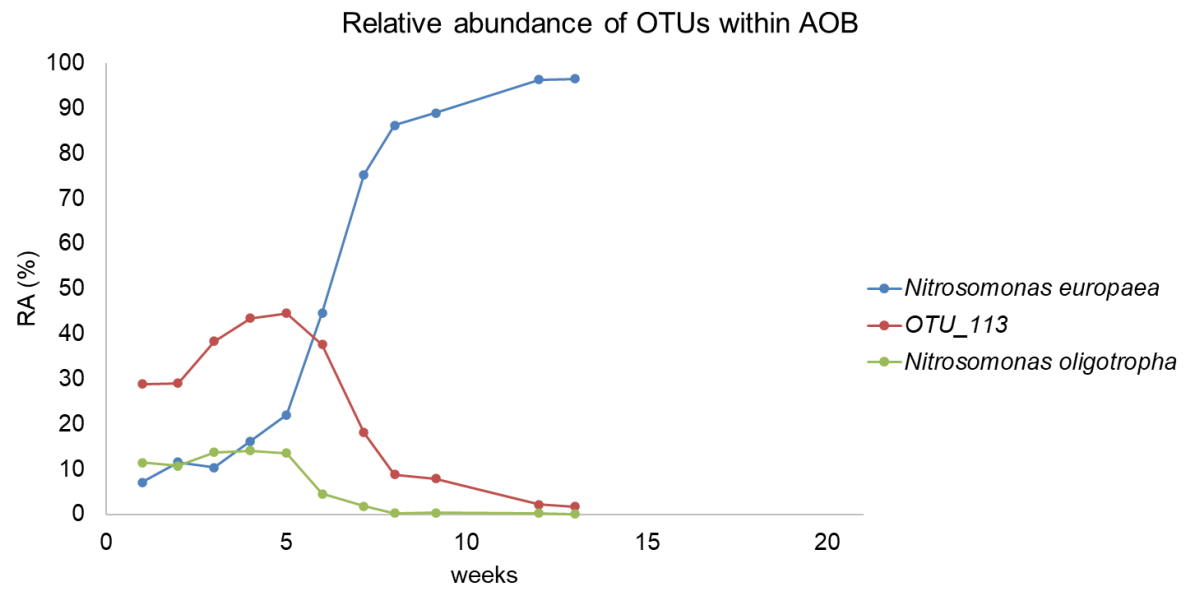


Fig. 6B Relative abundance of OTUs within AOB based on 16S rRNA reads. There were 24 OTUs detected to be affiliated with family *Nitrosomonadaceae*, and three major OTUs were plotted.

To estimate the absolute biomass concentration of different microbes, the relative abundance of 16S rRNA was multiplied by the total VSS (Fig.5C). Using this approach, it was estimated that the AOB concentration decreased from $8*10^{-3}$ to $2.4*10^{-3}$ g/L in week 1~5 then increased to $1.4*10^{-2}$ g/L in week 13. Concentration of AOB started to surpass family *Nitrospiraceae* from week 7. Family *Nitrospiraceae* decreased a little from $1.7*10^{-2}$ to $3.7*10^{-3}$ g/L in week 1~13. The AMX concentration dropped 3.5 orders from $9.0*10^{-2}$ to $4.0*10^{-5}$ g/L in week 1~8, and then rose to $8.8*10^{-4}$ g/L in week 13.

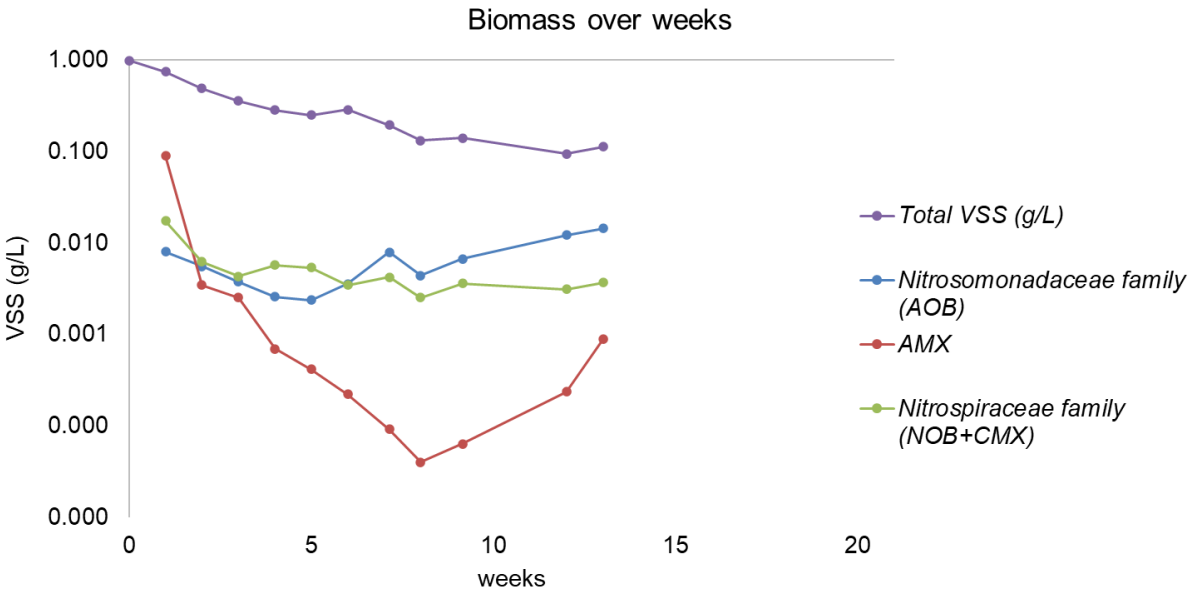


Fig 6C. Biomass concentration of *Nitrosomonadaceae* (AOB), *Kuenenia* (AMX), *Nitrospiraceae* (NOB and CMX), calculated by VSS * 16S rRNA relative abundance. The y-axis is on a logarithmic scale.

4.4. Abundance of other microbes

Besides AOB, *Nitrospira*, and AMX, the abundance of other bacteria was also investigated (Fig.7A & 7B). The top two OTUs which had most reads in week 13 were genus *Ignavibacterium* and genus *Denitratisoma*. The biomass concentration of them were also estimated by total VSS * 16S rRNA relative abundance.

Ignavibacterium was the most abundant microbe, climbing steadily from 12% in week 1 to 47% in week 8, then decreasing to 38% in week 13. The VSS of *Ignavibacterium* increased a bit to 110 mg/L in week 1~8, then decreased to 40 mg/L in week 13.

The second most abundant microbe, *Denitratisoma*, increased from 0.5 to 5%. The VSS of *Denitratisoma* rose from 3.5 mg/L in week 1 to 15 mg/L in week 2 and then descended to 6.0 mg/L in week 13. *Denitratisoma* and family *Nitrospiraceae* had the same order of magnitude of relative abundance, and their VSS showed similar little decrease over time.

The rest of the OTUs were combined as “Others” and saw a steady decrease from 82% in week 2 to 40% in week 13. The VSS of “Others” decreased from 540 to 50 mg/L.

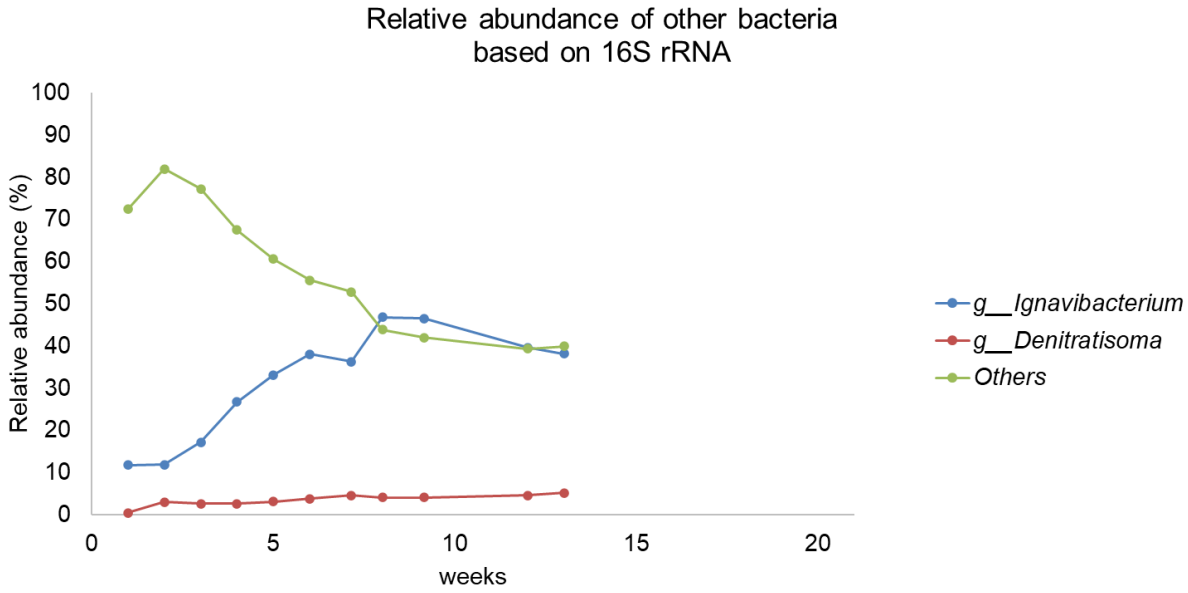


Fig.7A Relative abundance of other bacteria. Genus *Ignavibacterium* and genus *Denitratisoma* were the top two OTUs of the relative abundance in week 13. The rest OTUs were collectively called “Others”.

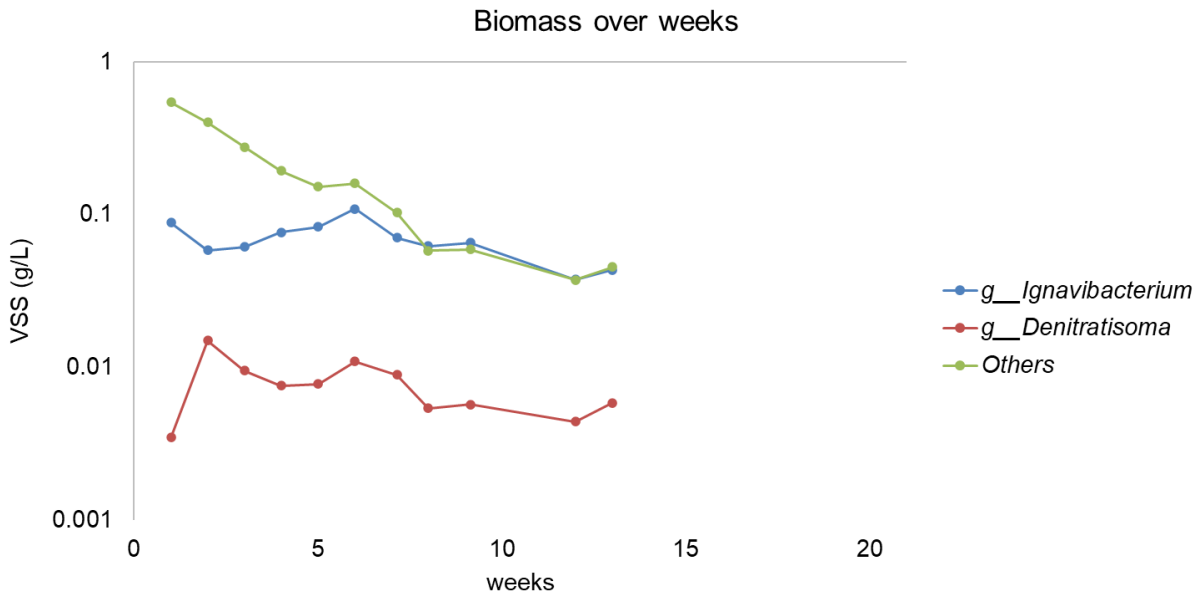


Fig.7B Biomass concentrations of other bacteria calculated by VSS * 16S rRNA relative abundance. The y-axis is on a logarithmic scale.

5. Discussion

5.1. Nitrogen conversions established in oxygen limiting conditions

A membrane bioreactor operated as a sequencing batch reactor was successfully started up to investigate ammonium degradation in conditions of severe oxygen limitation. Besides small amounts of oxygen, nitrate was supplied to the reactor to investigate the potential use of nitrate as electron acceptor in the ammonium removal process. After approximately 13 weeks, a stable conversion was established in the system. The conversion observed concerned the oxidation of ammonium to dinitrogen gas, with the production of small amount of nitrate as side-product with a nitrate yield on ammonium of approximately 0.1 mol/mol. The increase in NO_3^- concentration suggests that the hypothesized comammox conversion involving nitrate reduction to nitrite was not dominant. Instead, the conversion observed was close to the theoretical stoichiometry of a coculture consisting of AOB and AMX.

The attribution of the overall conversion observed to the combined activity of both AOB and AMX was only partly confirmed by the development of the microbial community structure in time. Whereas the relative abundance of the AOB family of *Nitrosomonadacea* increased in time as expected, the relative abundance of AMX decreased first, but increased from week 8 on. To our surprise, OTUs affiliated with the family *Nitrospiraceae* were effectively maintained in the reactor and the relative abundance remained largely constant. What role the *Nitrospiraceae* have in the overall conversion remains unclear.

The eventual reason why the CMX conversion proposed was not observed in this work remains to be elucidated. Of course, it can be true that no microorganism exists that is capable of using nitrate besides oxygen for ammonium oxidation to nitrite. This would limit the ecological role of CMX to full oxidation of ammonium to nitrate, but it would also make it very difficult to explain why Van Kessel found CMX *Nitrospirae* in her enrichment study. Another explanation can be found in the time used for the experiment: Even though the bioreactor was in operation for more than 140 days it may have been inadequate for nitrate reducing CMX to become a dominant process. The first enrichment of comammox under hypoxia took one year (Daims et al., 2015). Another group observed comammox started to accumulate after 200 days of cultivation (Roots et al., 2019). Given the generation time (SRT) in the bioreactor of approximately 80 days, it may take more generations for nitrate reducing CMX to become a dominant factor in the conversions observed. Other factors limiting the proliferation of nitrate reducing CMX may include deficiencies in the medium or the use of an inadequate inoculum.

Although the hypothesized CMX* reaction was not observed, the development over time with respect to nitrogen removal, oxygen consumption, and microbial community development are highly interesting and shed some light on nitrogen removal in hypoxic conditions.

5.2. Development of the conversion - Oxygen Transfer Rate (OTR) increased

In time the ammonium uptake rate in the process increased from 0.23 mM/d in week 4 to 0.67 mM/d in week 20 (Fig.2). Since the nitrate production rate remained largely unaffected in this period, this suggests a significant increase in oxygen uptake rate (Fig.4, OTR calculated from N conversion). This is a remarkable observation since we assumed that oxygen limitation occurred at all times, and at a liquid concentration oxygen of 0 mg/L, a constant oxygen uptake rate is to be expected. The main cause might be explained as follows:

Initially the oxygen was mainly used for heterotrophic oxidation of inactive biomass, and as inactive biomass dwindled, the system transitioned toward oxidation of more ammonium to nitrite. This possibility is supported by VSS measurement, where there was obvious biomass loss. initially. Later, the depletion of organic matter shifted the microbial community towards consuming more ammonia. This is further supported by 16S rRNA data, where heterotroph *Ignavibacterium* became dominant initially and later decreased in population (Fig.7). It has been shown that *Ignavibacterium* is heterotroph, so it is possible that the initial OTR was consumed by *Ignavibacterium* to degrade VSS (Iino, 2014; Iino et al., 2010).

Estimation of OTR consumed by VSS supports this speculation:
Since the VSS loss seemed to start to narrow down from week 8, and *Ignavibacterium* kept increasing until week 8, the VSS loss until week 8 was taken to estimate the OTR.
The VSS loss until week 8 was ~ 0.4 g/L. Assume VSS has formula $CH_{1.8}O_{0.5}N_{0.2}$, and the carbon is oxidized to CO_2, there would be 4.2 electrons transfer. The average OTR consumed by VSS would be:

$$\frac{0.4\ gVSS/L}{8\ weeks * \ 7\frac{days}{week} * 24.6\ gVSS/mol} * \frac{4.2\ mol_{O2}}{4\ \ mol_{VSS}} * 1000\frac{mmol}{mol} = 0.3\frac{mmol}{L_{reactor} * d}$$

The estimated 0.3 mmol/L-reactor/d of OTR consumed by VSS matches the OTR increase calculated from N conversion over the process, which is also ~0.3 mmol/L-reactor/d.

5.3. Development of the Microbial Community

5.3.1 Among AOB, Only *Nitrosomonas europaea* was enriched

AOB was enriched by about one order, (Fig.5) matching the increase of NH_4^+ consumption rate from 0.23 to 0.70 mM/d in week 4~13 (Fig.2). Among the detected AOB, only *Nitrosomonas europaea* was enriched, indicating that it has the highest affinity for O_2 among AOB. In this study, the saturated DO* was ~4.4 μM, and the real DO would be below this value. Considering the K_m = 1-15 μM O_2 in literature (Laanbroek &Gerards, 1993), this suggests that *N. europaea* might be an important nitrifier in hypoxic conditions.

Relative abundance of *Nitrosomonas oligotropha* decreased, probably because it has lower oxygen affinity (K_m = 38.1 +/- 13 μM) (H. D.Park &Noguera, 2007). However, this explanation contradicts with Gieseke, Purkhold, Wagner, Amann, &Schramm, 2001, who found *Nitrosomonas oligotropha* dominated the deeper layers of a phosphate-removing biofilm where DO < 3.4 μM.

5.3.2 NOB relative abundance remained almost unaffected

The *Nitrospiraceae* relative abundance remained stagnant around 2~3% in week 4~13. The typical NOB *Nitrospira defluvii* accounted for >99.5% of *Nitrospiraceae* OTUs, suggesting that NOB was dominant among *Nitrospiraceae* and no sign of CMX being enriched. Estimated NO_3^- production by NOB and AMX together matches the measured NO_3^- production, so the NOB was likely doing its typical job, oxidizing nitrite to nitrate, instead of other reaction during week 4~13 (Box 1, next page).

However, it remained unclear why NOB population stayed nearly unchanged. In theory, NOB should compete for oxygen with AOB and for nitrite with AMX. Ma et al. tried to enrich AMX and suppress NOB by low DO (average DO = 4.7 μM) in SBR, however, they were unable to eliminate NOB, and found NOB to maintain 2-2.6% of all bacteria detected by FISH (Ma et al., 2015). Paul Roots et al. used low DO but could not suppress the full nitrification either (Roots et al., 2019). Even though NOB *Nitrospira* has been reported to have comparable oxygen affinity (K_O = 4-17 μM) as AOB (K_O = 1-15 μM), it harvests less energy per N or per O_2 than AOB (Theory 2.1, Table 1). (Blackburne, Vadivelu, Yuan, &Keller, 2007; R.Manser, Gujer, &Siegrist, 2005; RetoManser, Gujer, &Siegrist, 2005; M. R.Park, Park, &Chandran, 2017) (Laanbroek &Gerards, 1993) Furthermore, NOB *Nitrospira*'s nitrite affinity (K_m = 9-27 μM, (Nowka, Daims, &Spieck, 2015; Schramm, DeBeer, Van DenHeuvel, Ottengraf, &Amann, 1999) is lower than AMX *Keunenia* (K_m = 0.2-3 μM, (Egli et al., 2001; van derStar et al., 2008a,b)). It is remarkable that NOB could survive while competing with AOB and AMX.

Box 1. Estimation of NO_3^- produced by NOB and AMX

NOB

NO_3^- produced by NOB can be estimated from its growth rate. The growth rate is assumed to be the same as its biomass removal rate due to sampling, because biomass concentration seemed stagnant (Fig. 6C). The biomass concentration was estimated by VSS times 16S rRNA relative abundance. Although 16S rRNA relative abundance usually does not represent the real relative abundance of species, the 16S rRNA relative abundance might be close to the real relative abundance of NOB. It is because 2-3% 16S rRNA in this study matches other research's 2~2.6% detected by FISH with similar condition (Ma et al., 2015).

In week 4~13 the estimated *Nitrospiraceae* concentration around 4 mg/L (Fig.6C), and every week ~120 mL of sludge was sampled out of the ~1450 mL reactor. Assuming all *Nitrospiraceae* was NOB, the weekly NOB biomass loss should be: 4 * 120 / 1450 = 0.33 mg/L/week. To compensate for the biomass loss, the growth rate equals the loss rate, so the NO_3^- production rate due to NOB growth is:

$$\frac{0.331\ mg/L/week\ *\ 44}{7\ d/week\ *\ 24.6\ g/mol} = 0.085\ mM/d$$

The 44 comes from the stoichiometry of NOB metabolism, where 1 mole of biomass production is coupled with 44 moles of NO_3^- production. NOB metabolism (See Theory 2.5, NOB Met):

$$0.2\ NH_4^+ + 44\ NO_2^- + CO_2 + 20.9\ O_2 + 0.6H_2O \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 44\ NO_3^- + 0.2\ H^+$$

AMX

According to AMX metabolism (See Theory 2.5, AMX Met):

$$9.3\ NH_4^+ + 11.2\ NO_2^- + CO_2 \rightarrow 9.1\ N_2 + 2.1\ NO_3^- + CH_{1.8}O_{0.5}N_{0.2} + 17.7\ H_2O + 0.2\ H^+$$

1 mole of biomass production is coupled with 2.1 moles of NO_3^- production, and 18.4 moles of N are removed [9.1 (N_2) * 2 + 0.2 ($CH_{1.8}O_{0.5}N_{0.2}$) =.18.4 N]. So, the ratio of NO_3^- production to N removal is 2.1/18.4. The N removal rate was between 0.15 ~ 0.7 mM/d, and this multiplied by the 2.1/18.4 ratio corresponds to 0.017 ~ 0.08 mM/d of NO_3^- production, under the assumption that the nitrogen removal was only done by AMX.

Estimated total NO_3^- production by NOB and AMX

= 0.085 mM/d (NOB) + 0.017~0.08 mM/d (AMX)

= 0.10~0.17 mM/d

This range matches the measured 0.09~0.14 mM/d NO_3^- production rate in week 4~13 (except for the 0.24 mM/d outlier of week 8).

5.3.3 AMX decreased and then increased

AMX population initially dropped and increased later (Fig.6). Probably the inoculated AMX was excessive and a large portion was decayed. One cause for the initial drop could be that the NO_2^- was too scarce to maintain the large amount of the inoculated AMX. The medium recipe contained no NO_2^-, whereas the AMX mother reactor had 27 mM/d supply rate. There could be other reasons that lead to the drop initially, but they were not the focus of this study, as long as the AMX population increased eventually.

5.3.4 Other bacteria seemed to play important roles in the N conversions

It is unexpected to observe that the dominant microbe was not the conventional AOB, AMX or NOB. Instead, *Ignavibacterium* dominated, and *Denitratisoma* had the comparable relative abundance to NOB.

The *Ignavibacterium* relative abundance increased from 12% to 47% in week 1~8, and decreased later in week 8~13 (Fig.7). This somewhat fits with the trend of VSS loss, where the reactor kept losing biomass until around week 8 (Fig.5). *Ignavibacterium* has been described as a heterotrophic bacterium, potentially consuming organic compounds produced upon cell lysis (Iino, 2014; Iino et al., 2010). In the initial weeks it probably consumed significant portion of OTR to degrade organic matter as discussed in 5.2, and later it might use nitrate or nitrite to consume organic matter.

6. Conclusions and Recommendations

6.1. Conclusions

Van Kessel's enrichment of comammox under hypoxic condition and our biochemistry model lead to the hypothesis that in oxygen limiting conditions, comammox oxidizes one ammonium by one nitrate and one oxygen, producing two nitrite, by using the enzymes AMO, HAO, and reversing NXR:

$$NH_4^+ + NO_3^- + O_2 \rightarrow 2\ NO_2^- + H_2O + 2\ H^+$$

And the nitrite produced by comammox makes it form cross-feeding symbiosis with anammox, which is predicted to be the most thermodynamically favorable conversion under oxygen limitation and presence of ammonium and nitrate.

A membrane bioreactor operated as a sequencing batch reactor was successfully started up to investigate ammonium degradation in conditions of severe oxygen limitation. During 140 days of cultivation, the system developed to the conversion of AOB+AMX, but the hypothesized CMX* + AMX conversion was not achieved. The community was enriched toward lower NO_3^- production / NH_4^+ uptake ratio, indicating the community and N conversion developed toward utilizing more ammonium under oxygen limitation. *N.europaea,* an AOB, was enriched in this study, which had DO < 4.4 μM, suggesting it might play an important role in nitrification in very low DO conditions.

6.2. Recommendations

1. Continue running the reactor to see if the hypothesized comammox reaction would appear and investigate what other *Nitrospiracaea* are doing in oxygen limiting conditions.

2. Continue monitoring how *Ignavibacterium* and *Denitratisoma* develop with N conversions to understand their contribution to denitrification. It is expected that they will keep decreasing until the population reaches equilibrium with the organic in the medium.

3. To accelerate the investigation of comammox's behaviour under hypoxia, it is recommended to use enriched comammox inoculum, and see if it survives the cultivation and whether it is doing the hypothesized conversion.

4. Gas flow controller might shift over time and had higher flow rate or higher O_2% as can be seen from OTR calculated from in/offgas. In addition, both OTR calculated from in/offgas and N conversion are greater than the maximum OTR estimated by k_La (0.278 mM/d). To have more accurate OTR measurement, gas flow rate and ingas composition should be checked regularly. Recirculation of gas supply could also improve the OTR measurement, by causing the offgas composition to have more pronounced difference form the ingas.

7. References

APHA. (1998). Standard methods for the examination of water and wastewater (20th ed.) Washington DC: American Public Health Association

Blackburne, R., Vadivelu, V. M., Yuan, Z., &Keller, J. (2007). Kinetic characterisation of an enriched Nitrospira culture with comparison to Nitrobacter. *Water Research*, *41*(14), 3033–3042. https://doi.org/10.1016/j.watres.2007.01.043

Costa, E., Pérez, J., &Kreft, J.-U. (2006). Why is metabolic labour divided in nitrification? *Trends in Microbiology*, *14*(5), 213–219. https://doi.org/10.1016/J.TIM.2006.03.006

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., …Wagner, M. (2015). Complete nitrification by Nitrospira bacteria. *Nature*, *528*(7583), 504–509. https://doi.org/10.1038/nature16461

Dimitri Kits, K., Sedlacek, C. J., Lebedeva, E.V., Han, P., Bulaev, A., Pjevac, P., …Wagner, M. (2017). Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature*, *549*(7671), 269–272. https://doi.org/10.1038/nature23679

Egli, K., Fanger, U., Alvarez, P. J. J., Siegrist, H., Van derMeer, J. R., &Zehnder, A. J. B. (2001). Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. *Archives of Microbiology*, *175*(3), 198–207. https://doi.org/10.1007/s002030100255

Gieseke, A., Purkhold, U., Wagner, M., Amann, R., &Schramm, A. (2001). Community Structure and Activity Dynamics of Nitrifying Bacteria in a Phosphate-Removing Biofilm. *Applied and Environmental Microbiology*, *67*(3), 1351–1362. https://doi.org/10.1128/AEM.67.3.1351-1362.2001

Hoekstra, M. (2017). *Mainstream anammox, potential & feasibility of autotrophic nitrogen removal*. https://doi.org/10.4233/UUID:94B5831B-D22D-4FB2-8122-4D4300AE4526

Iino, T. (2014). The family ignavibacteriaceae. In *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea* (Vol. 9783642389542, pp. 701–703). https://doi.org/10.1007/978-3-642-38954-2_143

Iino, T., Mori, K., Uchino, Y., Nakagawa, T., Harayama, S., &Suzuki, K. I. (2010). Ignavibacterium album gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from microbial mats at a terrestrial hot spring and proposal of Ignavibacteria classis nov., for a novel lineage at the periphery of green sulfur bacteria. *International Journal of Systematic and Evolutionary Microbiology*, *60*(6), 1376–1382. https://doi.org/10.1099/ijs.0.012484-0

Kartal, B., vanNiftrik, L., Keltjens, J. T., Op den Camp, H. J. M., &Jetten, M. S. M. (2012).

Anammox-Growth Physiology, Cell Biology, and Metabolism. In *Advances in Microbial Physiology* (Vol. 60, pp. 211–262). https://doi.org/10.1016/B978-0-12-398264-3.00003-6

Kleerebezem, R., &VanLoosdrecht, M. C. M. (2010, January). A generalized method for thermodynamic state analysis of environmental systems. *Critical Reviews in Environmental Science and Technology*, Vol. 40, pp. 1–54. https://doi.org/10.1080/10643380802000974

Laanbroek, H. J., &Gerards, S. (1993). Competition for limiting amounts of oxygen between Nitrosomonas europaea and Nitrobacter winogradskyi grown in mixed continuous cultures. *Archives of Microbiology*, *159*(5), 453–459. https://doi.org/10.1007/BF00288593

Lawson, C. E., &Lücker, S. (2018). Complete ammonia oxidation: an important control on nitrification in engineered ecosystems? *Current Opinion in Biotechnology*, *50*, 158–165. https://doi.org/10.1016/J.COPBIO.2018.01.015

Lücker, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B., …Daims, H. (2010). A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(30), 13479–13484. https://doi.org/10.1073/pnas.1003860107

Ma, B., Bao, P., Wei, Y., Zhu, G., Yuan, Z., &Peng, Y. (2015). Suppressing nitrite-oxidizing bacteria growth to achieve nitrogen removal from domestic wastewater via anammox using intermittent aeration with low dissolved oxygen. *Scientific Reports*, *5*(1), 1–9. https://doi.org/10.1038/srep13048

Manser, R., Gujer, W., &Siegrist, H. (2005). Membrane bioreactor versus conventional activated sludge system: Population dynamics of nitrifiers. *Water Science and Technology*, *52*(10–11), 417–425. https://doi.org/10.2166/wst.2005.0719

Manser, Reto, Gujer, W., &Siegrist, H. (2005). Consequences of mass transfer effects on the kinetics of nitrifiers. *Water Research*, *39*(19), 4633–4642. https://doi.org/10.1016/j.watres.2005.09.020

Nowka, B., Daims, H., &Spieck, E. (2015). Comparison of oxidation kinetics of nitrite-oxidizing bacteria: Nitrite availability as a key factor in niche differentiation. *Applied and Environmental Microbiology*, *81*(2), 745–753. https://doi.org/10.1128/AEM.02734-14

Park, H. D., &Noguera, D. R. (2007). Characterization of two ammonia-oxidizing bacteria isolated from reactors operated with low dissolved oxygen concentrations. *Journal of Applied Microbiology*, *102*(5), 1401–1417. https://doi.org/10.1111/j.1365-2672.2006.03176.x

Park, M. R., Park, H., &Chandran, K. (2017). Molecular and Kinetic Characterization of

Planktonic Nitrospira spp. Selectively Enriched from Activated Sludge. *Environmental Science and Technology*, *51*(5), 2720–2728. https://doi.org/10.1021/acs.est.6b05184

Roots, P., Wang, Y., Rosenthal, A. F., Griffin, J. S., Sabba, F., Petrovich, M., …Wells, G. F. (2019). Comammox Nitrospira are the dominant ammonia oxidizers in a mainstream low dissolved oxygen nitrification reactor. *Water Research*, *157*, 396–405. https://doi.org/10.1016/J.WATRES.2019.03.060

Schramm, A., DeBeer, D., Van DenHeuvel, J. C., Ottengraf, S., &Amann, R. (1999). Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: Quantification by in situ hybridization and the use of microsensors. *Applied and Environmental Microbiology*, *65*(8), 3690–3696. https://doi.org/10.1128/aem.65.8.3690-3696.1999

Van DeGraaf, A. A., DeBruijn, P., Robertson, L. A., Jetten, M. S. M., &Kuenen, J. G. (1996). Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology*, *142*(8), 2187–2196. https://doi.org/10.1099/13500872-142-8-2187

van derStar, W. R. L., Miclea, A. I., vanDongen, U. G. J. M., Muyzer, G., Picioreanu, C., &vanLoosdrecht, M. C. M. (2008). The membrane bioreactor: A novel tool to grow anammox bacteria as free cells. *Biotechnology and Bioengineering*, *101*(2), 286–294. https://doi.org/10.1002/bit.21891

Van DerStar, W. R. L., Van DeGraaf, M. J., Kartal, B., Picioreanu, C., Jetten, M. S. M., &VanLoosdrecht, M. C. M. (2008). Response of anaerobic ammonium-oxidizing bacteria to hydroxylamine. *Applied and Environmental Microbiology*, *74*(14), 4417–4426. https://doi.org/10.1128/AEM.00042-08

vanKessel, M. A. H. J., Speth, D. R., Albertsen, M., Nielsen, P. H., Op den Camp, H. J. M., Kartal, B., …Lücker, S. (2015). Complete nitrification by a single microorganism. *Nature*, *528*(7583), 555–559. https://doi.org/10.1038/nature16459

VanNieuwenhuijzen, A. F., VanBentem, A. G. N., Buunnen, A., Reitsma, B. A., Uijterlinde, C. A., &VanNieuwenhuijzen, A. A. F. (2008). *The limits and ultimate possibilities of technology of the activated sludge process*. https://doi.org/10.2166/wst.2008.545

8. Appendix

8.1. Comammox medium recipe

1. Concentrated stock solutions preparation (in demi water):

		g/L	mol/L_conc
Mg^{2+}	MgSO4*7H2O	160.34	0.6505
Ca^{2+}	CaCl2*2H2O	239.95	1.6322
Fe^{2+}	FeSO4*7H2O	9.12	0.0328
	EDTA·2Na·2H_2O	6.36	0.0171

They were prepared in a 1L volumetric flask

2. TES

	weight (g)	mol/L_TES
EDTA·2Na·2H_2O	19.07	0.051230
$ZnSO_4$ · 7 H_2O	0.4456	0.001550
$CoCl_2$ · 6 H_2O	0.2396	0.001845
$MnSO_4$ · H_2O	0.8550	0.005059
$CuSO_4$ · 5 H_2O	0.2651	0.001062
$(NH_4)_6Mo_7O_{24}$ · 4 H_2O	0.2243	0.000181
$NiCl_2$ · 6 H_2O	0.1978	0.000832
$HNaSeO_3$	0.0887	0.000588
H_3BO_3	0.0147	0.000238
Na_2WO_4 · 2 H_2O	0.0538	0.000163

about ~ NaOH 1.5 g was added to adjust the pH to 6

The chemicals were dissolved in 1L demi water in a volumetric flask.
After adding all chemicals into demi water, the pH was 3.83, and there were undissolved solids despite stirred vigorously. After the pH was adjusted to 6.03 by adding 15 pellets (~1.50g) of NaOH, all the solids dissolved.

3. The comammox medium

Medium volume		**~20 L**
K_2HPO_4	[g]	2.95
KH_2PO_4	[g]	0.44
$(NH_4)_2SO_4$	[g]	13.21
$NaNO_3$	[g]	3.94
TES	[mL]	31.25
Mg^{2+}	[mL]	15.6
Ca^{2+}	[mL]	7.80
Fe^{2+}	[mL]	62.5
yeast extract	[g]	0.070
water + chemical Total Weight [kg]	[kg]	20.0128

The pH of the medium was 7.12. The medium was then autoclaved in 121 ^oC overnight. The medium bottle was wrapped in aluminium foil to prevent light.

The ion concentration in the medium is arranged in the following table:

	mM
NH_4^+	10.0
NO_2^-	0
NO_3^-	2.3
$H_2PO_4^-/HPO_4^{2-}$	1.0
Cl^-	1.3
SO_4^-	5.6
Na^+	2.6
K^+	1.9
HCO_3^-	0
Ca^{2+}	0.64
Fe^{2+}	0.10
Cu^{2+}	1.66E-03
Mg^{2+}	0.51
Zn^{2+}	2.42E-03
Mn^{2+}	7.90E-03
Fe^{3+}	0
Co^{2+}	2.88E-03
Ni^{2+}	1.30E-03
H_3BO_3	3.71E-04
SeO_3^{2-}	9.17E-04
SeO_4^{2-}	0
WO_4^{2-}	2.55E-04
MoO_4^{2-}	1.98E-03
EDTA	0.13
I^-	0
yeast extract	3.5 mg/L

8.2. Selection of medium

The purpose of this section is to explain how I decided the recipe of medium and what I learned from reviewing papers.

My first thought was to get the same medium as van Kessel's, but it was impossible for us to get the aquaculture recirculation water. Furthermore, to calculate NH_4^+ and NO_3^- conversion rates, it would be much easier to use synthetic wastewater.

Since this study focuses on nitrification, the first thing to be determined was the NH_4^+ and NO_3^- concentration. It took one year for van Kessel to enrich CMX with 0.5 mM NH_4^+, but in order to shorten the enrichment to a few months, hoping to finish this within the master thesis time, we decided to use 10 mM NH_4^+. Then, based on the stoichiometry for
AMX + CMX*
$2.7\ NH_4^+ + 0.6\ NO_3^- + 0.2\ CO_2 + O_2 \rightarrow 1.7\ N_2 + 0.24\ CH_{1.8}O_{0.5}N_{0.2} + 4.2\ H_2O + 2.1\ H^+$
the NO_3^- / NH_4^+ is 0.6/2.7, so NO_3^- = 10 *0.6/2.7 = 2.3 mM

The next step was to decide the minerals recipe. While Daims enriched CMX with synthetic medium, they used $CaCO3_{(s)}$ to maintain pH, which does not work with continuous systems. In addition, they did not explain how they decided the concentrations of the minerals. Therefore, I looked up several papers, trying to find out the reasons behind using those concentrations of minerals. After reading so much, I realized that I cannot just find a universal medium or any explanation on why the recipes were so. I also learned that although the compositions differed, all the media worked, so I do not have to worry about using a "wrong" medium, I can just pick one.

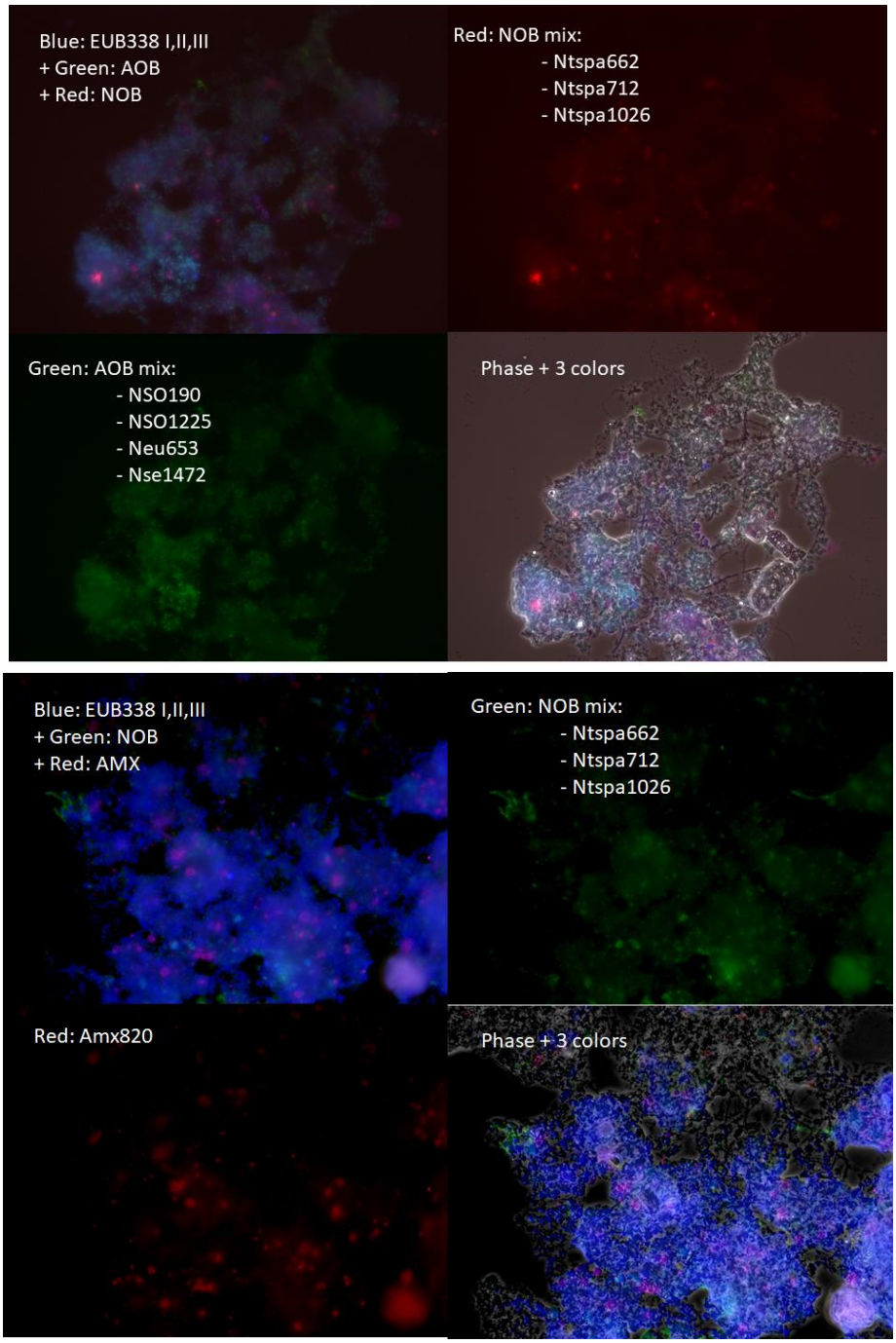
As you can see from Table 4 in the next page, NH_4^+ vary between 0.5~120mM, NO_2^- 0~75 mM, phosphate 0.2~6.2; some studies did not add Ni^{2+}, WO_4^{2-}, or I^- ; Some use Fe^{2+}, some use Fe^{3+} ; some use SeO_3^{2-}, some use SeO_4^{2-}. There were many variations, so it was too difficult to tell which one is the "best" medium. The only conclusion was that they all worked, what I should do is just try it.

Since I was aiming for AMX + CMX, I decided to adapt the medium recipe from Dr. Michele Laureni's AMX medium (Solar-Jafra et al. 2020), which has been cultivating the mother AMX for 10 years. Their NH_4^+ is 75 mM, while I was going for 10 mM, so I lowered the phosphate from their 6.2 mM to my 1 mM. The rest of the minerals remained the same. Other than that, I added 3.5 mg/L yeast extract to the medium, which might facilitate growth (Hoekstra, 2017).

Table 4. Summary of N conversion culture media

mM	Daims et al., 2015	Van de Graaf et al., 1996	Hoekstra, 2017	Soler-Jofra et al., 2020	This study	Kampschreur, 2010	van der Star et al., 2008
NH_4^+	0.5 and then 1 mM	5.0	75	75	10.0	7.5	120
NO_2^-	0	5.0	0	75	0	0	120
NO_3^-	0	0	0	0	2.3	0	100 and then 0
$H_2PO_4^-$ /HPO_4^{2-}	0.37	0.20	5.2	6.2	1.0	0.53	0.18
Cl^-	11	2.5	112	1.3	1.3	11	
SO_4^-	0.21	3.7	1.4	38	5.6	0.14	
Na^+	10	5.1	37	75	2.6	3.7	
K^+	1.4	5.2	3.9	11	1.9	0.39	
HCO_3^-	CaCO3	5.0	0	0	0	0	15
Ca^{2+}	CaCO3	1.2	0.55	0.64	0.64	0.0	4.1
Fe^{2+}	0.0036	0.018	0	0.10	0.10	0	0.045
Cu^{2+}	1.2E-04	1.0E-03	1.3E-03	1.6E-03	1.66E-03	1.8E-06	
Mg^{2+}	0.20	1.2	1.4	0.51	0.51	0.14	1.6
Zn^{2+}	5.1E-04	1.5E-03	4.6E-03	2.3E-03	2.42E-03	4.4E-06	
Mn^{2+}	2.0E-04	5.0E-03	5.6E-03	7.9E-03	7.90E-03	6.4E-06	
Fe^{3+}	0	0	6.1E-02	0	0	5.8E-05	
Co^{2+}	6.2E-04	1.8E-03	1.3E-02	2.9E-03	2.88E-03	1.2E-05	
Ni^{2+}	1.0E-04	8.0E-04	0	1.3E-03	1.30E-03	0	
H_3BO_3	8.1E-04	2.3E-04	2.7E-02	3.5E-04	3.71E-04	2.5E-05	
SeO_3^{2-}	1.1E-05	0	0	9.3E-04	9.17E-04	0	
SeO_4^{2-}	0	5.7E-04	0	0	0	0	
WO_4^{2-}	1.2E-05	0	0	2.6E-04	2.55E-04	0	
MoO_4^{2-}	3.0E-04	9.1E-04	2.7E-03	1.9E-03	1.98E-03	2.6E-06	
EDTA	0	0.054	0.30	0.13	0.13	0.00028	0.050
I^-	0	0	1.2E-02	0	0	1.1E-05	
yeast extract			3.5 mg/L		3.5 mg/L	10 mg/L	

8.3. FISH images



FISH sample from week 16. The magnification was x400.

FISH shows that there were AOB, NOB, AMX, but the signal was weak and blurry. Maybe it is because:

- I did not use competitive probes so there was nonspecific binding
- I did not grind them and they were flocs which had many layers.
- the probes decayed as they are generally susceptible.

To improve FISH, future work can try adding competitive probes and potter the cells to disintegrate flocs. And remember to add a scale bar.

8.4. FISH Probes

	Probe	Color	Sequence 5' --> 3'	Specificity	Formamide concentration
	Probe mix 1				
	EUB338_Cy5	Cy5	GCTGCCTCCCGTAGGAGT	most Bacteria	0-50%
	EUB338-II/III	Cy5	GCWGCCACCCGTAGGTGT	*Planctomycetales/Verrucomicrobiales*	0-50%
	AMX11 (AMX820)	Cy3	AAAACCCCTCTACTTAGTGCCC	Genera *Brocadia, Kuenenia*	40%
NOB mix	S-*-Ntspa-0712-a-A-21-fluos	Fluos	CGCCTTCGCCACCGGCTCTCC	most members of the phylum *Nitrospirae*	50%
	Ntspa1026	Fluos	AGCACGCTGGTATTGCTA	*Nitrospira moscoviensis*, activated sludge clones A4 and A11	20%
	S-G-Ntspa-0662-a-A-18-fluos	Fluos	GGAATTCCGCGCTCCTCT	genus *Nitrospira*	35%
	Probe mix 2				
	EUB338_Cy5	Cy5	GCTGCCTCCCGTAGGAGT	most Bacteria	0-50%
	EUB338-II/III	Cy5	GCWGCCACCCGTAGGTGT	*Planctomycetales/Verrucomicrobiales*	0-50%
NOB mix	S-*-Ntspa-0712-a-A-21-Cy3	Cy3	CGCCTTCGCCACCGGCCTTCC	most members of the phylum *Nitrospirae*	50%
	S-G-Ntspa-0662-a-A-18-Cy3	Cy3	GGAATTCCGCGCTCCTCT	genus *Nitrospira*	35%
	Ntspa1026	Cy3	AGCACGCTGGTATTGCTA	*Nitrospira moscoviensis*, activated sludge clones A4 and A11	20%
AOB mix	Nso190-fluos	Fluos	CGATCCCCTGCTTTTCTCC	*Betaproteobacterial* ammonia-oxidizing bacteria	55%
	NSO1225-fluos	Fluos	CGCGATTGTATTACGTGTGA	*Betaproteobacterial* ammonia-oxidizing bacteria	35%
	NEU653	Fluos	CCCCTCTGCTGCACTCTA	Most halophilic and halotolerant *Nitrosomonas spp*.	40%
	Nse1472-fluos	Fluos	ACCCCAGTCATGACCCCC	*Nitrosomonas europea, N. halophila, N. eutropha, Kraftisried-Isolat Nm103*	50%

8.5. Results of Reactor 2

The hypothesized CMX* conversion was not observed, because the majority of NH_4^+ uptake went to NO_3^- production and the NO_3^- production rate kept increasing. The N conversions show no sign of evolving toward higher NH_4^+ consumption or N removal under timeframe of 80 days.
On day 27, HRT was changed from 2 days to 1 day, and the gas supplied was changed from 50 mL/min 4%O_2 5%CO_2 91%N_2 and 50 mL/min N_2 to 100 mL/min 4%O_2 5%CO_2 91% N_2.

Regarding the structure of microbial community, the 16S rRNA data is not yet available due to Covid-19.