

Sustained nitrogen loss in a symbiotic association of Comammox *Nitrospira* and Anammox bacteria

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ABSTRACT

The discovery of anaerobic ammonia-oxidizing bacteria (Anammox) and, more recently, aerobic bacteria common in many natural and engineered systems that oxidize ammonia completely to nitrate (Comammox) have significantly altered our understanding of the global nitrogen cycle. A high affinity for ammonia ($K_{m(\text{app}), \text{NH}_3} \approx 63 \text{ nM}$) and oxygen place Comammox *Nitrospira inopinata*, the first described isolate, in the same trophic category as organisms such as some ammonia-oxidizing archaea. However, *N. inopinata* has a relatively low affinity for nitrite ($K_{m, \text{NO}_2} \approx 449.2 \mu\text{M}$) suggesting it would be less competitive for nitrite than other nitrite-consuming aerobes and anaerobes. We examined the ecological relevance of the disparate substrate affinities by coupling it with the Anammox bacterium Candidatus *Brocadia anammoxidans*. Synthetic communities of the two were established in hydrogel granules in which Comammox grew in the aerobic outer layer to provide Anammox with nitrite in the inner anoxic core to form dinitrogen gas. This spatial organization was confirmed with FISH imaging, supporting a mutualistic or commensal relationship. The functional significance of interspecies spatial organization was informed by the hydrogel encapsulation format, broadening our limited understanding of the interplay between these two species. The resulting low nitrate formation and the competitiveness of Comammox over other aerobic ammonia- and nitrite-oxidizers sets this ecological cooperation apart and points to potential biotechnological applications. Since nitrate is an undesirable product of wastewater treatment effluents, the Comammox-Anammox symbiosis may be of economic and ecological importance to reduce nitrogen contamination of receiving waters.

1. Introduction

Anaerobic ammonia-oxidizing (Anammox) bacteria occupy a unique niche due to their ability to anaerobically oxidize total ammonia (TN, NH_3 and NH_4^+) along with nitrite to form dinitrogen gas (Jetten et al., 2009; Kartal et al., 2010, 2014; Kuypers et al., 2003; Strous et al., 1999). They are widely distributed in oxygen depleted environments across both marine and freshwater habitats where they often partner with aerobic ammonia-oxidizing archaea (AOA) to obtain the nitrite required for growth (Gao et al., 2018; Oshiki et al., 2016; Yan et al., 2012). This association is thought to be favored in oligotrophic environments such as the Oxygen Minimum Zones (OMZs) (Lam et al., 2007; Pitcher et al., 2011; Straka et al., 2019b; Yan et al., 2012) by the high affinities of the marine AOA for ammonia and oxygen (ranging from an extremely low

$K_{m(\text{app}), \text{NH}_3} \approx 3 \text{ nM}$ for the marine *Nitrosopumilus maritimus* to a $K_{m(\text{app}), \text{NH}_3} \approx 5 \mu\text{M}$ for the terrestrial moderate thermophile *Nitrosotenuis uzoniensis*) (Kits et al., 2017; Könneke et al., 2014; Leininger et al., 2006; Martens-Habben et al., 2009; Qin et al., 2017; Wuchter et al., 2006). The unique physiology of Anammox has also found utility in nitrogen removal from high TN wastewater, where they generally partner with putatively r-strategist ammonium-oxidizing bacteria (AOB) having generally lower affinities for ammonium ($K_{m(\text{app}), \text{NH}_4^+} \approx 500 \mu\text{M}$ NH_4^+) but much higher growth rates than the AOA (Jia and Conrad, 2009; Kits et al., 2017; Martens-Habben et al., 2009). In these systems the organisms generally grow together as biogranules, in which growth of oxygen-consuming AOB near the surface functions to sustain an inner Anammox sub-oxic core (Hawley et al., 2014; Lauren et al., 2015). The discovery of complete ammonia-oxidizing *Nitrospira* species

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(Comammox) (Daims et al., 2015; van Kessel et al., 2015) which possess ammonia ($K_{m(\text{app})}$, $\text{NH}_3 \approx 63 \text{ nM}$) (Kits et al., 2017) and oxygen affinities likely near that of AOA, suggests that they may also couple with Anammox in low TN environments and have possible functions in nitrogen removal in other treatment applications.

Comammox species are common in many natural and engineered systems, including forest and agricultural soils, freshwater and brackish sediments, and waste and drinking water treatment plants (Camejo et al., 2017; Fowler et al., 2018; Pinto et al., 2016; Pjevac et al., 2017; Spasov et al., 2020; Xia et al., 2018; Yang et al., 2020). Comammox organisms produce nitrate as a product of ammonia oxidation with nitrite as an intermediate (Daims et al., 2015; van Kessel et al., 2015). The co-occurrence of two Comammox species (*N. nitrosa* and *N. nitrificans*) with Anammox bacteria (*Brocadia*) in an enrichment culture suggested that the intermediate nitrite is feeding Anammox hence yielding in a functional relationship between these two organisms (van Kessel et al., 2015). Comammox was also found to coexist with AOB and Anammox in a sequencing batch reactor for sludge digester liquor treatment and responsible for ~25% nitrogen removal (Wu et al., 2019). Although it is as yet unclear how much Comammox contributes to TN and nitrite oxidation in Water Resources Recovery Facilities (WRRFs), recent analyses suggest that *Nitrospira* can become the dominant ammonia oxidizer in WRRFs operated at low dissolved oxygen (DO) concentrations, and so offer more energy efficient nitrification (Roots et al., 2019; Spasov et al., 2020).

Currently municipal waste-water treatment plants generate approximately \$2 billion in annual electric costs of which pumping and aeration are responsible for about two thirds of facilities' energy consumption (Lemar and De Fontaine, 2017). While energy demand of water reclamation facilities is expected to grow with the population increase and water quality standards improvements, Anammox technology offers a ~50% reduction in aeration cost in comparison to conventional treatment systems (van Dongen et al., 2001). However, the ubiquitous lithotrophic nitrite-oxidizing bacteria (NOB) create a significant obstacle for Anammox implementation in mainstream full-scale waste-water treatment installations due to their competition with Anammox for the NO_2^- supplied by conventional AOB species (Daims et al., 2016; Kent et al., 2019; Koops and Pommerening-Röser, 2001; Third et al., 2001). The higher growth rate of characterized Comammox bacteria relative to AOA (Kits et al., 2017) suggest they would function well in the mainstream. Therefore, a pairing of Anammox with an organism such as Comammox that can supply NO_2^- and have high affinities for both dissolved oxygen and TN would suppress NOB and achieve low effluent nitrogen concentrations. This pairing would offer an operational advantage in treatment systems relying on nitrification by lowering aeration requirements. Lower production of the greenhouse gas nitrous oxide (N_2O) by Comammox relative to AOB would offer a secondary advantage (Kits et al., 2019). However, in order for the Comammox-Anammox pairing to stably cooperate a delicate balancing of TN and oxygen supply is imperative.

Mathematical models have indicated that oxygen and TN concentrations are major niche differentiating factors for Anammox and AOA or Anammox and AOB. For example, the association between AOB/AOA and Anammox is often disrupted by NOB competing with Anammox for nitrite (Kent et al., 2019; Third et al., 2001), resulting in a lower conversion of ammonia to N_2 and an increase in nitrate formation. Thus, there is clear need to resolve competitive and cooperative relationships among Comammox, Anammox, AOA, AOB, and NOB in order to develop a better predictive understanding of their roles in the global nitrogen cycle and to better define possible applications in engineered systems (Straka et al., 2019b). The Comammox relationship with Anammox is of specific interest since both bacteria utilize ammonia and nitrite and are active in low DO environments. However, comparative laboratory analyses of organisms that function cooperatively at the boundary between oxic and anoxic environments have been limited for lack of appropriate experimental systems. Suspended microbial cultures do not form the

oxygen gradient essential for partnership. Chemostat studies must balance the conflicting nutrient requirements for cooperative growth and are prone to washout. We here use synthetic community assemblies of Comammox *N. inopinata* and Anammox (dominant species *Candidatus Brocadia anammoxidans*) immobilized in hydrogel granules to evaluate environmental conditions supporting their partnership.

The hydrogel format, which is a three-dimensional network of hydrophilic polymers, has been successfully implemented to immobilize active nitrifying and denitrifying bacteria (Santos et al., 1996; Wijffels et al., 1995), denitrifying cultures (Xu et al., 2017), anaerobes originating from hydrothermal vents (Landreau et al., 2016) (Ali et al., 2015), and Anammox (Ali et al., 2015; Landreau et al., 2020). The gels can also be formed to resemble naturally occurring biogranules (Flemming and Wingender, 2010; Flemming et al., 2007), in which Comammox and Anammox can be entrapped in a matrix of extracellular polymeric substances (EPS) that promotes the formation of stable nutrient gradients and microbial interactions on a micrometer scale. Using a synthetic hydrogel set-up to achieve conditions for both aerobic and anaerobic growth, we now demonstrate the formation of a stable partnership in which Comammox competitively excludes the AOB to supply nitrite to Anammox and, in turn, Anammox lowers nitrite to a non-inhibitory concentration. This is the first clear evidence for possible ecological relevance of an association that should be useful for various biotechnological applications.

2. Materials and Methods

2.1. Microbial strains and growth

Comammox *Nitrospira inopinata* strain was incubated in limited mineral media aerobically at 37°C pH 7.5 in the presence of 1mM NH_4^+ (Daims et al., 2015). Anammox biomass was obtained from a waste-water treatment plant in Rotterdam Sluisjesdijk (the Netherlands) and was maintained anaerobically in a plug-flow glass column supplied with 1mM NH_4^+ and 1.3 mM NO_2^- and operated at 30°C. The dominant Anammox organism in this sludge was reported to be *Candidatus Brocadia anammoxidans* (van der Star et al., 2007). The mineral media used for Anammox growth had following composition (van de Graaf et al., 1996): 2.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mM CaCl_2 , 5 mM NaHCO_3 , 0.2 mM KH_2PO_4 , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 9.0 mg, $\text{EDTA} \cdot 2\text{Na}$ 5 mg. Trace elements solution (per 1L ddH₂O): EDTA, 15 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.43 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.24 g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.99 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25 g; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.22 g; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.19 g; $\text{NaSeO}_4 \cdot 10\text{H}_2\text{O}$, 0.21 g; H_3BO_4 , 0.014 g.

2.2. Hydrogel beads fabrication

A 10% polyvinyl alcohol (PVA) (w/v) and 2% sodium alginate (SA) (w/v) solution in water was prepared and sterilized as previously described (Landreau et al., 2020). Comammox *Nitrospira inopinata* culture was concentrated 100-fold via 0.1 μm tangential filtration cassette (Millipore Sigma). Comammox viability after filtration was assessed via incubation in the mineral medium with subsequent quantification of nitrification activity. Granules enriched in Anammox were disaggregated using a blender for 2 minutes and resuspended in the mineral medium. Suspensions of Comammox and Anammox bacteria were mixed in a 1:1 ratio and then interspersed with the PVA-SA polymer solution to achieve 6% PVA and 1% SA concentrations. Using the 0.1 mm diameter tips PVA-SA-bacteria mixture was dropped into a 2% (w/v) barium chloride (BaCl_2) bath in order to form spherical hydrogel particles. The hydrogel beads were allowed to harden in the BaCl_2 solution for 1 hour before rinsing several times in a sodium chloride solution (0.9%) to remove excess polymer. All solutions and medium used for the hydrogel bead production were prepared anaerobically to minimize Anammox exposure to oxygen. The granules were incubated in batch bottles containing an ammonia-mineral media (Daims et al., 2015) without organic carbon and their activity was monitored for 86

days. Three sets of Comammox-Anammox beads were set up as biological replicates and incubated in parallel. The co-culture was oxygenated with ca. 4 mgO₂/L 3 times per week and ammonium was replenished when depleted. The microbial activity was measured using colorimetric analysis of nitrogen species concentration in the growth media throughout the incubation period. Two additional batch bottles were set up to separately assess the independent growth of Comammox and Anammox bacteria, respectively. Dissolved oxygen concentration was monitored with oxygen sensor spots (PyroScience GmbH, OXSP5).

2.3. Microbial activity measurements

Ammonium (NH₄⁺), nitrite (NO₂⁻), and nitrate (NO₃⁻) concentrations were measured using a colorimetric method with Gallery™ Automated Photometric Analyzer (ThermoFisher Scientific, Waltham, MA U.S.A.) with the Total Oxidized Nitrogen (TON)-Nitrite and Ammonia reagents calibrated using sodium nitrite (NaNO₂) and ammonium chloride (NH₄Cl) standards. Samples were processed immediately after collection.

2.4. Molecular analysis of microbial community composition

Genomic DNA was extracted from PVA-SA hydrogel beads by bead-beating and from growth media (two series of 25 seconds each, Bullet Blender 5 homogenizer) and subsequent purification using a Power Biofilm kit (DNeasy Power Biofilm kit, Qiagen) following manufacturer instructions. DNA extraction and analysis are described in Supplementary materials.

2.5. Fluorescence in situ hybridization

Fixation and hybridization of the bacterial hydrogel beads samples was carried as previously described (Nielsen et al., 2009) with the following modifications. Hydrogel beads were fixed in 4% para-formaldehyde for 3–16 hours at 4°C and washed with Ethanol/PBS (50:50 w/v) three times. If not processed immediately, samples were stored in PBS/Ethanol 50:50 solution at -20°C. Fixed hydrogel beads were submerged in OCT Compound (Agar Scientific) or NEG-50™ (Richard-Allan Scientific™) overnight and then frozen at -20°C for 3–16 hours prior to sectioning. Thin 20–30 nm sections of hydrogel beads were produced using a micro-cryotome (Cryostar NX50™) at -20°C and mounted on gelatin coated Teflon-covered glass slides. Thin sections were dried in the oven at 46°C for 10 minutes, sequentially dehydrated in 50, 80, 95% ethanol, air dried, and stored at -20°C. Using Liquid Blocker Pap Pen (Life Technologies, Carlsbad, CA, USA) a hydrophobic barrier was applied to glass slides containing thin cryosections. Oligonucleotide probes are summarized in Suppl. Table 2 and were applied in equimolar concentration to the thin sections and incubated at 46°C for 2 hours. All probes were purchased from Eurofins, Louisville, KY. The slides were mounted in the Vectashield antifade mounting medium (Cole-Parmer). A confocal laser scanning microscope Zeiss LSM700 (Carl Zeiss, Germany) equipped with an Axio cam 503 mono camera was used to visualize hybridized cryosections with 488, 543, and 633 nm lasers. A 10x/0.45 objective was used to image large regions of the sections. 40x/1.3 and 100x/1.46 Oil Ph3 plan-apochromat oil objectives were used in order to obtain higher-resolution images. The images were collected in the Zen Blue software.

2.6. Quantitative PCR

In order to quantify Comammox, Anammox, AOB, and NOB in hydrogel beads a quantitative PCR (qPCR) analysis was performed. All specific primers and thermocycling conditions for qPCR assays are described in the reference articles (Suppl. Table 2) and were performed on Roche LightCycler 480 high-throughput real-time PCR system in white LightCycler 480 multiwell tubes and associated LightCycler 480

transparent caps (Roche Molecular Systems, Inc, Pleasanton, CA, United States). Triplicate reactions were performed for each sample. Amplification was performed according to protocols described by Fowler et al. (2018) (Fowler et al., 2018). Details are described in Supplementary materials.

3. Results

3.1. Comammox and Anammox bacteria establish cooperation within synthetic granules in batch cultures

A hydrogel formulation was developed to entrap high numbers of the only available Comammox pure culture *N. inopinata* and an Anammox enrichment (dominant organism *Candidatus Brocadia anammoxidans*) in synthetic polyvinyl alcohol – sodium alginate (PVA-SA) hydrogel beads. Comammox-Anammox beads demonstrated a constant rate of ammonium consumption over the study period of 86 days, resulting in complete nitrogen removal from the system (Fig. 1a). Since no external nitrite was supplied, Comammox activity was the sole source of nitrite for Anammox. We did not observe measurable nitrite or nitrate accumulation throughout the incubation period as would be expected from Comammox (Daims et al., 2015; van Kessel et al., 2015) or Anammox (van de Graaf et al., 1996; Strous et al., 1999) metabolism alone. Beads containing only Comammox stoichiometrically oxidized ammonia to nitrate, with transient accumulation of nitrite (Fig. 1b), and beads containing only the Anammox enrichment converted ammonium to N₂ when supplied with ammonia and nitrite at the ratio of 1:1.3 (Fig. 1c).

3.2. qPCR supports Anammox and Comammox cooperation in hydrogel beads

Total bacterial abundance, as measured by qPCR targeting the 16S rRNA gene, ranged from 10⁵ to 10⁶ gene copies ng⁻¹ of granule material sampled at the beginning and the end of the incubation period from both entrapped and planktonic communities (Suppl. Fig. S1). Quantification of the *amoA* of Comammox and Betaproteobacterial AOB, and of Anammox 16S rRNA genes, demonstrated that Comammox and Anammox bacteria dominated the ammonia-oxidizer population in the hydrogel beads (Fig. 2). In contrast, Betaproteobacterial AOB were near the limit of detection in all hydrogel samples and persisted only in the fraction of non-immobilized planktonic bacteria (Fig. 2). The planktonic fraction contained mostly heterotrophic species (Suppl. Fig. S1) and low amounts in *amoA* of Comammox *Nitrospira inopinata* and 16S rRNA gene copies of Anammox if compared to the bead fraction indicating that Comammox and Anammox were retained in the bead fraction (Fig. 2).

3.3. 16S rRNA sequencing demonstrated Comammox *N. inopinata* and Anammox presence after the incubation period while AOB and NOB remained low but constant

The community composition of PVA-SA hydrogels at the beginning and end of incubation was investigated using amplicon sequencing of the V4-V5 region of the 16S rRNA gene (Suppl. Fig. S2). In total 409 operational taxonomic units (OTUs) were identified. There was a substantial increase in the relative abundances of OTUs assigned to *Xanthomonadales*, *Burkholderiales*, *Rhodocyclales* and *Rhizobiales* over time, indicating growth conditions favorable to heterotrophic organisms even in the absence of supplied organic carbon in the media (Suppl. Fig. S2). The increase in abundance of OTUs for heterotrophic populations resulted in overall decreasing relative abundances for OTUs assigned to '*Candidatus Brocadiaceae*' (Anammox), *Nitrospira inopinata* (Comammox), *Nitrosomonas* (AOB), *Nitrosopumilis* or *Nitrososphaera* (AOA), and other *Nitrospira* (NOB) that were not assigned to the *Nitrospira inopinata* OTU between the initial biomass and after 86 days (Suppl. Fig. S3). The Comammox OTU accounted for 20.3% reads at the beginning of the incubation and averaged 5.15% (± 7.73% S.D., n=3) at termination.

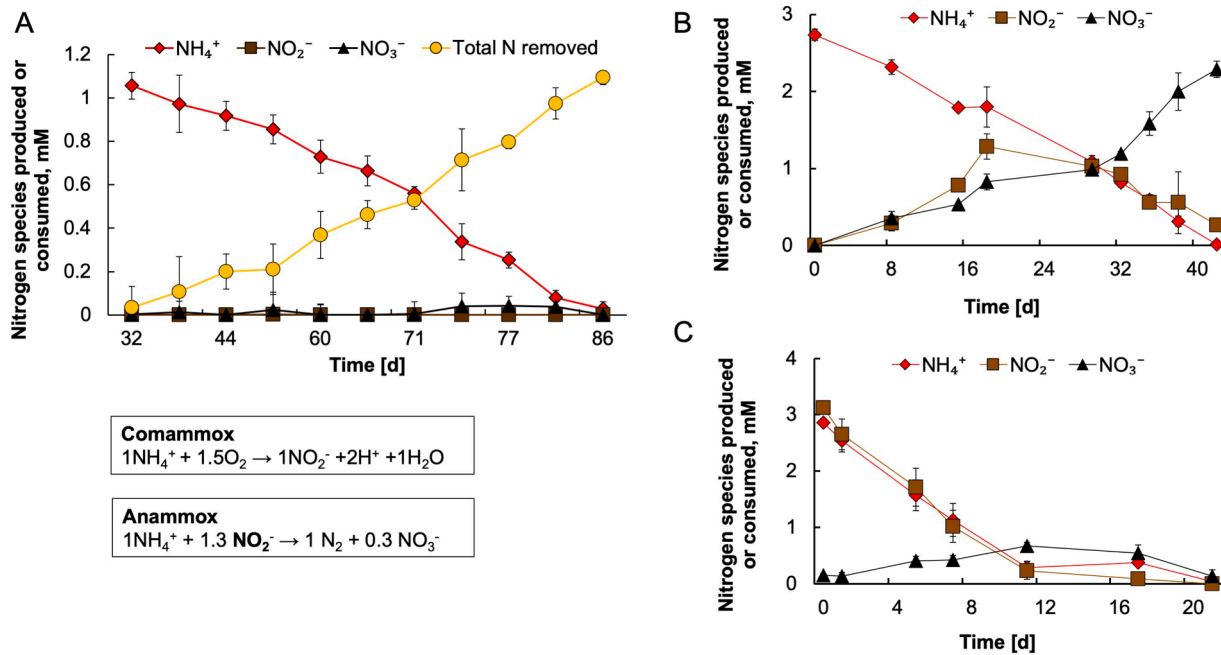


Figure 1. Comammox and Anammox cooperation within hydrogel beads. (a) Immobilized Comammox and Anammox hydrogel beads demonstrated cooperation at 4 mgO₂/L and with ammonium as sole source of energy, electrons, and nitrogen in three biological replicates (n=3). (b) In the absence of Anammox the gel immobilized Comammox was capable of further oxidizing nitrite to nitrate. (c) In the absence of Comammox, the established Anammox anabolic reaction pathway was demonstrated.

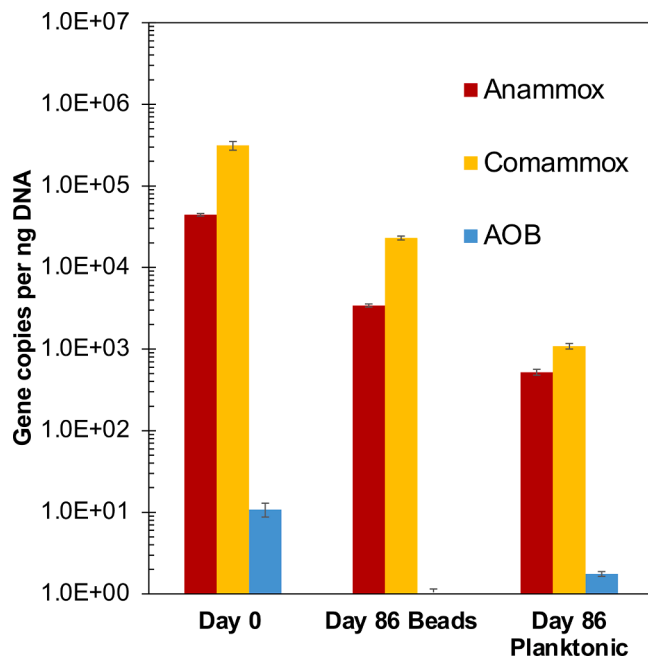


Figure 2. Abundance of Comammox, Anammox, and canonical AOB in hydrogel beads and in the planktonic fraction determined by qPCR of *amoA* genes (Comammox, AOB) and 16S rRNA genes (Anammox). Gene copy numbers per ng of total extracted DNA are shown for samples taken at the start of the incubation (day 0) and after 86 days. Genetic material corresponding to the initial biomass (day 0) was obtained directly after immobilization through the same extraction method as the day 86 biomass samples. Gene copy numbers were adjusted according to their average occurrence (Suppl. Table 3).

Canonical AOB, AOA, and NOB accounted for only 0.060%, 0.010%, and 0.020% of the reads initially and remained low throughout the experimental period, with final average relative abundances of 0.010%,

0.017%, and 0.0033% respectively (S.D. $\leq 0.01\%$) after 86 days of cultivation under nitrifying conditions (Suppl. Fig 3). A single OTU assigned to *N. inopinata* was the only nitrifier OTU of substantial relative abundance. Thus, it can be inferred that it alone was responsible for the observed nitrification activity (Fig. 1a-b). Together this indicated that Comammox was responsible for partial nitrification in support of Anammox. The observed apparent loss of Anammox in PVA-SA hydrogels after 86 days, as represented by 7.68% of the reads in the initial beads and 0.32% ($\pm 0.05\%$ S.D., n=3) of the final biomass (Suppl. Fig. S3), likely reflected a combination of degradative loss in the nutrient-limited interior and by oxygen exposure near the outer boundary of the granules, increased heterotroph abundance, and poor extraction of DNA from Anammox cells embedded deep within hydrogel

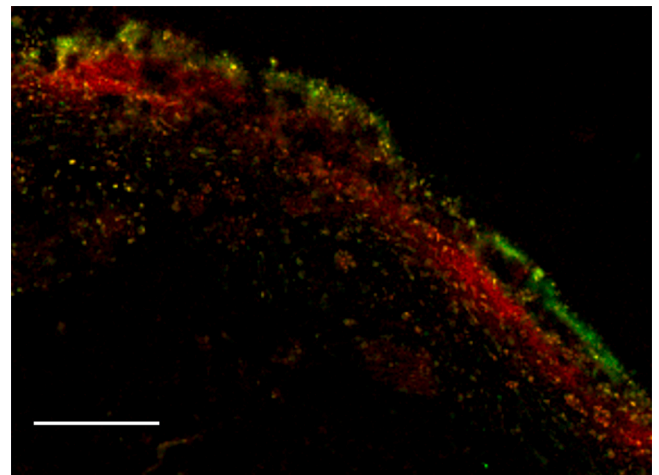


Figure 3. Comammox *Nitrospira* and Anammox demonstrate spatial segregation within hydrogel beads. Simultaneous *in situ* hybridization of a bead section with Cy3-labeled probe AMX368 (red; Anammox), fluorescein-labeled probe Ntsp662 (green; *Nitrospira*), and Cy5-labeled probe Nso1225 (blue; Betaproteobacterial AOB). The bar represents 100 μm .

granules. Additionally, PCR biases for the v4-v5 16S rRNA gene primers may preferentially amplify other taxa that increased in abundance during incubation, skewing relative abundance data (Orschler et al., 2019).

3.4. Comammox and Anammox species are spatially segregated within hydrogel beads

Given the efficient removal of nitrogen by this community, we further examined the population structure using rRNA-targeted FISH imaging and qPCR. Fluorescent probe AMX368-hybridized Anammox bacteria were detected in the initial bead samples as well as at the end of the incubation period (Fig. 3), confirming Anammox persistence within hydrogel beads. Following initial bead fabrication, cells were dispersed in the polymer matrix while they mainly appeared on the peripheral layer of the beads after 86 days of incubation (Fig. 3). While the Comammox organisms *N. inopinata* (green) was randomly distributed just after immobilization, it formed a thin layer at or near the hydrogel surface following the incubation period. In particular, Comammox cells resided in between the oxygenated edge of the hydrogel beads and the Anammox cells (red), which were situated in a deeper (presumably anoxic) layer. Visually, we observed a higher abundance (not quantified) of Anammox using FISH probes than from either amplicon or qPCR amplicon analysis. Probe Nso1225 (specific for Betaproteobacterial AOB) did not produce a detectable fluorescent signal (Fig. 3, Suppl. Fig. S5).

4. Discussion

The ability of Comammox microorganisms to survive under low substrate concentrations (Kits et al., 2017) allows them to occupy oligotrophic environments. Cooperation with Anammox bacteria has been reported in bioreactor studies (van Kessel et al., 2015; Lückner et al., 2010). Here we used a unique hydrogel format to pair Comammox and Anammox in a granule-like structure engineered to promote the formation of nutrient gradients theorized to foster their co-occurrence within natural habitats. Microbial activities in natural biofilms or biogranules generate substrate gradients, allowing aerobic organisms (like Comammox) to grow on the biofilm periphery and anaerobes (like Anammox) in the oxygen depleted adjacent inner layers (Aqeel et al., 2019; Weber et al., 2007). The hydrogel format promotes a similar relationship in a controlled laboratory setting. Therefore, we fabricated synthetic hydrogel granules harboring a single species of Comammox, *Nitrospira inopinata*, and an enrichment of Anammox related to *Brocadia*. The beads remained intact throughout the 86-day incubation period, as previously shown in our study demonstrating retention of active AOA in hydrogel constructs (Landreau et al., 2020). Cooperation was confirmed by a nearly total nitrogen removal and negligible production of nitrate (Fig. 1). This is the first reported encapsulation of Comammox organism alone or combined with Anammox consortium into hydrogels. Previously mentioned advantages of synthetic hydrogels combined with kinetic and metabolic potential of Comammox bacteria therefore suggest a practical application of Comammox / Comammox co-cultures in manufacturing highly oxygen efficient (Wang et al., 2019) hydrogels for nitrogen removal in engineered systems.

In order to confirm that observed ammonia oxidation and nitrogen removal resulted from the activity of Comammox and Anammox organisms, and not from other ammonia oxidizers, we conducted a population analysis by sequencing the hydrogel beads samples. 16S rRNA analysis of microbial populations from the initial beads versus those incubated for 86 days beads revealed that *N. inopinata* remained the major aerobic ammonia oxidizer. Other nitrifying organisms AOA, AOB, and NOB accounted for only a small fraction of the OTUs (<0.06%) and slightly decreased in relative abundance after the incubation of 86 days (Suppl. Fig. S3). These data contrasts with a previous study of a sequencing batch reactor reporting 0.1% relative abundance of

Anammox and high relative abundances of AOB (18% vs. less than 1 % of Comammox) (Wu et al., 2019), which did not fully support the proposed cooperation between Comammox and Anammox. In our study, however, Comammox was demonstrated to be responsible for partial nitrification in the constructed hydrogel beads, as deduced from the virtual absence of other known ammonia-oxidizing organisms. On the other hand, the presence of heterotrophic populations originating from the Anammox enrichment culture (Suppl. Fig. S2) may also have been of functional significance.

Members of the *Ignavibacteria* taxa, some of which are capable of performing dissimilatory nitrate reduction to ammonia (DNRA) under anoxic conditions (Han et al., 2020), remained stable throughout the batch test. Also of possible relevance to nitrogen loss was the activity of populations assigned to four proteobacterial orders, *Xanthomonadales*, *Burkholderiales*, *Rhodocyclales*, and *Rhizobiales*, all of which are known to contain species capable of facultative anaerobic heterotrophic denitrification (Finkmann et al., 2000; Lycus et al., 2017). Low nitrate concentrations in the effluent might in part be attributed to the activity of heterotrophic denitrifiers, relying on organic carbon from degradation of alginate, biomass decay (Okabe et al., 2005), or small organic acids produced by autotrophic organisms (Martiny et al., 2005; Rittmann et al., 1994). Alginate did not appear to provide a significant source of carbon since bead integrity remained stable throughout the study period and the observed nitrate consumption was inconsistent with alginate being a significant carbon source for denitrifying heterotrophs (Suppl. Table. 1). Although it is unclear what nutrients supported heterotrophic growth, the high abundance of aerobic heterotrophs suggests that oxygen and not nitrate served as the primary terminal electron acceptor (as suggested by retention of *Sinabacteraceae* (Zhou et al., 2008) and *Chloroflexi* (Tian et al., 2015) bacteria), which would lead to a competition between heterotrophs and *N. inopinata* for oxygen in the outer edges of the hydrogel granules, and utilizing nitrate (if any) within the anaerobic core volume of the granules. Therefore, it cannot be excluded that some nitrate (produced by Comammox or Anammox) was first reduced to nitrite by denitrifiers, and then utilized by Anammox. A more detailed analysis of the nitrogen flow through this system with N-labeled compounds is warranted in order to untangle the multiple N-cycle process that are / may be co-occurring in this system.

PCR-based methods are known to have potential bias due to unequal amplification and cloning efficiency particularly for Anammox detection using both 16S rRNA regions and functional genes amplification (Cai et al., 2020). Additionally, PCR biases for the v4-v5 16S rRNA gene primers may preferentially amplify other taxa that increased in abundance during incubation, skewing relative abundance data (Orschler et al., 2019). Thus, in addition to confirming by qPCR the retention of Comammox and Anammox, and an insignificant contribution of AOB and NOB (Fig. 3), we demonstrated their expected spatial relationship using PCR-independent fluorescent probes specific to the associated bacterial families (Fig. 3). The fluorescent Ntsp662 oligonucleotide probe specific to *Nitrospira* showed that Comammox cells were most abundant in the outer layer of the beads, as dictated by the oxygen dependence of aerobic ammonia oxidation. The few Comammox cells observed deeper in the granule interior may be an artifact of introducing bacteria into the interior during the slicing procedure, possibly reflecting the ability of Comammox to grow at lower substrate concentrations typically present in deeper regions or inactive Comammox retaining high ribosome content detectable by FISH. The Anammox bacteria were detected using fluorescent Amx368 probe and were distributed in small aggregates throughout the inner core space (similarly to (Landreau et al., 2016)) but mostly clustered immediately below Comammox towards the edge of the bead where substrate supply is highest. Predicted high affinity for oxygen therefore can explain Comammox spatial localization within the outer layer of the hydrogel beads and supports a cooperative relationship between Comammox and Anammox in sub-oxic environments, demonstrating their possible ecological relevance.

A few variables in our cooperation model can potentially affect the performance of Comammox-Anammox cooperation. Since kinetic parameters define the fitness of the strains under specific conditions (Straka et al., 2019a), a low total ammonia concentration can be limiting to Anammox bacteria leading to slower conversion rates especially if the Anammox species was obtained from an ammonia laden system as it is typically the case for wastewater side stream treatment systems (Smeulders et al., 2020). Additionally, Comammox and Anammox may have differential sensitivity to various abiotic factors such as temperature, pH, and salinity affecting both Comammox and Anammox performance. Thus, a delicate balance of these parameters has to be achieved for a stable cooperation. In order to grow Comammox and Anammox in low ammonia environments (such as the mainstream) at high rate a future goal must be to enrich high affinity strains from the environment in large quantities instead of using low affinity strains (from the side stream) and apply them at low substrate environments (Straka et al., 2019a).

Together these results suggest that nitrite was consumed by Anammox and not further oxidized by Comammox. This is in alignment with reported nitrite affinities of $K_{m(\text{app})}$, $\text{NO}_2 \approx 48 \mu\text{M}$ for Anammox and $K_{m(\text{app})}$, $\text{NO}_2 \approx 449.2 \mu\text{M}$ for *N. inopinata*, suggesting that a higher rate of nitrite consumption by Anammox provided a competitive advantage. We also determined the nitrite half-inhibition constant ($K_{i,\text{NO}_2} = 0.31 \pm 0.19 \text{ mM}$ nitrite) of *N. inopinata* and at concentrations similar to those measured in the running experiment ($<0.1 \text{ mM}$) we did not observe a noticeable inhibition effect (Suppl. Fig. S4). This suggests a symbiotic partnership in which Anammox maintains nitrite concentrations at non-inhibitory levels and Comammox serves Anammox by consuming oxygen and providing an anaerobic environment while also supplying nitrite. However, a novel Comammox species, Candidatus *Nitrospira kreftii*, was recently identified displaying physiological characteristics distinct from *N. inopinata*. Particularly, Candidatus *N. kreftii* has a higher nitrite affinity ($K_{m(\text{app})}$, $\text{NO}_2 \approx 13 \mu\text{M}$) altering its potential interactions with other nitrifiers and denitrifiers (Sakoula et al., 2021). Thus, for specific engineering solutions, it is important to understand the kinetic parameters of different Comammox species. Follow-up studies using other Anammox and Comammox species (once available in culture) should be carried out to further investigate their co-existence and activities in bioreactors. The stability of Comammox-Anammox association is suggestive of other interactions, such as the release of small organic compounds (e.g., formate) from autotrophic Anammox (Freitag et al., 1987; Koch et al., 2015; Lawson et al., 2017) serving as electron donors for heterotrophs or the possible mixotrophic lifestyle of Comammox. In fact, some *Nitrospira* strains (*Nitrospira moscovinensis*) have been reported capable of utilizing formate along with nitrate (Koch et al., 2015) and it cannot be excluded that Comammox can also follow an anaerobic lifestyle in which formate and nitrate (both produced by Anammox) could give them a contemplative edge over other nitrifiers. Low nitrate accumulation sets the cooperation described here apart from previously studied systems of AOB/AOA and Anammox or AOB/AOA and NOB, in which significant nitrate was produced. While Comammox co-culture with Anammox acts essentially similar to AOB-Anammox system by generating nitrite for Anammox consumption, there are advantages of using Comammox over AOB in water treatment facilities. Comammox can possibly outcompete AOB as they likely have a higher affinity for oxygen than many AOB (Roots et al., 2019). However, Comammox affinity for oxygen may be very similar to canonical NOB since both groups belong to *Nitrospira* and share terminal oxidases (Palomo et al., 2018). Therefore, it remains to be investigated if the presence of Comammox *Nitrospira* deters NOB species that usually cause operational problems in AOB-Anammox reactors by producing undesirable nitrate as a final product. Our results demonstrate very little NOB growth in hydrogels after 86 days of incubation supporting this hypothesis. From this perspective, Comammox may be better adapted than AOA or AOB for these engineered systems and could therefore be paired with Anammox for possibly complete N removal in these systems.

5. Conclusions

- Our results suggest that the recently described complete ammonia-oxidizing organism *N. inopinata* can establish a cooperative relationship with anaerobic ammonia-oxidizing bacteria in a synthetic granule format.
- The spatial organization of the two organisms was consistent with their physiology, demonstrating that even though these organisms may not be optimal partners they do establish a relationship that points to ecological relevance.
- This novel cooperation was documented by the low nitrate formation and the competitiveness of the Comammox organism over other aerobic ammonia- and nitrite-oxidizers.
- Since nitrate is an undesirable product in the effluent of wastewater treatment plants, the Comammox-Anammox symbiosis may be of economic and ecological importance to reduce nitrogen contamination of receiving waters. However, heterotrophic denitrification contribution needs to be further evaluated.

CRedit authorship contribution statement

Ekaterina Y. Gottshall: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Sam J. Bryson:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Kathryn I. Cogert:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Matthieu Landreau:** Methodology, Writing – review & editing. **Christopher J. Sedlacek:** Resources, Writing – review & editing. **David A. Stahl:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Holger Daims:** Resources, Writing – review & editing. **Mari Winkler:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2021.117426](https://doi.org/10.1016/j.watres.2021.117426).

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