Simultaneous Anaerobic and Aerobic Ammonia and Methane Oxidation under Oxygen Limitation Conditions

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ABSTRACT Methane and ammonia have to be removed from wastewater treatment effluent in order to discharge it to receiving water bodies. A potential solution for this is a combination of simultaneous ammonia and methane oxidation by anaerobic ammonia oxidation (anammox) bacteria and nitrite/nitrate-dependent anaerobic methane oxidation (N-damo) microorganisms. When applied, these microorganisms will be exposed to oxygen, but little is known about the effect of a low concentration of oxygen on a culture containing these microorganisms. In this study, a stable coculture containing anammox and N-damo microorganisms in a laboratory scale bioreactor was established under oxygen limitation. Membrane inlet mass spectrometry (MIMS) was used to directly measure the *in situ* simultaneous activity of N-damo, anammox, and aerobic ammonia-oxidizing microorganisms. In addition, batch tests revealed that the bioreactor also harbored aerobic methanotrophs and anaerobic methanogens. Together with fluorescence *in situ* hybridization (FISH) analysis and metagenomics, these results indicate that the combination of N-damo and anammox activity under the continuous supply of limiting oxygen concentrations is feasible and can be implemented for the removal of methane and ammonia from anaerobic digester effluents.

IMPORTANCE Nitrogen in wastewater leads to eutrophication of the receiving water bodies, and methane is a potent greenhouse gas; it is therefore important that these are removed from wastewater. A potential solution for the simultaneous removal of nitrogenous compounds and methane is the application of a combination of nitrite/nitrate-dependent methane oxidation (N-damo) and anaerobic ammonia oxidation (annamox). In order to do so, it is important to investigate the effect of oxygen on these two anaerobic processes. In this study, we investigate the effect of a continuous oxygen supply on the activity of an anaerobic methane- and ammonia-oxidizing coculture. The findings presented in this study are important for the potential application of these two microbial processes in wastewater treatment.

KEYWORDS anaerobic methane oxidation, anammox, nitrification, methane oxidation, oxygen exposure, wastewater treatment, ammonia oxidation

Aerobic removal of organic carbon and nitrogen from wastewater requires large amounts of oxygen, energy, and space, which make it an energy intensive and costly process (1). Furthermore, organic carbon is oxidized to the climate-active waste product CO_2 or converted to biomass, contributing to global warming and solid waste generation, respectively. Conversely, in anaerobic wastewater treatment, most of the organic carbon is converted into valuable biogas (methane), resulting in less sludge production. Consequently, for anaerobic wastewater systems, operational costs are considerably lower and the produced biogas can be used for electricity generation (1).

However, one drawback of using anaerobic wastewater treatment is the remaining dissolved methane after methane is harvested from the gas phase (2). This dissolved methane escapes from these anaerobic treatment systems to the atmosphere and has a significant environmental impact because methane is a greenhouse gas with radiative forcing that is 28 times higher than that of CO_2 on a 100-year scale (3).

Recently, several anaerobic microbial processes, which oxidize methane or ammonia while reducing nitrogen oxides, were discovered. In the anaerobic ammonia oxidation (anammox) process, bacteria use nitrite as their terminal electron acceptor to convert ammonia into mainly N_2 and some nitrate (equation 1) (4). In parallel with this, two distinct groups of microorganisms, collectively termed nitrite/nitrate-dependent anaerobic methane oxidation (N-damo) microorganisms, can oxidize methane coupled to nitrite or nitrate reduction. Nitrite-dependent methane oxidation is performed by bacteria ("*Candidatus* Methylomirabilis spp."), which reduce nitrite to N_2 via NO without producing N_2O (equation 2) (5, 6). On the other hand, nitrate-dependent methane oxidation is carried out by archaea ("*Candidatus* Methanoperedens spp.") by coupling the oxidation of methane to the reduction of nitrate to nitrite (equation 3) (7). A combination of these microbial methane and ammonia oxidation processes can be applied for the simultaneous removal of ammonia and methane from wastewater streams (8, 9).

$$\text{Anammox bacteria:} \quad 1NH_4^+ + 1.3NO_2^- \rightarrow 1N_2 + 0.3NO_3^- \qquad (1)$$

$$\text{N-damo bacteria:} \quad 3CH_4 + 8NO_2^- \rightarrow 3CO_2 + 4N_2 \qquad (2)$$

$$\text{N-damo archaea:} \quad CH_4 + 4NO_3^- \rightarrow CO_2 + 4NO_2^- \qquad (3)$$
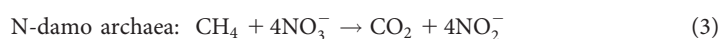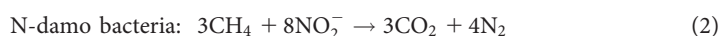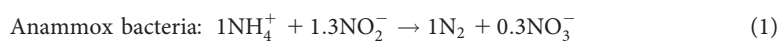
Under laboratory conditions, anammox and N-damo microorganisms can be grown together in stable cocultures, which are able to convert ammonia and methane simultaneously (10–13). In these laboratory-scale systems, the electron acceptor in the form of nitrate and/or nitrite is fed externally to the cultures. However, these compounds cannot be added to full-scale wastewater treatment plants due to economic and environmental concerns. Furthermore, N-oxide concentrations present in wastewater cannot support anaerobic ammonia and methane oxidation in full-scale installations. Therefore, nitrate and/or nitrite has to be microbially produced *in situ* for a successful application of these processes (8). It was previously shown that nitrite can be produced by aerobic ammonia-oxidizing bacteria (AOB), which form stable cocultures with anammox bacteria in both laboratory-scale bioreactors and full-scale wastewater treatment plants (14, 15). In such bioreactors, limiting amounts of oxygen are added for the conversion of part of the ammonia to nitrite (partial nitritation), which is then subsequently used by anammox bacteria. The application of these partial-nitritation anammox (PNA) bioreactors at full scale has been accepted as a more cost-effective and environmentally friendly method to remove ammonia from wastewater (1, 16). It has recently been put forward that, with the addition of methane-oxidizing microorganisms, such bioreactors can simultaneously remove ammonia and methane (8, 11, 12). Furthermore, several modeling studies have explored the feasibility of such bioreactors in laboratory and full-scale applications (17–21).

The key to the successful application of a combined N-damo/anammox system lies in the controlled addition of oxygen and the resulting conversion of ammonia to nitrite, which would then support both anaerobic methane and ammonia oxidation. However, this oxygen addition should be strictly controlled, since too much oxygen would induce the growth of aerobic nitrite-oxidizing (NOB) and methane-oxidizing (MOB) microorganisms (11, 22). Furthermore, high concentrations of oxygen inhibit N-damo and anammox microorganisms (11, 23). Altogether, the efficient and successful application of these systems depends on the controlled addition of oxygen and the constant and sufficient supply of nitrite/nitrate for anammox and N-damo bacteria. The supply of oxygen, ammonium, and methane to a culture that contains aerobic and anaerobic ammonia-, nitrite-, and methane-oxidizing microorganisms will result in a competition for all supplied and produced substrates, thereby creating an interesting
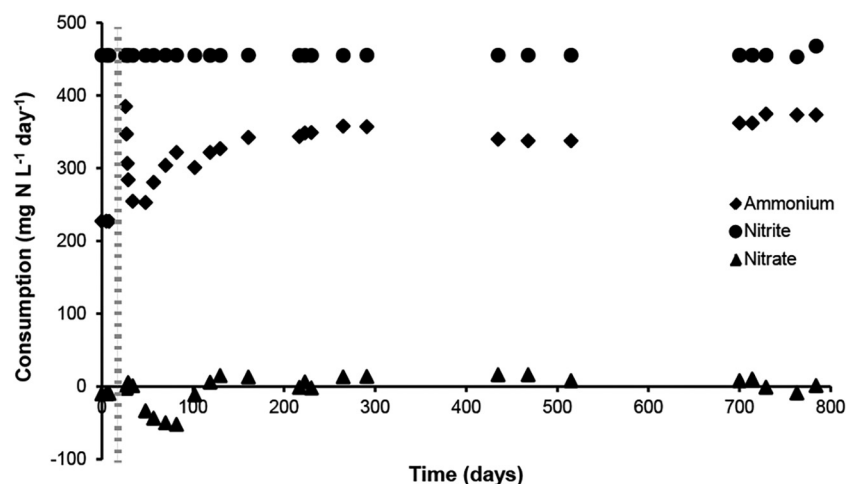
FIG 1 Consumption of ammonium (diamonds), nitrite (circles), and nitrate (triangles) by the reactor. Directly after the addition of oxygen and the increase of the ammonium concentration in the medium, nitrate was produced and is plotted here as a negative consumption. The start of the addition of oxygen is indicated with the dotted line.

interplay between several clades of microorganisms. In order to apply the simultaneous removal of ammonia and methane under oxygen-limited conditions, it is imperative to understand the microbial interactions in these bioreactors.

Here, we describe a laboratory-scale sequencing batch reactor (SBR) containing an anammox/N-damo coculture. Limiting amounts of oxygen were introduced to this culture, and the microbial interactions between the different clades of ammonia- and methane-oxidizing microorganisms were investigated using stable ^{15}N-isotopes, *ex situ* batch assays, and membrane inlet mass spectrometry (MIMS). The community composition was studied by fluorescence *in situ* hybridization (FISH) and 16S rRNA gene analysis. Taken together, results of this study show that aerobic ammonia oxidizers and methanotrophs can be simultaneously active with anammox and N-damo microorganisms in the presence of limited amounts of oxygen.

RESULTS

Establishing an oxygen-limited anammox/N-damo coculture. A 3-liter laboratory-scale SBR containing both nitrate- and nitrite-dependent anaerobic methane-oxidizing microorganisms (N-damo) and anammox bacteria was used to establish an oxygen-limited coculture. The initial SBR bioreactor simultaneously removed ammonium, nitrite, and nitrate; most nitrite was removed by anammox bacteria (a detailed description of the culture can be found in Stultiens et al. [13]). In order to stimulate the growth of aerobic ammonia-oxidizing bacteria for *in situ* nitrite production, oxygen was added via the gas phase. The effect on the overall performance and microbial composition of the culture was studied by combining activity assays with FISH and metagenomics. Over time, the oxygen concentration was gradually increased to a final concentration of 5.4% in the influent gas. The dissolved oxygen concentration remained below the detection limit of the oxygen probe ($<3.1\,\mu$M), indicating that virtually all supplied oxygen was consumed by the culture. The ammonium concentration in the medium was increased parallel to the increasing oxygen supply in order to support the growth of aerobic ammonia oxidizers in the culture, and ammonia and nitrite were immediately consumed by the culture (Fig. 1). Initially, nitrate accumulation was observed after increasing the ammonium concentration in the medium (see Fig. S1 in the supplemental material). Most likely, the increased production of nitrate was due to nitrite oxidation carried out by anammox bacteria, which received more ammonium and consequently produced more N_2 and nitrate. After 82 days, nitrate reduction increased, resulting in consumption of the produced nitrate (Fig. 1). The nitrate supplied externally via the medium was also consumed, which corresponded to a rate of 27 ± 2 mg

N-NO$_3^-$ · liter^{-1} · day^{-1}. Ammonium was consumed at an average rate of 325 ± 44 mg N-NH$_4^+$ · liter^{-1} · day^{-1}. The concentration of nitrite was always below the detection limit of our assay (2 μM); all supplied nitrite was thus consumed by the culture, which corresponds to a conversion of 456 ± 2 mg N-NO$_2^-$ · liter^{-1} · day^{-1}.

Microbial composition of the oxygen-limited coculture. Before the addition of oxygen, the culture was dominated by N-damo bacteria (Fig. 2). The addition of oxygen resulted in the presence of two types of aggregates/flocs. The flocs settled within 5 to 10 min after the stirring stopped, whereas the granules immediately began settling during the settling phase of the bioreactor. The granules mainly consisted of methane-oxidizing *Methanoperedens*-like archaea and anammox bacteria. After the addition of oxygen, the *Methanoperedens*-like archaea increased in abundance, whereas *Methylomirabilis*-like bacteria slowly disappeared from the culture. The presence of N-damo bacteria and archaea and anammox bacteria was confirmed by analysis of the amplicon sequencing of 16S rRNA gene sequences at two different time points (2 and 9 months after the start of the supply of oxygen, respectively) (Table 1). The relative number of *Methylomirabilis*-like bacterial reads in the sequencing data decreased, confirming the FISH analysis, while *Methanoperedens*-like archaea increased in number. Aerobic ammonia oxidizers were not visible with FISH, but their presence was confirmed by the presence of *Nitrosomonas* 16S rRNA sequences in the metagenome at both time points (Table 1).

In situ **activity measurements in the reactor.** In order to determine the performance of different microbial groups within the N-damo/anammox coculture when supplied with oxygen, one of the nitrogen substrates was added to the medium as a ^{15}N-labeled compound (ammonium, nitrite, or nitrate), and production of isotopically labeled gasses (dinitrogen gas and nitrous oxide) was monitored in the liquid phase of the reactor using membrane inlet mass spectrometry (MIMS). When ^{15}N-labeled ammonium was added, single-labeled ^{14}N^{15}N (^{29}N$_2$) dinitrogen gas was produced, indicating the activity of anammox bacteria. In addition, double-labeled ^{15}N^{15}N (^{30}N$_2$) dinitrogen gas was detected, indicating combined activity of aerobic ammonia-oxidizing and anammox bacteria that resulted in the production of ^{30}N$_2$ (Fig. 3A). When ^{15}N-labeled nitrite was fed to the reactor, both ^{29}N$_2$ and ^{30}N$_2$ were detected, indicating that anammox and other N$_2$-producing microorganisms were active (Fig. 3B). Addition of ^{15}N-labeled nitrate (Fig. 3C) led to the production of ^{29}N$_2$, whereas almost no ^{30}N$_2$ production was observed, suggesting that nitrate-dependent N$_2$ production (complete denitrification processes) was negligible in the coculture. In addition, N$_2$O production was not detected in any of these activity assays (Fig. S1).

Batch activity assays. To determine the potential for aerobic ammonia and methane oxidation by the coculture, batch assays were performed. In incubations supplemented with oxygen (6%) and ammonium, nitrite was produced at a rate of 50 ± 0.001 nmol NO$_2^-$ ml · culture^{-1} · h^{-1} (Fig. 4A). Aerobic methane oxidizers were immediately active in batch incubations at a maximum rate of 2.0 ± 0.4 μmol CH$_4$ · ml culture^{-1} · h^{-1} (Fig. 4B). Interestingly, batch incubations also showed production of some methane (Fig. 5), which could be inhibited by the addition of 25 mM 2-bromoethanesulfonate (BES; a known inhibitor of methanogenesis [24]).

DISCUSSION

Several studies have explored the feasibility of a combination of partial nitration, anammox, and N-damo (25–27), but little is known about the effect of oxygen on the stability of such a coculture. This knowledge is important; when a combination of N-damo and anammox processes is applied, oxygen is required for the internal production of nitrite from ammonium, which is needed for both anammox and N-damo activity. In order to study the effect of oxygen on these anaerobic microbial processes and thus the feasibility of the application of anammox and N-damo, limiting amounts of oxygen were supplied to a coculture containing anammox and N-damo microorganisms. Virtually all supplied oxygen was consumed by the culture; ammonium, nitrite, and nitrate were removed by the culture with average rates of 325 ± 44 mg N-NH$_4^+$ · liter^{-1} · day^{-1}, 456 ± 2 mg N-NO$_2^-$ · liter^{-1} · day^{-1}, and 27 ± 2 mg N-NO$_3^-$ · liter^{-1} · day^{-1}, respectively. After the increase of the ammonium
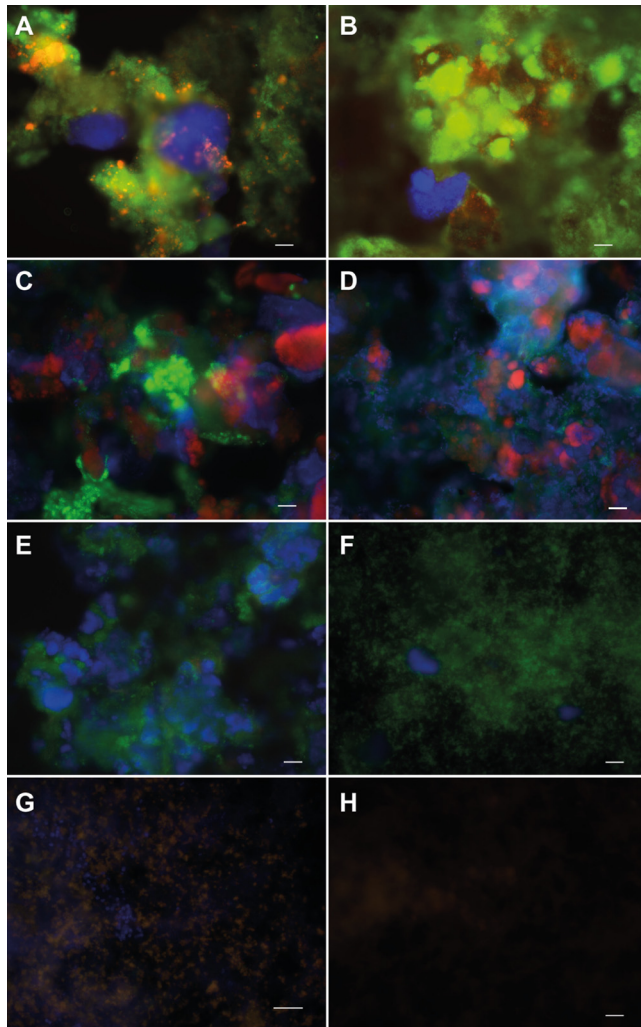
FIG 2 Representative fluorescence *in situ* hybridization (FISH) pictures of the enrichment culture under anaerobic (A and B) and oxygen-limited (C to H) conditions. Bars, 20 μm. (A and B) Samples taken from an N-damo/anammox coculture before oxygen addition. In red, anammox bacteria are visible (Cy3, AMX820), and archaea are visualized in blue (Cy5, ARCH915). *Methylomirabilis* spp. constitute the majority of the biomass and are visible in green (FLUOS, DAMO1027). (C) Sample taken 292 days after oxygen addition. The percentage of *Methanoperedens*-like archaea (red, DARCH641) and anammox bacteria (blue, AMX820) increased, while the relative abundance of *Methylomirabilis* spp. (green, DAMO1027) decreased. (D) Sample taken 732 days after oxygen addition. The percentage of *Methylomirabilis* spp. (green, DAMO1027b) decreased further. *Methanoperedens*-like archaea (red, DARCH641), and anammox bacteria (blue, AMX820) became the dominant microorganisms in the culture. (E) Sample of granules sampled 800 days after the start of oxygen-limited culturing conditions. Anammox bacteria (green, AMX820) and archaea (blue, ARCH915) are the dominant microorganisms in the granules. *Methylomirabilis* spp. (red, DAMO1027) could not be detected. (F) Sample of flocculent biomass taken 800 days after the start of oxygen-limited culturing conditions. Archaea (blue, ARCH915) are nearly absent in the flocculent, while anammox biomass (green, AMX820) is less dense than in the granules. Like in the granules, no *Methylomirabilis* spp. (red, DAMO1027) was detected in the flocs. (G) Sample of flocculent biomass taken 800 days after the start of oxygen-limited culturing conditions. Next to anammox bacteria, visible in yellow/orange (Cy3, PLA46; Fluos, AMX820), other bacteria present in the flocculent biomass are visible in blue (EUBmix). (H) Negative control.

concentration in the medium and the consequent increase of anammox activity, an accumulation of nitrate was observed. However, this additional nitrate was eventually consumed by the culture as well, most probably by N-damo archaea, which also increased in number. N-damo bacteria decreased in abundance, potentially because of the competition for nitrite with anammox bacteria; it has been shown before that *Methylomirabilis*-like bacteria have a lower affinity for nitrite than that of anammox bacteria (17, 28).

TABLE 1 16S rRNA sequences obtained from nitrogen- and methane-cycling microorganisms[a]

Group	No. of 16S rRNA reads at:		Group or genus	Normalized for total reads at:	
	T1	T2		T1	T2
Archaea	296	7,588	*Methanoperedens* spp.	688	2,920
			Methanogens	22	125
			Other *Archaea*	12	66
Nitrifiers	11	37	*Nitrosomonadacea*	27	15
Methanotrophs	47		*Methylobacterium*	2	0
			Methylomonas	0	17
			Methylocystis	105	107
			Methylosinus	2	9
			Methylovirgua	5	0
Anammox	2,948	4,831	*Brocadiaceae*	7,169	1,979
			Scalinduaceae	22	2
N-damo bacteria	3,963	1,716	*Methylomirabilis* spp.	9,666	704

[a]Samples were taken 2 (T1) and 9 (T2) months after the start of the supply of oxygen. For T1, 13.757 reads were analyzed and for T2, 33.555 reads.

The active addition of oxygen to the culture made it possible for aerobic microorganisms to become more active. Using MIMS, production of $^{30}N_2$ upon the addition of ^{15}N-labeled ammonium could be detected, which can only occur if ammonia-oxidizing microorganisms are actively producing ^{15}N-labeled nitrite that can subsequently be used by anammox bacteria. These microorganisms are indeed present in the culture, since nitrite production was observed in aerobic batch incubations with ammonium. However, the observed nitrite production was very low, and no significant decrease in the concentration of ammonium could be measured in these batch incubations. The supplied conditions in the batch incubation and in the reactor were very different, possibly explaining the low activity. In the reactor, the activity of the ammonia-oxidizing microorganisms must be higher in order to explain the observed production of $^{30}N_2$. The potential methane oxidation activity was measured in batch assays; however, it is difficult to conclude how much methane and oxygen are consumed by aerobic methanotrophs in the bioreactor. Surprisingly, batch incubations of the coculture also showed production of some methane. Anammox bacteria have been observed before in methanogenic sludge, but bacteria in these reactor systems were kept under strict anoxic conditions (29, 30). Apparently, the granules provide an environment without oxygen in which the methanogens can thrive.

It is known that aerobic nitrifiers can produce N_2O under oxygen-limited conditions. In our incubations, we did not observed production of N_2O. This is in contrast to a study by Liu et al. (31), in which some production of N_2O was observed in a coculture containing anaerobic and aerobic ammonia- and methane-oxidizing microorganisms. The reactor setups in their study and in ours were different, Liu et al. separated the addition of methane and oxygen by using a membrane reactor in which methane was added via the membrane and oxygen via the gas supply. No $^{30}N_2$ production was detected upon the addition of labeled nitrate, implying that there is only little or even no activity of denitrifying microorganisms, which has been described before in cocultures containing N-damo and anammox microorganisms (32). In contrast, $^{30}N_2$ production was observed when nitrite was added as the labeled substrate. This might be due to the activity of denitrifying microorganisms; however, these microorganisms would most likely also produce $^{30}N_2$ when ^{15}N-labeled nitrate would be supplied. $^{30}N_2$ from labeled nitrite can also be produced from N-damo bacteria. However, FISH analysis has showed that the relative abundance of these microorganisms is very low. Another explanation would be that part of the ^{15}N-labeled nitrite is converted into nitrate by anammox bacteria. This labeled nitrate can subsequently by used by N-damo archaea,
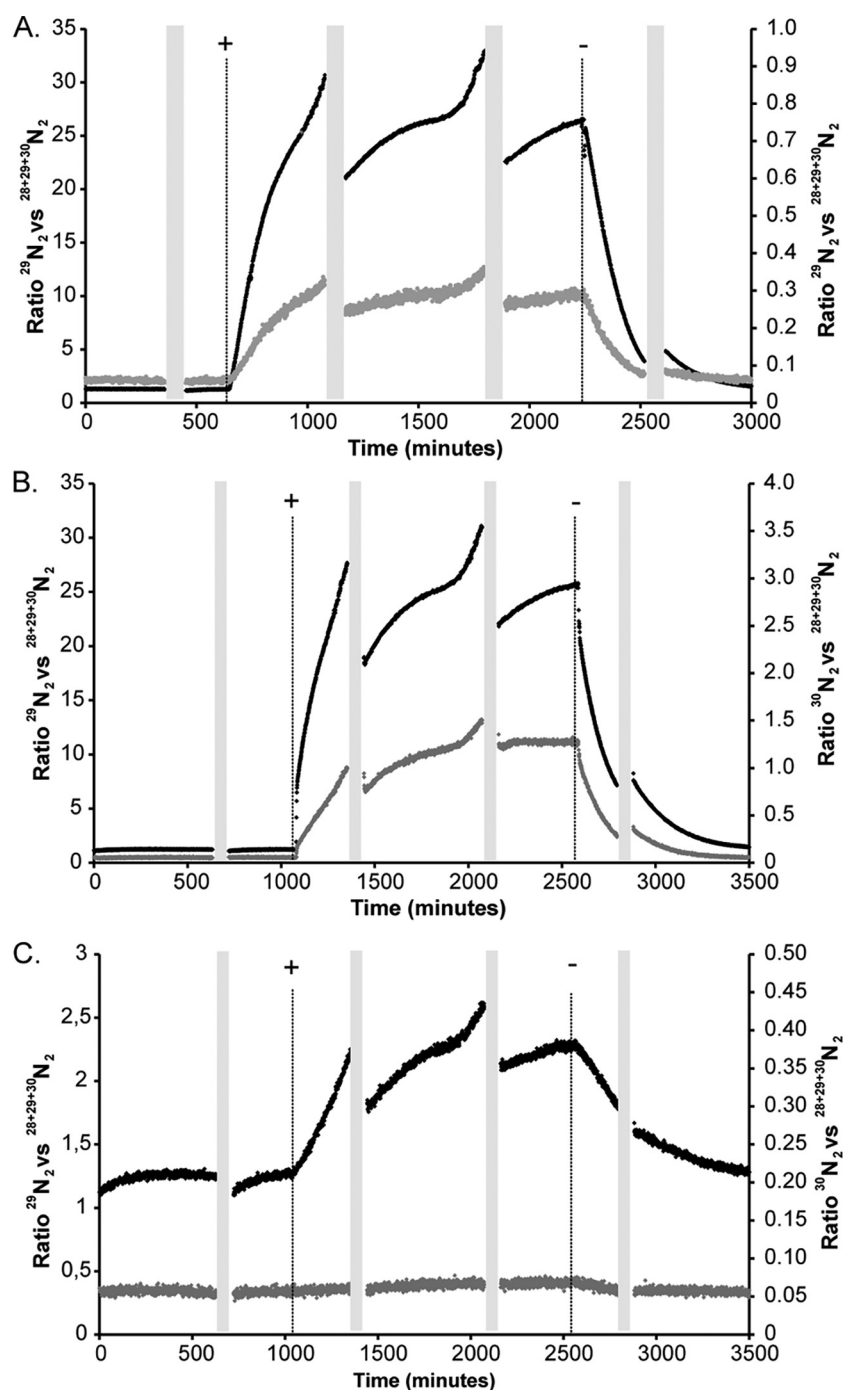
FIG 3 Labeled dinitrogen gas production by the culture measured using membrane inlet mass spectrometry (MIMS) when the culture was fed medium containing ^{15}N-labeled ammonium (A), ^{15}N-labeled nitrite (B), or ^{15}N-labeled nitrate (C). The reactor was running normally, and sequencing batch reactor (SBR) cycles are indicated with gray bars. Time points at which medium containing ^{15}N-labeled substrates was connected and disconnected are indicated by the dotted lines and symbols (+ and −). Production of both ^{29}N$_2$ (black) and ^{30}N$_2$ (gray) was measured and plotted as ratio of total nitrogen gas ($^{28+29+30}$N$_2$).

which can perform dissimilatory nitrate reduction to ammonium (DNRA), resulting in the production of labeled ammonium (7, 33).

In order to apply a combination of N-damo and anammox processes in wastewater treatment, nitrite has to be produced microbially in the treatment plant, a process which requires oxygen. In this study, we show that combined activity of N-damo and anammox organisms
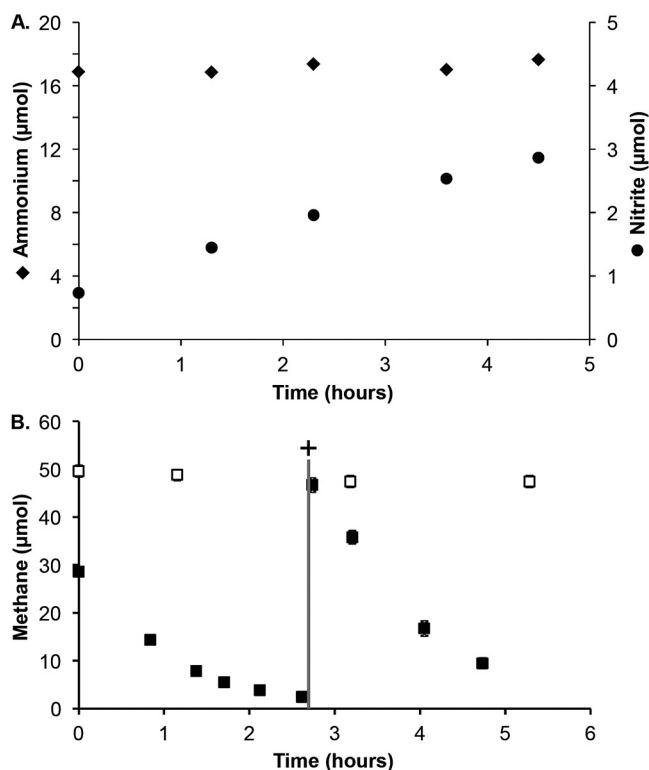
FIG 4 Potential ammonium and methane oxidation by the culture. (A) In the presence of ammonium and oxygen, nitrite (circles) was produced by the culture. (B) In the presence of oxygen, methane (closed symbols) was rapidly consumed by the culture; methane was not consumed when oxygen was not present (open symbols). After 2.5 h, additional methane was added (indicated by the dotted line).

is feasible under the continuous supply of oxygen without the production of N_2O. This study thus shows that a combination of these microbial processes can possibly be applied to simultaneously remove nitrogen and methane from wastewater.

MATERIALS AND METHODS

Ethics statement. This article does not contain any studies with human participants or animals performed by any of the authors.

Oxygen-limited anaerobic methane- and ammonia-oxidizing coculture. An anaerobic methane- and ammonia-oxidizing culture was cultivated in a 3-liter sequencing batch reactor, as described by Stultiens et al. (13). During the filling period, the culture was continuously flushed at $10\,ml \cdot min^{-1}$ with
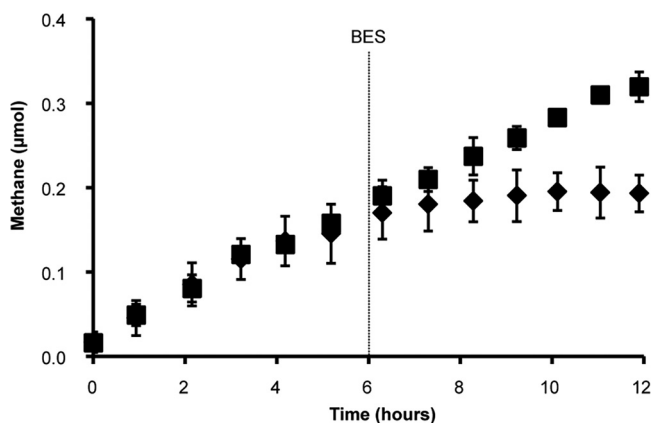

FIG 5 Production of methane in batch incubations with and without 25 mM 2-bromoethanesulfonate (BES). BES was added after 3 h of incubation (indicated by the dotted line).

a mixture of CH_4/CO_2 (95%/5%) and compressed air. The final oxygen concentration in the supplied gas was 5.4%, which resulted in a measured dissolved oxygen concentration that was below the detection limit of the dissolved O_2 sensor ($\leq 3.1\,\mu M$). The mineral medium fed to the culture was supplemented with 20 to 35 mM NH_4Cl, 1.5 mM $NaNO_3$, and 40 mM $NaNO_2$, and 1.3 liters were fed to the culture per day. Roughly 1 month after the start of oxygen addition, the ammonium concentration in the medium was increased from 20 mM to 40 mM to stimulate the growth of aerobic ammonia oxidizers. Liquid samples were taken regularly and were centrifuged for 5 min at maximal speed. The supernatant was stored at $-20°C$ until further analysis.

Fluorescence *in situ* hybridization (FISH). To fix cells for FISH, biomass was harvested by centrifugation for 5 min at $20,000 \times g$. The obtained pellet was washed with phosphate-buffered saline (PBS; 10 mM Na_2HPO_4/NaH_2PO_4 [pH 7.5] and 130 mM NaCl). Subsequently, the biomass was fixed in paraformaldehyde (4%) and hybridized with fluorescently labeled probes as described by Ettwig et al. (6). Probes were Cy3, Cy5, or Fluos labeled and are specified in Table 1. Eub 338, Eub 338 II, and Eub 338 III were mixed in equimolar solution (EUBmix) and used as such. For Bet42a, Gam42a, and Ntspa712, unlabeled competitor probes were used to prevent unspecific hybridization. After hybridization, slides were air-dried and embedded in Vectashield that included 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA). Subsequently, slides were examined and images obtained by utilization of an Axioplan 2 epifluorescence microscope equipped with a digital camera, in combination with the AxioVision software package (Zeiss, Germany).

Metagenome analysis. Biomass samples (1.5 ml) were taken from the oxygen-limited anaerobic methane- and ammonia-oxidizing culture (1.6 liters) and centrifuged for 5 min at $20,000 \times g$. Subsequently, genomic DNA was isolated from the pellet using the PowerSoil kit (MO Bio, Carlsbad, CA), the ammonium acetate method (34), and the cetyltrimethylammonium bromide (CTAB) extraction method (35). Libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Genomic DNA (1 ng) was used for enzymatic fragmentation, which was followed by incorporation of the indexed adapters and amplification. The prepared libraries were purified using the AMPure XP beads (Beckman Coulter, Indianapolis, IN) and checked for size distribution and quality using a high-sensitivity DNA kit in combination with the Agilent 2100 Bioanalyzer. Quantity of the DNA was determined using the Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit (Thermo Fisher Scientific, Inc., Waltham, MA). Subsequently, libraries were pooled, denatured, and sequenced using the Illumina MiSeq sequence machine (San Diego, CA). Paired-end sequencing of 2×300 bp was performed using the MiSeq reagent kit v3 (San Diego, CA) according to the manufacturer's protocol. To study biodiversity, 16S rRNA gene sequences were extracted and analyzed using CLC Workbench with the Silva Database version 132 as a reference.

Batch assay determining methanogenic activity. The headspace of 120-ml serum bottles was exchanged with argon, and 20 ml biomass from the oxygen-limited anaerobic methane- and ammonia-oxidizing culture (1.6 liters) was added to each bottle. Subsequently, the headspace was flushed with argon for another 10 min. CO_2 was added to the anaerobic headspace to a final headspace concentration of 4%. In parallel with incubations without additions, incubations were carried out with either 10 mM or 25 mM BES, added after 3 h of incubation. All incubations were performed in duplicate. Bottles were incubated in a shaking incubator at 30°C and 150 rpm. Gas samples were taken every hour and liquid samples every 2 h. At each time point, presence of nitrite and/or nitrate was also checked using test strips (Merck, Germany).

Batch assay determining aerobic ammonia- and methane-oxidizing activity. The headspace of 60-ml serum bottles containing 10 ml biomass from the oxygen-limited anaerobic methane- and ammonia-oxidizing culture (1.6 liters) was exchanged with argon/CO_2 (90%/10%). Subsequently, O_2 was added to the headspace to a final headspace concentration of 6%. Incubations for the determination of ammonia-oxidizing activity were supplemented with 2 to 3 mM NH_4^+, and incubations for testing aerobic methanotrophy with 2% CH_4. In addition, incubations without oxygen were prepared as negative controls. All treatments were done in triplicate. Bottles were incubated in a shaking incubator at 30°C and 150 rpm. Gas and liquid samples were taken every one to 2 h. For liquid samples, 0.5 ml liquid was extracted from the incubations with a syringe and transferred to an Eppendorf cup. Samples were centrifuged for 1 min at $20,000 \times g$, and the supernatant was transferred to a clean cup and stored at $-20°C$ until further analysis. At each time point, the presence of nitrite was also checked using test strips (Merck, Germany). Afterwards, protein samples (2×1 ml) were taken per bottle and spun down for 5 min at $20,000 \times g$, and the pellet was stored at $-20°C$ until further analysis.

Real-time gas measurements using membrane inlet spectrometry. An HPR-40 membrane inlet mass spectrometer (Hiden Analytical, UK) was used for real-time gas measurements by inserting the membrane inlet mass spectrometry (MIMS) probe directly in the bioreactor. Reactor configuration remained normal to avoid any artifacts in the activity due to changes in substrate supply (both via the gas and medium supply). Labeled substrates (^{15}N-labeled ammonium, nitrite, or nitrate) were added by replacing the unlabeled substrate by an isotopically labeled substrate in the standard medium. Medium was than supplied to the reactor under the standard configuration, after which the production of gasses was measured.

Analytical methods. Liquid samples taken during activity tests were centrifuged for 1 min at $20,000 \times g$, and the supernatant was stored at $-20°C$ until further analysis. Ammonium was determined colorimetrically using a modified orthophataldialdehyde assay (36), and nitrite by the sulfanilamide reaction. Nitrate was measured by reducing it to nitrite at 60°C (30 min) using a saturated solution of VCl_3 in HCl. The produced nitrite was measured using the sulfanilamide reaction. To measure the protein content of the biomass, 1.5-ml biomass samples were taken in triplicate and centrifuged for 5 min at

maximum speed. Supernatant was removed, and the pellet was subsequently homogenized in 0.5 M NaOH, boiled at 90°C for 30 min, and neutralized with 0.5 M HCl. Next, protein content was determined by the bicinchoninic acid assay (Pierce, USA). Methane was measured using gas chromatography (HP 5890 gas chromatograph with a flame ionization detector and Porapak Q column). N_2, O_2, CO_2, and N_2O production was analyzed using gas chromatography (Agilent 6890 and Porapak Q column, 80°C) in combination with mass spectrometry (Agilent 5975, quadruple inert MS).

Data availability. The obtained 16S rRNA reads have been submitted to the Sequence Read Archive (SRA) under BioProject accession number PRJEB37137.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

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