

Nitrate-dependent anaerobic methane oxidation (N-DAMO) as a bioremediation strategy for waters affected by agricultural runoff

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Abstract

Agricultural drainage ditches are subjected to high anthropogenic nitrogen input, leading to eutrophication and greenhouse gas emissions. Nitrate-dependent anaerobic methane oxidation (N-DAMO) could be a promising remediation strategy to remove methane (CH₄) and nitrate (NO₃[−]) simultaneously. Therefore, we aimed to evaluate the potential of N-DAMO to remove excess NO₃[−] and decrease CH₄ release from agricultural drainage ditches. Microcosm experiments were conducted using sediment and surface water collected from three different sites: a sandy-clay ditch (SCD), a freshwater-fed peatland ditch (FPD), and a brackish peatland ditch (BPD). The microcosms were inoculated with an N-DAMO enrichment culture dominated by *Candidatus Methanoperedens* and *Candidatus Methyloirabilis* and supplemented with ¹³CH₄ and ¹⁵NO₃[−]. A significant decrease in CH₄ and NO₃[−] concentration was only observed in the BPD sediment. In freshwater sediments (FPD and SCD), the effect of N-DAMO inoculation on CH₄ and NO₃[−] removal was negligible, likely because N-DAMO microorganisms were outcompeted by heterotrophic denitrifiers consuming NO₃[−] much faster. Overall, our results suggest that bioaugmentation with N-DAMO might be a potential strategy for decreasing NO₃[−] concentrations and CH₄ emission in brackish ecosystems with increasing agricultural activities where the native microbial community is incapable of efficient denitrification.

Keywords: N-DAMO, nitrate, eutrophication, methane emissions, bioaugmentation

Introduction

Lakes, rivers, wetlands, ditches, and ponds are strongly affected by agricultural practices, becoming more eutrophic and anoxic, consequently releasing a substantial amount of nitrous oxide (N₂O) and methane (CH₄) into the atmosphere (Walter et al. 2007, O'Connor et al. 2010, Peacock et al. 2021). Both N₂O and CH₄ are potent greenhouse gases, significantly contributing to the global temperature increase (Jackson et al. 2019, Rosentreter et al. 2021). It has been estimated that in The Netherlands, 330 000 km of ditches may be responsible for 16% of national CH₄ emissions (Koschorreck et al. 2020). Methane produced in anoxic environments via methanogenesis can be consumed via either aerobic or anaerobic methanotrophs, which are thus important players to lower emissions. Especially, nitrate-dependent anaerobic methane oxidation (N-DAMO) is relevant in this respect, as this process removes both CH₄ and nitrogen compounds at the same time. N-DAMO was first discovered in 2006 in the sediment of a Dutch freshwater canal (Raghoebarsing et al. 2006). Research on enrichment cultures showed that *Candidatus Methanoperedens nitroreducens* was coupling anaerobic CH₄ oxidation to the reduction of NO₃[−] (Equation 1; Haroon et al. 2013). In this process, produced nitrite (NO₂[−]) can be further reduced to dinitrogen gas

(N₂) by *Candidatus Methyloirabilis oxyfera* while oxidizing CH₄ through an intra-aerobic methane oxidation pathway (Ettwig et al. 2010; Equation 2).



Several freshwater eutrophic ecosystems and peatlands have been shown to harbor N-DAMO communities (Liu et al. 2015, Shen et al. 2015, Welte et al. 2016, Yang et al. 2018, Shi et al. 2022), indicating that fertilization may stimulate the abundance of *Ca. Methyloirabilis* sp. in rice paddy fields, potentially decreasing CH₄ emissions from these methanogenic environments (Shen et al. 2021, Wang et al. 2022, Yang et al. 2022). Consequently, the N-DAMO process gained a lot of attention in recent years as a potential mitigation strategy to decrease both greenhouse gas emissions and the accumulation of nitrogen originating from agricultural activities (Contreras et al. 2022, Gómez-Gallego 2022). Until today, however, no laboratory studies had been conducted to evaluate the potential of the N-DAMO process in CH₄ and NO₃[−] removal as a bioremediation strategy. Therefore, this work aimed to evaluate whether bioaugmentation with an N-DAMO enrich-

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ment culture consisting of *Ca. Methanoperedens* and *Ca. Methyloirabilis* could decrease CH_4 emissions and NO_3^- concentrations. For that purpose, microcosm experiments supplemented with $^{13}\text{CH}_4$ and $^{15}\text{NO}_3^-$ were conducted using sediments and surface water collected from three different drainage ditches influenced by agricultural runoff. The nitrogen species evolution, CH_4 concentration, and $^{13}\text{CO}_2$ formation were followed over time. Additionally, the genomic potential of the inoculum and microbial community composition in the original sediment and the microcosm sediment was analyzed using metagenomics and 16S rRNA amplicon sequencing, respectively.

Materials and methods

Study sites, sampling, and field data collection

Sediment and surface water samples were collected from three agriculture-impacted areas in The Netherlands. The selected sites consisted of a sandy-clay ditch (SCD) located in the Ooijpolder, a former floodplain of the River Rhine, a freshwater-fed peatland ditch (FPD) surrounded by peat meadows in agricultural use in Aldeboarn, and a brackish peatland ditch (BPD) located in Polder Westzaan that is within a “Natura 2000” protected area under agricultural influence and subjected to re-salinization to restore brackish ecological values (Fig. 1). The three selected sites differ substantially due to their location, history, sediment type and composition, land use, and yearly N regime (Table 1); nevertheless, all three sites are under strong anthropogenic pressure mainly due to intensive agriculture activities.

In each of the sites, we collected sediment samples ($n = 9$) from the upper part of the sediment profile targeting organic layers in 1.8-mm-thick transparent PVC tubes (diameter 6 cm and height 60 cm), using a UWITEC sediment corer (UWITEC, Mondsee, Austria), while the surface water samples were collected from the water column above the sediment. In each of the sites, we measured CH_4 and CO_2 fluxes using a transparent closed chamber (height 30 cm and diameter 28.8 cm) floating on top of the water column connected to a Los Gatos (LGR®) Ultra-Portable Greenhouse Gas Analyzer. A fraction of the surface water (40 mL) and well-mixed sediment samples (15 mL) of each site were frozen (-20°C), to measure the nutrient concentration in the water column and to extract DNA, respectively. The water was filtered through 0.45 μm pore size filters before chemical analysis. Dissolved N species (NO_3^- , NO_2^- , NH_4^+) were measured colorimetrically using a Bran + Luebbe system and Seal Model III system continuous auto-analyzer.

Inoculum source and characteristic

The N-DAMO enrichment culture was propagated from the original enrichment using sediment from Twentekanaal canal (The Netherlands; Raghoebarsing et al. 2006) fed with extra NO_3^- in bioreactor systems (Arshad et al. 2015). In April 2021, the culture mainly consisted of *Ca. Methanoperedens nitroreducens* BLZ2 (~33%) and *Ca. Methyloirabilis oxyfera* (~27%; Fig. S1). To determine the CH_4 oxidation and NO_3^- reduction potential of the culture, we screened for the functional genes involved in CH_4 and N cycling of the top (>1% mapped reads) most abundant metagenome-assembled genomes (Fig. S1). The analyzed metagenomic dataset was first reported in Schoemerich et al. (2022; “Bioreactor 1”), available under NCBI Bioproject ID PRJNA850006. See Supplementary Materials for more details on metagenomic analysis. For the microcosm’s inoculation, 30 mL of culture was anoxically withdrawn from the bioreactor and washed three times

with sterile medium (without NO_3^-) in an anaerobic glovebox. Washed biomass was then used to inoculate the microcosm.

Microcosm setup and sampling procedure

To measure microbial activity and assess the effect of N-DAMO addition on the N-concentration and CH_4 emissions, we set up microcosms experiment with three different treatments: (1) Control—sediment without any additions; (2) NO_3^- —sediments supplemented with 3 mM $\text{Na}^{15}\text{NO}_3^-$ and 10 mL $^{13}\text{CH}_4$; and (3) $\text{NO}_3^- + \text{N-DAMO}$ —sediment supplemented with 3 mM $\text{Na}^{15}\text{NO}_3^-$, 10 mL $^{13}\text{CH}_4$, and 0.40 ± 0.0081 g (dry weight) of N-DAMO enrichment culture. All the treatments were prepared in quadruplicates using 30 g of homogenized slurry sediment and 40 mL of surface water mixed in 120 mL sterile serum bottles. The microcosms were set up in the anoxic glovebox (<10 ppm O_2), where anoxic $\text{Na}^{15}\text{NO}_3^-$ and inoculum were added. The bottles were closed and crimped to ensure anoxic conditions and prevent oxygen intrusion. Afterward, the headspace was exchanged with N_2/CO_2 and $^{13}\text{CH}_4$ was injected into all treatments except the control. All the microcosms were kept in the dark at 17°C . Sampling was performed using a sterile syringe and needle. The first time point was measured immediately after inoculation and supplementation of $^{13}\text{CH}_4$ and $^{15}\text{NO}_3^-$. Measurements continued two to three times a day while NO_3^- was being actively reduced. Reduction of NO_3^- was followed using NO_3^- strips (MQuant, STEP Systems) and later measured spectrophotometrically. Depending on the NO_3^- reduction rate, the incubation time varied between sediment types from lasting several days (FPD and SCD) to several weeks (BPD). To capture the microbial community actively involved in NO_3^- reduction and CH_4 oxidation, halfway through the experiment, one bottle of each treatment was sacrificed for DNA extraction. Consequently, the final number of replicates per treatment was three at the end of the incubations.

Gas measurements

The $^{13}\text{CO}_2/^{12}\text{CO}_2$, $^{46}\text{N}_2\text{O}/^{44}\text{N}_2\text{O}$, and $^{30}\text{N}_2/^{28}\text{N}_2$ ratios were determined by gas chromatography coupled to mass spectrometry (Trace DSQ II, Thermo Finnigan, Austin, TX, USA), and the concentration of CH_4 was quantified by gas chromatography with flame ionization detection (Hewlett Packard HP 5890 Series II Gas Chromatograph, Agilent Technologies, CA, USA). The pressure was measured at each time point using a portable pressure meter (GMH 3100, GHM Messtechnik, Regenstauf, Germany) to account for the compressed gas in the headspace.

Water chemistry

One milliliter of liquid sample was withdrawn anoxically at each time point for the NO_3^- , NO_2^- , and NH_4^+ quantification using colorimetric methods. The Griess assay was used to quantify NO_3^- and NO_2^- , while the o-phthalaldehyde assay was used to determine the concentration of NH_4^+ (Meseguer-Lloret et al. 2002, Sun et al. 2003). Both assays were performed using 96-well plates and absorbance was measured on the spectrophotometer (Molecular Devices, Spectramax 190 Microplate Reader).

DNA extraction and amplicon sequencing

The sediment samples for the DNA extraction were collected in the field (in-situ sediment) and from all microcosms in the middle of incubation. DNA was extracted from one bottle of each treatment across all sediment types. In total, 15 DNA samples were obtained. The DNA extraction was performed using the PowerSoil DNA Extraction Kit (DNeasy PowerSoil Pro Kit, QIAGEN, Hilden,



Figure 1. Location of drainage ditches chosen for the microcosm experiment with the CH_4 and CO_2 fluxes and surface water parameters. Reported are NO_3^- and NH_4^+ concentrations, top and bottom pH, O_2 concentration, and temperature. By courtesy of Encyclopædia Britannica, Inc., copyright 2002; used with permission.

Table 1. Location, sediment type, land use, and sampling dates of the selected field sites.

Abbreviation	Location	Longitude	Latitude	Sediment	Land use	Sampling date
SCD	Ooijpolder	51.50400	5.54440	Clay/sand + organic top layer	Agricultural	14 April 2022
FPD	Aldeboarn	53.052324	5.902433	Freshwater peat	Agricultural	20 April 2022
BPD	Westzaan	52.480448	4.786335	Brackish peat	Nature reserve in agricultural area	02 February 2022

Germany), according to the manufacturer's protocol. The concentration of the DNA was quantified using the Qubit® 2.0 Fluorometer with DNA HS kits (Life Technologies, Carlsbad, CA, USA). 16S rRNA gene amplicon sequencing was done by Macrogen (Macrogen, Amsterdam, The Netherlands) using the Illumina MiSeq Next Generation Sequencing platform. Paired-end libraries were constructed using the Illumina Herculese II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina, Eindhoven, The Netherlands). Detailed information on primers and data analysis is provided in the Supplementary Materials. The raw sequence data and meta-data of the microcosms experiment have been deposited in the sequence read archive (SRA) database of the NCBI under the Bio-Project ID PRJNA918998.

Results and discussion

In-situ nitrogen measurements

At the time of measurement (May 2022), NO_3^- was only detected in water columns of BPD and FPD (3.0 and $2.5 \mu\text{M}$, respectively),

while no detectable NO_3^- was reported in SCD. Moreover, BPD had the highest NH_4^+ concentration ($12 \mu\text{M}$) compared to FPD ($4.7 \mu\text{M}$) and SCD ($5.7 \mu\text{M}$). As the samples were collected at the beginning of the growing season, the application of N-fertilizers was still rather low. Nevertheless, it is assumed that the majority of NO_3^- and NH_4^+ originated from agricultural runoff. Besides fertilizer application, organic matter degradation and mineralization in the sediment likely also contributed to high NH_4^+ concentrations in all ditches.

In-situ methane emission

Late spring CH_4 emissions varied greatly across the three different ditches (Fig. 1). The highest measured CH_4 fluxes were observed in FPD ($36.5 \pm 6.4 \text{ mg m}^{-2} \text{ d}^{-1}$), followed by the ditch BPD in Westzaan ($14.5 \pm 0.60 \text{ mg m}^{-2} \text{ d}^{-1}$) and SCD in the Ooijpolder ($7.7 \pm 1.6 \text{ mg m}^{-2} \text{ d}^{-1}$). The Ooijpolder SCD is characterized by a mineral sandy-clay layer with a relatively thin (15–30 cm) organic-rich top layer. The lower emission of CH_4 in this ditch was likely due to lower carbon (C) availability compared

to FPD and BPD. Also, the SCD and BPD were probably subjected to an aerobic breakdown of organic matter as a large amount of CO_2 was released from the water column. The FPD was a sink of CO_2 suggesting that in this ditch, a photosynthetic carbon fixation might have been greater than the decomposition of organic matter (Fig. 1). The FPD sediment released most CH_4 . This is not surprising as freshwater peatlands are known to be the largest natural source of atmospheric CH_4 worldwide and store disproportionate amounts of global soil carbon (Feng et al. 2020, Peacock et al. 2021). The high abundance of sulfate (SO_4^{2-}) and increased salinity was likely a reason why the CH_4 release was much lower in the BPD compared to the FPD.

A previous study conducted in the same region with a similar sediment type demonstrated that with increasing sea salt concentration (combination of salt and SO_4^{2-}), the porewater CH_4 concentrations significantly decrease (van Dijk et al. 2019). It was hypothesized that increased surface water salinity changed the conditions from methanogenic to SO_4^{2-} reducing as thermodynamically more favorable, decreasing the availability of fermentation products to fuel CH_4 production. Overall, the three ditches selected for this study represent distinct conditions suitable to study how N-DAMO activity may affect CH_4 emission and NO_3^- removal under various environmental conditions and whether inoculation with N-DAMO enrichment cultures has potential as a bioremediation strategy.

N-DAMO bioaugmentation promotes N removal in brackish sediment

The effect of N-DAMO inoculation on NO_3^- concentration was the most pronounced in the BPD microcosms. Nitrate reduction in microcosms supplemented with the N-DAMO enrichment culture proceeded much faster compared to uninoculated microcosms, completely removing added NO_3^- (3 mM) within 19 days of incubation (Fig. 2A). On the other hand, only one-third of the added NO_3^- (1 mM) was consumed in uninoculated microcosms after nearly 20 days of incubation. Therefore, it is evident that N-DAMO was the main process responsible for the complete removal of NO_3^- . The NO_3^- reduction driven by N-DAMO was, however, much slower in BPD compared to FPD and SCD where NO_3^- was removed within 80 and 60 hours after starting the incubation, respectively, and was much less dependent on the N-DAMO process as only a small fraction of CH_4 was oxidized (Figs. 2 and 3).

The slow NO_3^- reduction rate in BPD is likely because of the high salinity, which was previously shown to negatively affect N-DAMO activity, substantially decreasing denitrification potential (Chen et al. 2021, 2022). It was also shown that the intrusion of seawater negatively influences the denitrifying microbial community, decreasing the denitrification rate in freshwater ecosystems (Neubauer et al. 2019). It was hypothesized that the negative correlation between salinity and denitrification might be caused by higher sulfide and/or chloride levels (Rysgaard et al. 1999, Craft et al. 2009). The concentration of NO_2^- in uninoculated BPD microcosms increased at the beginning of the incubation reaching its maximum at day 8 ($98 \pm 20 \mu\text{M}$), however by the end of the experiment, it was completely gone. The N-DAMO inoculation clearly prevented the formation and accumulation of NO_2^- in water as it was quickly consumed at the beginning of the experiment (Fig. 2B). The NH_4^+ concentration was highest in N-DAMO inoculated microcosms compared to uninoculated microcosms, reaching nearly $790 \pm 70 \mu\text{M}$ at the end of

the experiment compared to uninoculated microcosms where it was $145 \pm 45 \mu\text{M}$ (Fig. 2C). It must be noted, however, that the N-DAMO inoculated microcosms had a higher concentration of NH_4^+ from the beginning of the experiment, although it is not clear why. It might be related to decaying organic matter and biomass added with the N-DAMO inoculum and subsequent mineralization processes. Moreover, it has been previously shown that N-DAMO communities dominated by ANME archaea are also capable of dissimilatory nitrate reduction to ammonium (DNRA; Arshad et al. 2015, Nie et al. 2021). Also, the N-DAMO enrichment culture used here appeared to be capable of DNRA as confirmed by the metagenomic analysis (Fig. S1). Therefore, it is possible that some of this NH_4^+ originated from NO_3^- reduction.

A substantial fraction of added NO_3^- in inoculated BPD microcosms was converted into N_2 gas as the ratio of $^{30}\text{N}_2/^{28}\text{N}_2$ increased from 1% to 6%, while in uninoculated microcosms, this ratio increased only to 1.3% (Fig. S2A). No NO_3^- was converted into N_2O , which was deduced from the ratio of $^{46}\text{N}_2\text{O}/^{44}\text{N}_2\text{O}$ (Fig. S2B). No increase of $^{46}\text{N}_2\text{O}/^{44}\text{N}_2\text{O}$ ratio suggests steady $\text{NO}-\text{N}_2\text{O}-\text{N}_2$ reduction mediated by *Ca. Methanoperedens* and side N_2O reducing community, or by *Ca. Methylomirabilis oxyfera*, via the conversion of two nitric oxide (NO) molecules to N_2 and O_2 bypassing N_2O formation (Fig. S1).

Freshwater ecosystems are known to have higher denitrification rates compared to brackish ones (Wang et al. 2018) and this was also found in our experiment. The freshwater SCD from Ooijpolder showed a very high denitrification potential as all NO_3^- was nearly completely depleted within 70 hours of incubation. The addition of the N-DAMO culture did not affect the denitrification rate as both inoculated and uninoculated microcosms performed similarly (Fig. 2D). In both cases, NO_2^- initially increased in concentration to $35 \mu\text{M}$ and afterward continuously decreased until it was nearly completely depleted after 53 hours (Fig. 2E). This is not surprising as the Ooijpolder from where the SCD sediment was collected is an agricultural area where denitrifying communities most likely are already well established. The initial NH_4^+ concentration in SCD microcosms inoculated with the N-DAMO enrichment cultures was higher ($128 \mu\text{M}$) compared to values in uninoculated microcosms ($81 \mu\text{M}$). By the end of the incubation, the NH_4^+ in bioaugmented microcosms was however very low ($9.3 \mu\text{M}$), while in control and NO_3^- supplemented microcosms it was still above $40 \mu\text{M}$ (Fig. 2F). The ratio of $^{30}\text{N}_2/^{28}\text{N}_2$ increased from 0% to 3%, in both inoculated and uninoculated microcosms (Fig. S2D) implying that similar denitrification rates occurred in both treatments.

Inoculation of FPD sediment with the N-DAMO enrichment culture slightly accelerated the removal of NO_3^- (Fig. 2G) but did not affect the final concentration of NO_2^- (Fig. 2H) and NH_4^+ (Fig. 2I). The ratio of $^{30}\text{N}_2/^{28}\text{N}_2$ in FPD increased from 0% to 3%, in both inoculated and uninoculated, microcosms indicating conversion of $^{15}\text{NO}_3^-$ into $^{15}\text{N}_2$ (Fig. S2G). The ratio of $^{46}\text{N}_2\text{O}/^{44}\text{N}_2\text{O}$ increased, however, only in uninoculated microcosms supplemented with NO_3^- , reaching its maximum of 2.3% after 71 hours (Fig. S2H). The production of N_2O implies that incomplete denitrification or NH_4^+ oxidation took place when NO_3^- was supplied to FPD sediment (Hallin et al. 2018). This is concerning as N_2O is a potent greenhouse gas, third in the rank after CO_2 and CH_4 . The N-DAMO bioaugmentation prevented the formation of N_2O possibly because *Ca. Methylomirabilis* can bypass N_2O production by reducing NO intra-aerobically (Ettwig et al. 2010; Fig. S1).

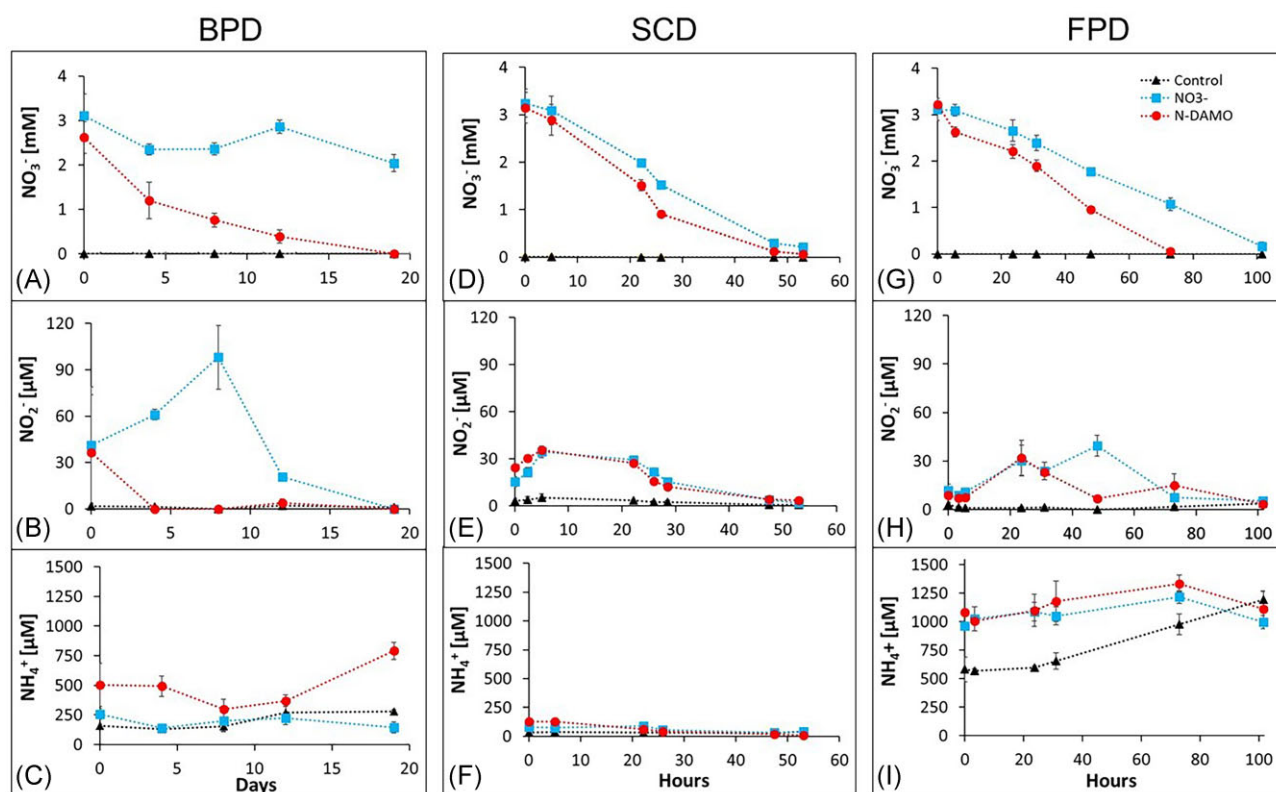


Figure 2. Evolution of N species over the microcosm incubation period. The concentration of nitrate (A, D, and G), nitrite (B, E, and H), and ammonium (C, F, and I). BPD, brackish peatland ditch; SCD, sandy-clay ditch; FPD, freshwater peatland ditch. Note different scales on the x-axis; BPD in days, SCD and FPD in hours. Methane was added to all microcosms besides control. Each measurement was performed in technical triplicate. Error bars represent the standard deviation of the biological triplicate.

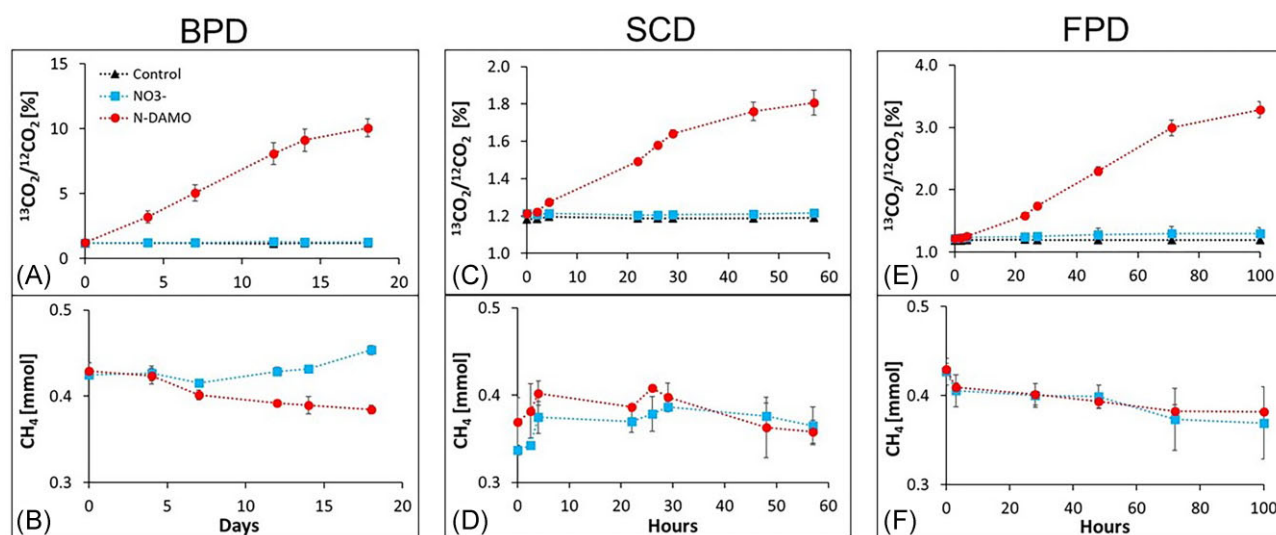


Figure 3. Methane oxidation and $^{13}\text{CO}_2$ production in the microcosm setups. The ratio of $^{13}\text{CO}_2/^{12}\text{CO}_2$ (A, C, and E; note the difference in y-axis scale) and CH_4 concentration (B, D, and F) over time. For a better presentation of the changes in CH_4 concentrations, the vertical axis starts at 0.3 mmol as no CH_4 was added to the control group. Methanogenesis was only observed in the FPD control setup where CH_4 was accumulating over time (Fig. S3). The error bars stand for the standard deviation between biological triplicates of each treatment. BPD, brackish peatland ditch; SCD, sandy-clay ditch; FPD, freshwater peatland ditch.

N-DAMO bioaugmentation increases CH_4 oxidation in brackish sediment

It has been suggested that increasing N input to various ecosystems may stimulate N-DAMO activity and reduce CH_4 emission to

the atmosphere (Shi et al. 2022, Wang et al. 2022). In our experiment, $^{13}\text{CO}_2$ was produced only in the presence of the N-DAMO enrichment culture (Fig. 3), implying that the native microbial community was not actively oxidizing CH_4 . Most CH_4 was oxi-

dized in the BPD microcosms, which was reflected in the highest increase of $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio from 1% to >10% (Fig. 3A). This was further confirmed by the decrease in the total amount of CH_4 , which dropped from 0.42 to 0.38 mmol (Fig. 3B). Overall, 10% of the added CH_4 was consumed after 20 days of incubation. The concentration of CH_4 in uninoculated microcosms however slightly increased over time, although in the control setup, we did not observe methanogenic activity and build-up of CH_4 . Overall, it can be concluded that bioaugmentation with the N-DAMO enrichment culture indeed promoted CH_4 oxidation and resulted in lower emissions of this greenhouse gas in BPD.

In the incubation with SCD and FPD sediment inoculated with N-DAMO, only a small amount of $^{13}\text{CO}_2$ was produced as the ratio of $^{13}\text{CO}_2/^{12}\text{CO}_2$ increased from 1.2% to 1.8% and to 3.2%, respectively (Fig. 3C and E). Similarly, there was no difference in CH_4 concentration between inoculated and uninoculated microcosms (Fig. 3D and F). This implies that heterotrophic NO_3^- reduction by the native microbial community, in both FPD and SCD sediments microcosm incubations, outcompeted N-DAMO microorganisms. The rapid depletion of NO_3^- and lack of alternatives led to a shortage of electron acceptors and consequently, CH_4 could not be oxidized. Previous studies showed that *Ca. Methanoperedens* and *Ca. Methyloirabilis* mediating N-DAMO have a high doubling time in the order of several weeks (Raghoebarsing et al. 2006, Vaksmaa et al. 2017), resulting in lower competitiveness compared to fast-growing denitrifiers.

N-DAMO archaea and bacteria persisted in the microbial community

The bioaugmentation with N-DAMO appeared to be persistent as introduced species were still present in the microbial community and survived over the incubation time, in all microcosms (Fig. 4). This is important as many bioinoculants used in agriculture have been shown to have low efficiency and survival rate over time (O'Callaghan 2016).

Generally, the core microbial taxa were similar between all sediment types (Fig. 4). All three sediments showed the presence of similar methanogenic archaea such as *Methanosaeta*, *Methanoregula*, and *Methanobacterium*. However, methanogenic archaea affiliating with *Methanosarcina* were only abundant in Ooijpolder SCD sediment where this taxon represented >9% of microbial community, while in peatland sediments of BPD and FPD its relative abundance was only 0.8% and 1.3%, respectively. Furthermore, the microbial community in BPD was characterized by a much higher relative abundance of *Bathyarchaeia* (~50%) compared to SCD (~20%) and FPD (~20%). The SCD was also the only sediment type that had a high relative abundance of *Nitrososphaera*, among which most genera are known ammonia oxidizers (Cao et al. 2013). It can also be noticed that for each treatment, the original sediment collected in the field, control sediment (unamended), and NO_3^- -amended sediment had nearly identical composition, meaning that the microbial community did not change much during the incubation and adding NO_3^- did not trigger changes in the microbial community. However, the inoculation with N-DAMO substantially changed the composition of the archaeal community. Specifically, in all sediments, *Ca. Methanoperedens* was by far the most dominating taxon (Fig. 4A). In the BPD microcosms, in the middle of the experiment, it represented >90% of the archaeal community. This suggests that (1) the abundance of archaea was generally low in BPD and (2) *Ca. Methanoperedens* adapted to the elevated salinity, which was previously suggested by Li et al. (2022). The relative abundance of *Ca. Methanoperedens* in the archaeal communities

of SCD and FPD was also high, 73% and 47%, respectively, implying that these archaea were responsible for the CH_4 oxidation and $^{13}\text{CO}_2$ formation in all inoculated sediments.

Similarly to the archaeal microbial community, the bacterial community was also very similar across all sediment types but also between the original sediment collected in the field, control sediment (unamended), and NO_3^- -amended sediment, halfway through the incubation (Fig. 4B). Although it was expected that denitrifying microorganisms would increase in relative abundance, it appeared that adding NO_3^- did not trigger changes in the microbial community. This may imply that the denitrifying community was already well established. Indeed, many microorganisms known to be involved in denitrification such as *Dechloromonas*, *Thiobacillus*, *Bacillus*, and *Denitratisoma* were detected in the microbial community in all sediment types.

The inoculation with the N-DAMO enrichment culture led to the introduction of *Ca. Methyloirabilis*, which represented 10%, 3%, and 3% of the bacterial community in BPD, SCD, and FPD, respectively. Therefore, it can be assumed that the conditions for *Ca. Methyloirabilis* were the most suitable in BPD, where the denitrification rate was the lowest. The concentration of NO_2^- , a necessary electron acceptor for *Ca. Methyloirabilis*, was for the most time nearly undetectable in BPD microcosms. This is most likely because NO_2^- was continuously consumed by *Ca. Methyloirabilis* as soon as NO_3^- was reduced, consequently keeping its concentration at a very low level. *Candidatus Methyloirabilis* probably also contributed to CH_4 oxidation and $^{13}\text{CO}_2$ formation in all inoculated microcosms.

It appeared that *Methylocystis* was introduced together with the N-DAMO enrichment culture as its abundance increased in all inoculated microcosms. This type II methanotroph was particularly abundant in the BPD setup where it represented 6.5% of the bacterial community in inoculated microcosms, while it was nearly undetectable in control and NO_3^- -supplemented setups. Also, *Rhizobiales* and *Denitratisoma* were found to be particularly enriched in N-DAMO inoculated BPD sediment, possibly contributing to N cycling, as many *Denitratisoma* species are known to be involved in NO_3^- reduction.

Conclusion and environmental implications

Agricultural runoff leads to increasing N content in surface waters. The input of nutrients further stimulates biomass production, eutrophication, fermentation, and, consequently, methanogenesis. As a result, agriculture-affected waters are strongly methanogenic and loaded with N compounds. There is an urgent need to restore the quality of these surface waters and decrease CH_4 emissions. Although many studies suggested that N-DAMO is an important sink of CH_4 , and might be a good bioremediation strategy, our study demonstrates that the effect of N-DAMO bioaugmentation is system specific. Inoculation with N-DAMO substantially contributed to the removal of NO_3^- and the decrease of CH_4 emission in brackish microcosms. It appeared that *Ca. Methanoperedens* and *Ca. Methyloirabilis* relative abundances were higher in the inoculated brackish sediment than in freshwater ones, implying their adaptability to saline conditions and competitiveness with the native microbial community. By contrast, in freshwater ecosystems, it appears that N-DAMO microorganisms are outcompeted by heterotrophic denitrifiers. Here, inoculation with N-DAMO microorganisms did not improve NO_3^- and CH_4 removal. This is most likely because in these freshwater ditches the denitrifying community is already well developed and outcompetes N-DAMO bacteria and archaea for the N substrates, thereby

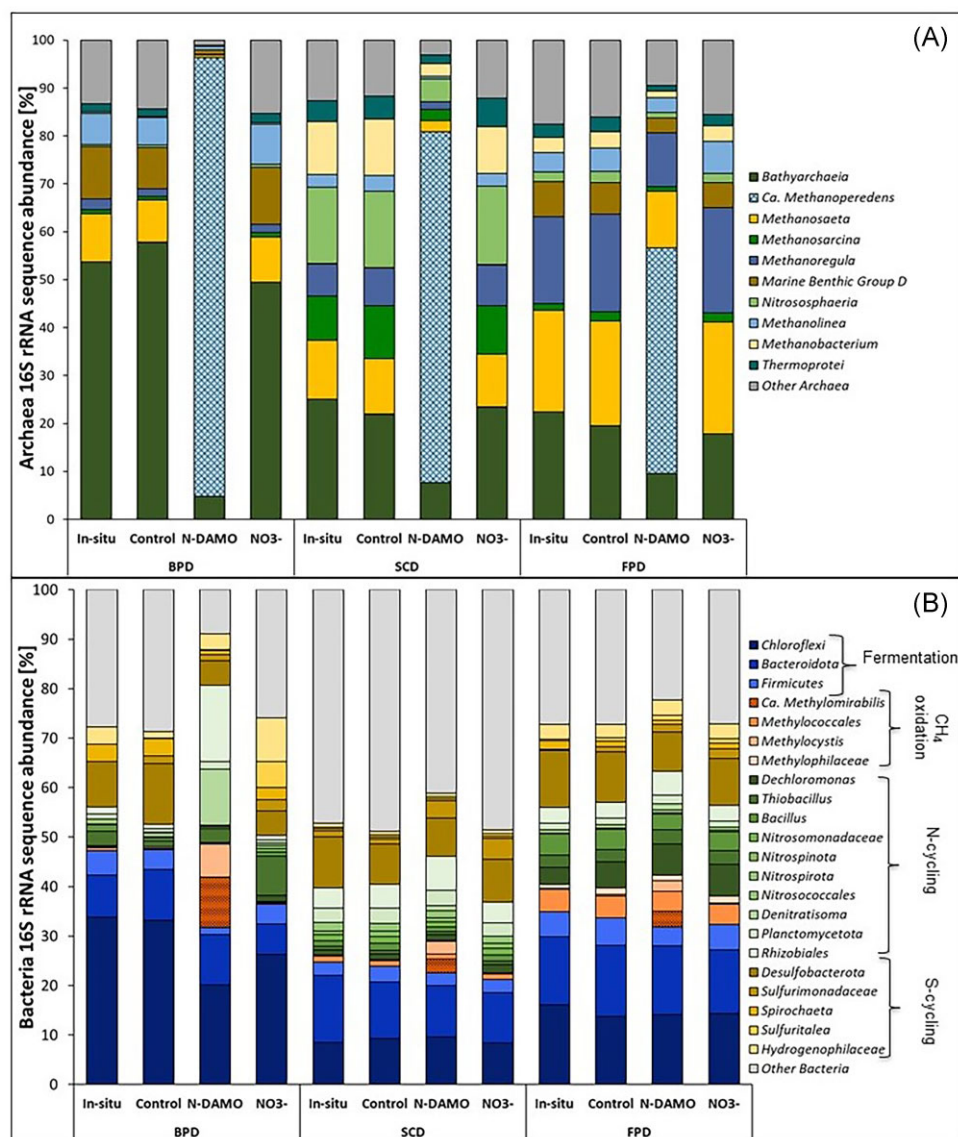


Figure 4. Archaeal (A) and bacterial (B) 16S rRNA gene sequence abundance. *In-situ* stands for the original sediment collected in the field; Control—sediment without any additions; N-DAMO—sediment inoculated with N-DAMO and amended with NO_3^- ; NO_3^- —sediment amended with NO_3^- . Control, N-DAMO, and NO_3^- represent communities half through the incubation period. The highest taxonomic level is presented with its putative function. BPD, brackish peatland ditch; SCD, sandy-clay ditch; FPD, freshwater peatland ditch.

preventing the mitigation of CH_4 emissions. However, under more realistic, environmental conditions where continuous but rather low NO_3^- supplies from agricultural runoff will take place, the N-DAMO community might be able to access some of the leached NO_3^- , possibly decreasing CH_4 emission. To test this hypothesis, a mesocosm or field experiment should be conducted over a prolonged period. Moreover, the introduction of anammox bacteria together with N-DAMO could further contribute to the removal of NH_4^+ and NO_2^- via conversion to N_2 . Overall, our results suggest that N-DAMO inoculation might be a potential strategy for climate-smart water quality protection and restoration in areas with increasing agricultural activities where the native microbial community is incapable of denitrification.

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Supplementary data

Supplementary data are available at [FEMSLE](https://femsle.onlinelibrary.com/) online.

Conflict of interest. None declared.

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