

Nitrate leaching and its implication for Fe and As mobility in a Southeast Asian aquifer

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Abstract

The drinking water quality in Southeast Asia is at risk due to arsenic (As) groundwater contamination. Intensive use of fertilizers may lead to nitrate (NO_3^-) leaching into aquifers, yet very little is known about its effect on iron (Fe) and As mobility in water. We ran a set of microcosm experiments using aquifer sediment from Vietnam supplemented with $^{15}\text{NO}_3^-$ and $^{13}\text{CH}_4$. To assess the effect of nitrate-dependent anaerobic methane oxidation (N-DAMO) we also inoculated the sediment with two different N-DAMO enrichment cultures. We found that native microorganisms and both N-DAMO enrichments could efficiently consume all NO_3^- in 5 days. However, CH_4 oxidation was observed only in the inoculated microcosms, suggesting that the native microbial community did not perform N-DAMO. In uninoculated microcosms, NO_3^- was preferentially used over Fe(III) as an electron acceptor and consequently inhibited Fe(III) reduction and As mobilization. The addition of N-DAMO enrichment cultures led to Fe(III) reduction and stimulated As and Mn release into the water. The archaeal community in all treatments was dominated by *Ca. Methanoperedens* while the bacterial community consisted of various denitrifiers. Our results suggest that input of N fertilizers to the aquifer decreases As mobility and that CH_4 cannot serve as an electron donor for NO_3^- reduction.

Keywords: arsenic, groundwater, iron, methane, N-DAMO, nitrate

Introduction

High arsenic (As) concentrations in groundwater are a global problem. It was estimated that as much as 150 million people worldwide might be affected by As-contaminated water exceeding the drinking water limit of $10\ \mu\text{g/L}$ recommended by the World Health Organization (WHO), with the vast majority of affected people (~94%) located in Asia (Podgorski and Berg 2020). Arsenic has been recognized as a group I human carcinogen by The International Agency for Research on Cancer (IARC). Long-term exposure to As-contaminated water or excessive As intake is a serious health hazard frequently leading to an increased risk of cancer, and cardiovascular and neurological diseases (Hughes 2002, Chen et al. 2009). Therefore, As pollution has become an alarming concern triggering a global research initiative aiming to understand the underlying biogeochemical mechanisms of As (im)mobilization in aquifers. Due to limited access to water treatment facilities and use of the untreated shallow groundwater as a primary drinking water source, As poisoning is particularly severe in rural areas of South and Southeast Asia (Carrard et al. 2019). Vietnam is among the most affected countries where As concentrations in drinking water from household water wells can reach $3050\ \mu\text{g/L}$, exceeding 300 times the WHO safe limit (Berg et al. 2007, Le Luu 2019). The problem of high As concentration in drinking water has not been solved yet, and it continues to be the largest mass poisoning of the human population in history (Sen and Biswas 2013).

Mobility of As is controlled by many factors including sediment geochemistry, evapotranspiration, flow-through conditions, pH, redox potential, microbial community, and ion availability

(Mladenov et al. 2014, Pipattanaojroenkul et al. 2021). Because of the strong affinity for As and adsorption ability of Fe(III) (oxyhydr)oxides, the reductive dissolution of Fe(III) minerals plays an important role in As groundwater accumulation (Yang et al. 2015). The coupling of reductive dissolution of Fe(III) (oxyhydr)oxides with organic carbon oxidation by microbial processes is considered the primary pathway for increasing dissolved As concentrations in aquifers of South and Southeast Asia (Fendorf et al. 2010). Several studies showed that the presence of Fe(III)-reducing microorganisms significantly increased the rate of Fe(III)-reduction and As mobilization (Islam et al. 2005a,b, Jiang et al. 2013, Glodowska et al. 2020). Arsenic is usually bound to the surface of Fe(III) (oxyhydr)oxide minerals in the form of As(V). When the Fe(III) mineral is reduced to dissolved or solid-phase Fe(II), As is also released from the Fe(III) minerals (Qiao et al. 2021). More crystalline Fe(III) minerals such as magnetite, goethite, or hematite, are generally less bioavailable for the microorganisms, and therefore are also less likely to release As. In contrast, ferrihydrite is a poorly crystalline mineral and thus more prone to reduction and more easily releases As into the water than other Fe(III) (oxyhydr)oxides (Das et al. 2014). Arsenic mobility and toxicity also depend on its oxidation state. Trivalent arsenite (As(III)) is generally more toxic and mobile, compared to pentavalent arsenate (As(V)) that has a higher affinity for Fe(III) minerals, and is usually retained in sediment (Malakar et al. 2021).

The nitrogen (N) cycle may change As mobility in groundwater by affecting the conversion of Fe(III) to Fe(II) (Fig. 1). Nitrogen is widely present in various environments, and its primary forms in

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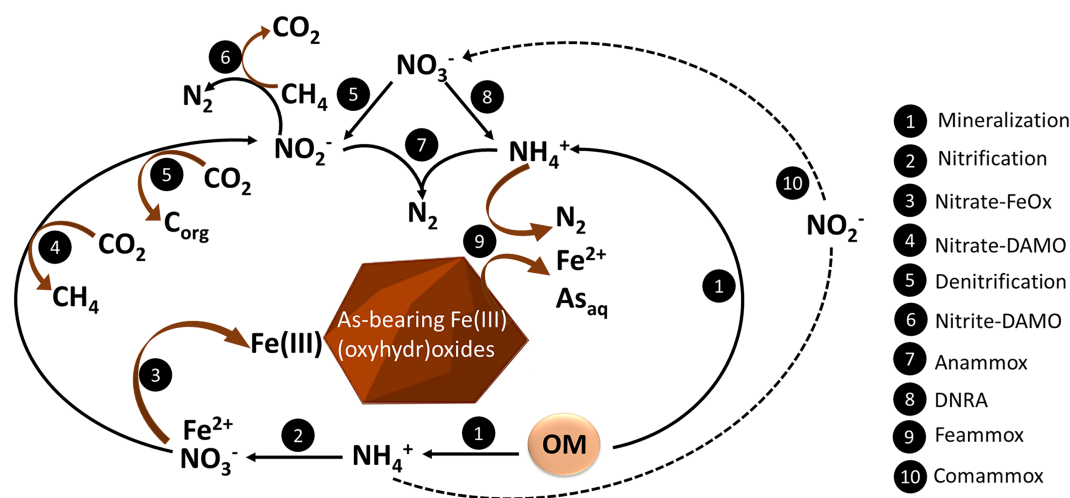
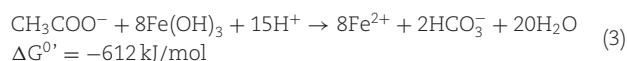
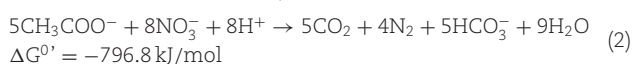
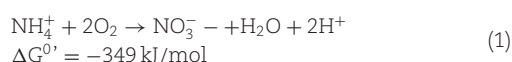


Figure 1. Possible nitrogen reactions in an aquifer and their effect on Fe and As mobility. Nitrate-dependent Fe^{2+} oxidation (Nitrate-FeOx), dissimilatory nitrate reduction to ammonium (DNRA), nitrate/nitrite-dependent anaerobic methane oxidation (Nitrate/Nitrite-DAMO).

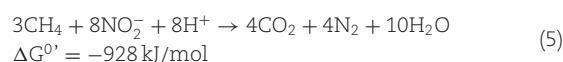
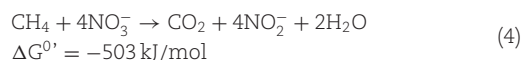
groundwater are nitrate (NO_3^-) and ammonium (NH_4^+), while nitrite (NO_2^-) is found at relatively low concentrations or is absent (Parvizishad et al. 2017). Due to increasing agricultural production and the excessive use of fertilizer, N can leach into the groundwater in the form of NH_4^+ or NO_3^- , increasing the total N content in the groundwater (Bijay-Singh and Craswell 2021). In the presence of oxygen (O_2), NH_4^+ can be oxidized to NO_3^- via nitrification (equation 1) by ammonia-oxidizing bacteria or archaea (Jetten et al. 1998, Könneke et al. 2005). Nitrification of NH_4^+ is the primary source of NO_3^- in aquifers (Umezawa et al. 2008). In nitrification-dominated environments, when both NO_3^- and Fe(III) are present in groundwater, heterotrophic microorganisms will likely preferentially utilize NO_3^- as an electron acceptor due to the higher Gibbs free energy change (equations 2 and 3) (Lovley and Phillips 1988, Hanson et al. 2013). Thus, the presence of NO_3^- can inhibit the reduction of Fe(III) (oxyhydr)oxides, preventing As mobilization to the aquifer, which in its pentavalent form remains stably adsorbed to the Fe(III) mineral (Weng et al. 2017). Moreover, a previous study showed that the addition of NO_3^- stimulates anoxic nitrate-dependent Fe(II) oxidation leading to a decrease in dissolved Fe(II) and As in groundwater (Harvey et al. 2002, Smith et al. 2017). This is because NO_3^- can oxidize Fe^{2+} to Fe^{3+} via biotic or abiotic reactions simultaneously co-precipitating dissolved As with Fe(III) minerals.



Due to the presence of clay minerals and overall net negative charge of soil and sediment particles, NO_3^- is more easily transported by water flow into the subsurface aquifer compared to the positively charged NH_4^+ (Köhler et al. 2006, Nieder et al. 2011). Consequently, heterotrophic denitrification may take place in anoxic underground aquifers (equation 3) (Austin et al. 2016). Denitrifying bacteria subsequently reduce NO_3^- to NO_2^- , nitric oxide (NO), nitrous oxide (N_2O), and ultimately to dinitrogen gas (N_2). Heterotrophic denitrifying bacteria and archaea usually couple the oxidation of organic matter with NO_3^- reduction. Autotrophic denitrifying bacteria however use NO_3^- to oxidize in-

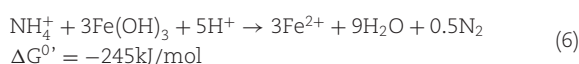
organic reduced compounds such as Fe(II), Mn(II) or As(III) (Weber et al. 2006, Li et al. 2020, Kappler et al. 2021). During this process, Fe(II) is oxidized and precipitate in form of poorly soluble Fe(III) (oxyhydr)oxides to which As(V) can preferentially adsorb (Hohmann et al. 2010).

Some microorganisms are capable of nitrate-dependent anaerobic methane oxidation (N-DAMO), a process mediated by ANME-2d archaea, specifically by *Candidatus Methanoperedens* (Raghoebarsing et al. 2006, Haroon et al. 2013). Since their discovery, several N-DAMO archaea have been enriched from anoxic freshwater sediments, digester sludge, and rice paddies (Hu et al. 2009, Arshad et al. 2015, Vaksmaa et al. 2017). The N-DAMO archaea reduce NO_3^- to NO_2^- while oxidizing methane (CH_4) to gain energy (equation 4). Nitrite-dependent methanotrophic bacteria named *Candidatus Methyloimrabilis* oxidize NO_2^- to N_2 at the expense of CH_4 (equation 5) (Ettwig et al. 2010).



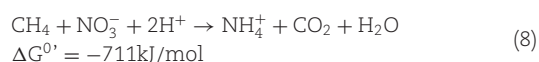
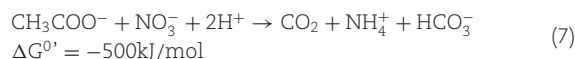
Together, the N-DAMO process might be particularly relevant for strongly methanogenic aquifers in agricultural areas where the intensive application of fertilizer leads to the NO_3^- accumulation, however, to date N-DAMO activity has not been confirmed in aquifer systems.

Feammox is one of the newly proposed pathways coupling NH_4^+ oxidation with Fe(III) reduction (equation 6) that could potentially lead to As release. Until now however, the contribution of Feammox to As groundwater contamination was only suggested based on a positive correlation between dissolved NH_4^+ , Fe and As (Gao et al. 2021) or a positive correlation between genes associated with ammonium oxidation (*hzsABC* and *hdh*) and Fe(III) reduction (*omcS*) (Xiu et al. 2022). Nevertheless, the Feammox process plays an important role in the N cycle in various ecosystems such as tropical forest soils, paddy fields, rivers, and lake sediments (Rios-Del Toro et al. 2018, Li et al. 2019). Although this process might be particularly relevant in Southeast Asian aquifers where high concentrations of NH_4^+ were reported, until now it remains unclear whether NH_4^+ is involved in As mobilization.

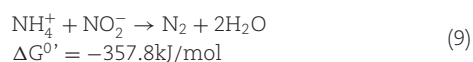


Moreover, Asammox—anaerobic ammonium oxidation coupled with As(V) reduction has been recently proposed in rice paddy soils (Zhang et al. 2022). This process could potentially increase the mobility of As since trivalent As is known to be generally more mobile than pentavalent As which tends to be easily adsorbed to Fe(III) minerals.

Ammonium, besides being produced by organic matter mineralization, can also originate from dissimilatory nitrate reduction to ammonium (DNRA) (equation 7). Many microorganisms from anoxic sediments can obtain energy via DNRA (Pandey et al. 2020). More importantly, N-DAMO archaea have also been shown to couple DNRA with CH₄ oxidation (equation 8) suggesting that anaerobic CH₄ oxidation might be coupled with NH₄⁺ production (Nie et al. 2021). It is particularly relevant when the DOC/NO₃⁻ molar ratio is high, then DNRA can replace denitrification as groundwater's main NO₃⁻ reduction pathway (Plummer et al. 2015).



Additionally, when NO₃⁻, NO₂⁻ and NH₄⁺ coexist in the redox interface, NO₃⁻ and NH₄⁺ can be converted into N₂ through the anammox process (equation 9).



To date, all identified anammox bacteria belong to the order 'Candidatus Brocadiales' within the phylum *Planctomycetes* (*Planctomycetota*) (Suarez et al. 2022). By conducting high-throughput sequencing of samples from aquifers around the world, Wang et al. estimated that anammox bacteria might be responsible for 80% of NO₃⁻ and NO₂⁻ removal at the global scale in these ecosystems (Wang et al. 2020).

Various N species can interact directly or indirectly with Fe(III) minerals. However, still very little is known about how the biological (trans)formation of N in an aquifer can affect the mobility of Fe and in consequence As. The input of N from the intensive application of fertilizers into methanogenic aquifers may stimulate N-DAMO processes, while DNRA may lead to the accumulation of NH₄⁺ and potentially Feammox. Therefore, our present work aimed to assess the potential for anaerobic CH₄ oxidation coupled to NO₃⁻ reduction in As-contaminated aquifer sediments, evaluate the transformation pathways of NO₃⁻, and investigate the possibility of Feammox potentially leading to As mobilization to groundwater. For this purpose, an As-bearing Fe(III)-rich sediment was anoxically incubated with ¹³CH₄ and supplemented with ¹⁵NO₃⁻. Additionally, the potential effect of N-DAMO on Fe and As (im)mobilization was studied by inoculating the sediment with N-DAMO enriched laboratory cultures. We monitored dissolved As concentration, Fe speciation, CH₄ and ¹³CO₂ concentrations as well as N species evolution over time. Furthermore, to assess the composition of the microbial community we performed 16S rRNA gene amplicon sequencing at the end of the experiment.

Materials and methods

Study site and sediment sample collection

The study site is located in a rural area of the Red River delta, in Van Phuc village, about 15 km south from Hanoi, Vietnam (20°55'18.7"N, 105°53'37.9"E). The area's geological, hydrochemical, and mineralogical characteristics have been studied previously (Berg et al. 2008, Eiche et al. 2017, Postma et al. 2017,

Stopelli et al. 2020). In brief, the north-western part of the studied area is characterized as a Pleistocene aquifer consisting of brownish-orange sands and groundwater with low dissolved Fe²⁺ (less than 0.5 mg/l) and As concentrations <10 µg/l (Fig. 2). The south-eastern part consists of younger grey Holocene sands and As groundwater concentrations varying between 200 and 600 µg/l often surpassing the WHO standard of 10 µg/L by a factor of 10–50. The concentration of dissolved Fe²⁺ is also high (10–20 mg/l) indicating strongly reducing conditions (van Geen et al. 2013). Furthermore, the Holocene aquifer is characterized by nearly flammable CH₄ concentrations (>50 mg/L) (Postma et al. 2017, Stopelli et al. 2021).

In November 2018, a drilling campaign took place and sediment cores (9 cm diameter) were collected by rotary drilling up to 46.5 m below the ground, at the redox transition zone (RTZ) located in the interface of Holocene As-contaminated and Pleistocene pristine aquifer (Fig. S1). The RTZ is subjected to intense geochemical and microbial activity which are suggested to be responsible for the As release to groundwater. Sediment samples were collected in water- and air-tight zip log bags (LamiZip, DAKLAPACK) with high barrier properties against oxygen and water vapor and protection against UV radiation to minimize sample alteration. All samples were flushed with N₂ immediately after sampling and cooled during transportation to minimize microbial activity. Afterward, all samples were stored at 4°C anoxically in the dark until further use.

Microcosms setup and incubation

For microcosm setups, the yellow-orange, less reduced sediment from 31 m depth was used as this type of sediment is known to have a higher content of Fe(III) minerals and As compared to the grey reduced sediment. The sediment from this depth is characterized by Kontny et al. (2021), briefly, the sediment contained about 27 mgFe/kg and 5.3 mgAs/kg. Microcosms were set up in 120 ml sterile glass serum bottles filled with 25 g of sediment and 50 ml synthetic groundwater medium (modified from Rathi et al. (2017); without As and Fe). Five different treatments were prepared in triplicates (Table 1): (i) amended with 0.2 ± 0.004 g (dry weight) biomass of N-DAMO(O) enrichment culture, 5 mM Na¹⁵NO₃ (final concentration) and 0.8 mM ¹³CH₄; (ii) abiotic control—the same composition as treatment 1 with additional 150 mM of sodium azide (NaN₃) to inhibit microbial activity; (iii) amended with 0.2 ± 0.004 g (dry weight) of N-DAMO(V) enrichment culture, 5 mM Na¹⁵NO₃ and 0.8 mM ¹³CH₄; (iv) only amended with 5 mM Na¹⁵NO₃ and 0.8 mM ¹³CH₄; (v) control group without any amendment. The N-DAMO(O) enrichment culture was obtained from an agricultural ditch in The Netherlands, and currently consists of *Ca. Methanoperedens nitroreducens* (~44%) and *Ca. Methyloirabilis* (~26%) (Raghoebarsing et al. 2006, Schoelmerich et al. 2022). The N-DAMO(V) culture was enriched from rice paddy soil from Vercelli, Italy, and consists mainly of *Ca. Methanoperedens* (~78%) (Vaksmas et al. 2017, Schoelmerich et al. 2022). Both cultures are grown in a continuous bioreactor under anoxic conditions with NO₃⁻ as electron acceptor and CH₄ as electron donor. Synthetic groundwater, Na¹⁵NO₃, and NaN₃ solution were gassed with N₂/CO₂ to remove dissolved O₂ before use. All microcosms were prepared anoxically in a glovebox (97% N₂ and 3% H₂) and closed with rubber stoppers and aluminum caps. The headspace gas was exchanged with N₂/CO₂ (9 : 1) until the final pressure of 1.83±0.05 bar to ensure CH₄ dissolution and anoxic conditions. Afterward, microcosms were kept in the dark at 30°C and shaken at 30 rpm for 65 days.

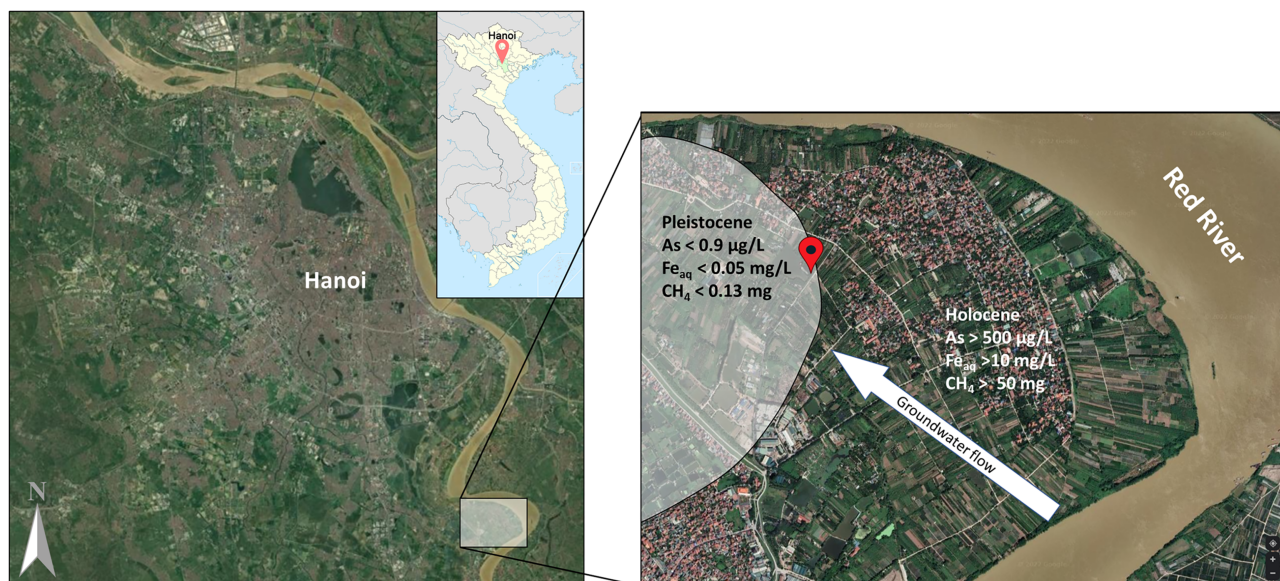


Figure 2. Satellite image of the study area (white square in the left image). Zoom into the drilling site (red pin) located in the redox transition zone at the interface of the Pleistocene and Holocene aquifer (right). Google Earth, Maxar Technologies

Table 1. Overview of microcosms setups used in the experiment.

Treatment	NAME	Inoculum	$^{15}\text{NO}_3$	$^{13}\text{CH}_4$	NaN_3
1	N-DAMO(O)	N-DAMO(O)	✓	✓	
2	Abiotic N-DAMO(O)	N-DAMO(O)	✓	✓	✓
3	N-DAMO(V)	N-DAMO(V)	✓	✓	
4	$\text{NO}_3^- + \text{CH}_4$		✓	✓	
5	Control				

Geochemical analyses

At each time point, 2 ml of slurry were withdrawn by using a sterile syringe and needle (\varnothing 1.20 × 40 mm) in an anoxic glove-box. Samples were centrifuged at 14000 rpm for 5 min. Afterward, 100 μL of the supernatant was mixed with 100 μL 1 M HCl to stabilize and dilute the sample for further Fe(II) quantification. One milliliter of supernatant was stabilized in 9 ml of 1% HNO_3 for As, Fe, and Mn, analysis by ICP-MS (8900, Agilent Technologies, USA). The remaining supernatant was transferred into an Eppendorf tube for NO_3^- , NO_2^- , and NH_4^+ quantification. Sediment was then digested with 1 ml 6 M HCl for 1 h, centrifuged for 5 min at 14000 rpm and 100 μL of the supernatant was diluted with 100 μL 1 M HCl. The concentrations of Fe(II) and total Fe were detected by the Ferrozine assay (Schäedler et al., 2018). The Griess assay was used to quantify NO_3^- and NO_2^- while the OPA assay was used to determine the concentration of NH_4^+ (Meseguer-Lloret et al. 2002, Sun et al. 2003).

The concentrations of $^{13}\text{CO}_2$, $^{15}\text{N}_2\text{O}$, and the ratio of $^{30}\text{N}_2/^{28}\text{N}_2$ 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, California, USA). Air pressure was also monitored at each sampling point by a portable pressure meter (GMH 3100, GHM Messtechnik, Regenstauf, Germany). The concentration of $^{13}\text{CO}_2$ and $^{15}\text{N}_2\text{O}$ in the headspace and the total amount of $^{13}\text{CO}_2$ and $^{15}\text{N}_2\text{O}$ in the incubation bottles were calculated following the formulas S1 and S2, respectively (Supplementary Materials).

DNA extraction and microbial community analysis

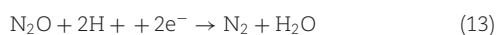
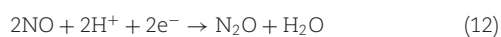
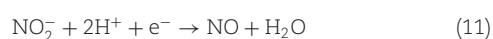
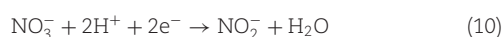
Sample for DNA extraction were collected from the original sediment used for the incubation and from microcosms at the end of incubation (65 days). The DNA extraction was performed using the PowerSoil DNA extraction kit (DNeasy PowerSoil Pro Kit, QIAGEN, Hilden, Germany), according to the manufacturer's protocol. An additional 500 μL of 10% (w/v) sterilized skim milk solution was added to the sediment sample to improve the DNA extraction yield (Hoshino and Matsumoto 2005). The concentration of DNA was quantified using Qubit® 2.0 Fluorometer with DNA HS kits (Life Technologies, Carlsbad, CA, USA). 16S rRNA gene amplicon sequencing was done by Macrogen (Amsterdam, The Netherlands) using the Illumina MiSeq Next Generation Sequencing platform. Paired-end libraries were constructed using the Illumina Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina, Eindhoven, Netherlands). Primers used for bacterial amplification were Bac341F (5'-CCTACGGGNGGCWGCAG-3'; (Herlemann et al. 2011) and Bac806R (5'-GGACTACHVGGGTWTCTAAT-3'; (Caporaso et al. 2012). Archaeal amplification was performed with primers Arch349F (5'-GYGCASCAGKCGMGAAW-3') and Arch806R (5'-GGACTACVSGGGTATCTAAT-3'; (Takai and Horikoshi 2000). For bacteria and archaea separately, reads were trimmed and removed based on quality (settings: left trim 17 and 20, truncation length 267 and 270, maxE 2), followed by denoising and dereplication (settings: error learning bases 1e10, pooling during denoising, overhang trimming during merging) Amplicon Sequence Variant (ASV) calling, and finally taxonomic assignment (SILVA version nr138 training set, (Quast et al. 2013) and read abundance

counting using DADA2 and its utilities (v1.22.0, (Callahan et al. 2016) in R (v4.1.2; R Core Team, 2019). After quality control and assignment of reads to ASVs, between 44679 and 115065 paired reads were assigned to a total of 770 archaea ASVs, and between 44710 and 80477 paired reads were assigned to a total of 944 bacteria ASVs. Further analyses and visualization of these count and taxonomic data were performed also using R or Excel. The raw sequence data and metadata of the microcosms experiment have been deposited at The Sequence Read Archive (SRA) database of the NCBI under the BioProject ID PRJNA887920.

Results and discussion

Nitrogen species evolution

In all microcosms supplemented with NO_3^- , except the abiotic one, NO_3^- was nearly completely removed within 5 days of incubation. The abiotic control (treated with NaN_3) showed no change in NO_3^- concentration over time (Fig. 3A). In the uninoculated microcosms, NO_3^- was reduced as efficiently as in N-DAMO inoculated microcosms. Previous studies from the same drilling site showed that the microbial community in sediment and water at 31 m depth has the potential for NO_3^- reduction (Glodowska et al. 2021, Glodowska 2021a). It was however surprising that the addition of N-DAMO cultures to the sedimentary native microbial communities showed a similarly high denitrification potential as native microbial community alone (equation 10). Methane in the N-DAMO inoculated microcosms, at least partially, served as an electron donor as concomitant NO_3^- reduction with decreasing $^{13}\text{CH}_4$ concentration and increasing $^{13}\text{CO}_2$ was observed (Fig. 4A, B). However, in the inoculated as well as uninoculated microcosms, the native microbial community likely utilized natural organic C still present in the sediment for the heterotrophic NO_3^- (Glodowska et al. 2020).



Although, the native microbial community in the microcosms supplemented with CH_4 and NO_3^- appeared to be capable of efficient NO_3^- reduction, at a similar rate as the two N-DAMO inoculated microcosms, it, however, showed only a limited ability to further reduce NO_2^- to other N-species (especially N_2) (equation 11, 12, 13). In these microcosms NO_2^- concentration rapidly increased to 1.12 mM within the first 2 days, dropped to 0.6 mM, and remained stable until the end of the experiment (Fig. 3B). Although our previous study showed that NC10 bacteria affiliating with *Ca. Methylomirabilis* that are known to reduce NO_2^- at the expense of CH_4 were present in the sediment and groundwater of this aquifer (Glodowska 2021a,b), their abundance in our experiment was probably too low to remove all NO_2^- . In the two N-DAMO inoculated treatments, NO_2^- was nearly undetectable during the whole incubation time as both N-DAMO cultures can efficiently reduce NO_2^- . Interestingly, after supplying an additional 5 mM of NO_3^- at the end of the experiment (after 65 days of incubation) the native microbial community was dormant, lost the ability to reduce NO_3^- , or depleted electron donor, as only small fraction of added NO_3^- was removed (Fig. S2). The tolerance of dif-

ferent microorganisms to NO_2^- greatly varies (Guo and Gao 2021), and the native denitrifying community may eventually have died due to prolonged exposure to relatively high concentrations of NO_2^- (0.6 mM).

Denitrification is a stepwise process in which three intermediate species are produced; NO_2^- , NO , and N_2O (equation 11, 12, 13) (Kuypers et al. 2018). In the NO_3^- and CH_4 supplemented microcosm, except for the accumulation of NO_2^- mentioned above, there was no significant accumulation of other N-intermediates and their concentration remained at very low levels until the end of the experiment (Fig. 3E and F). Only the ratio of $^{30}\text{N}_2/^{28}\text{N}_2$ in this treatment increased from 8 to 32% (Fig. 3D). There was also no significant accumulation of NH_4^+ in the NO_3^- and CH_4 supplemented microcosm implying that neither mineralization of residual organic matter or dead biomass, nor DNRA was taking place (Fig. 3C).

In the N-DAMO inoculated treatment, N_2 and NH_4^+ both began to increase immediately at the beginning of the experiment suggesting that both denitrification to N_2 and DNRA were taking place (Fig. 3D and C). Specifically, in the N-DAMO(O) and N-DAMO(V) inoculated microcosms, the $^{30}\text{N}_2/^{28}\text{N}_2$ ratio increased from 32 to 110% and from 5 to 127% at the end of the experiment, respectively (Fig. 3D). In addition to $^{30}\text{N}_2$, $^{29}\text{N}_2$ was also generated in the N-DAMO(O) and N-DAMO(V) treatments (reaching 2.4% and 2.2%, respectively) (Fig. S3), suggesting the activity of anammox bacteria. Two N-DAMO inoculated treatments converted about 20% of NO_3^- to NH_4^+ , which resulted in a NH_4^+ concentration of 0.95–0.85 mM, respectively, suggesting that DNRA is also an important way for N-DAMO microorganisms to reduce NO_3^- .

The concentration of NH_4^+ in the abiotic treatment increased considerably reaching almost 3 mM. This accumulation of NH_4^+ was most likely caused by the breakdown of NaN_3 , and its potential release from dead biomass. No clear patterns in $^{46}\text{N}_2\text{O}/^{44}\text{N}_2\text{O}$ and $^{31}\text{NO}/^{30}\text{NO}$ ratios and concentrations were reported (Fig. 3E, F and Fig. S4). Although previous studies have pointed out that DNRA is an important source of NH_4^+ in aquifers (Weng et al. 2017), our results suggest that NH_4^+ does not originate from bacterial NO_3^- reduction as NH_4^+ was generated only by the activity of inoculated N-DAMO enrichment cultures. Therefore, we suggest that the main source of NH_4^+ at the studied field site is the mineralization of buried organic matter and the potential infiltration of NH_4^+ from fertilizers. Further, we did not observe a parallel decrease in NH_4^+ concentration and an increase in Fe^{2+} , therefore it is unlikely that Feammox takes place in the Van Phuc aquifer.

CH_4 oxidation and CO_2 production

All the microcosms except a biotic control were supplied with 10 ml $^{13}\text{CH}_4$ (~ 0.8 mM). Only N-DAMO inoculated treatments however exhibited a considerable CH_4 decrease over time. A particularly pronounced drop in CH_4 concentrations was observed within the first 5 days of incubation which was also correlated with the formation of $^{13}\text{CO}_2$ (Fig. 4A and B) and NO_3^- reduction (Fig. 3A). It has to be borne in mind, however, that NO_3^- reduction also took place in the uninoculated microcosms, therefore only part of the denitrification activity can be attributed to N-DAMO microorganisms. Specifically, the content of CH_4 between day 0 and 6 decreased continuously in the N-DAMO(O) from 0.83 to 0.66 mM whereas in N-DAMO(V) CH_4 dropped from 0.93 to 0.78 mM.

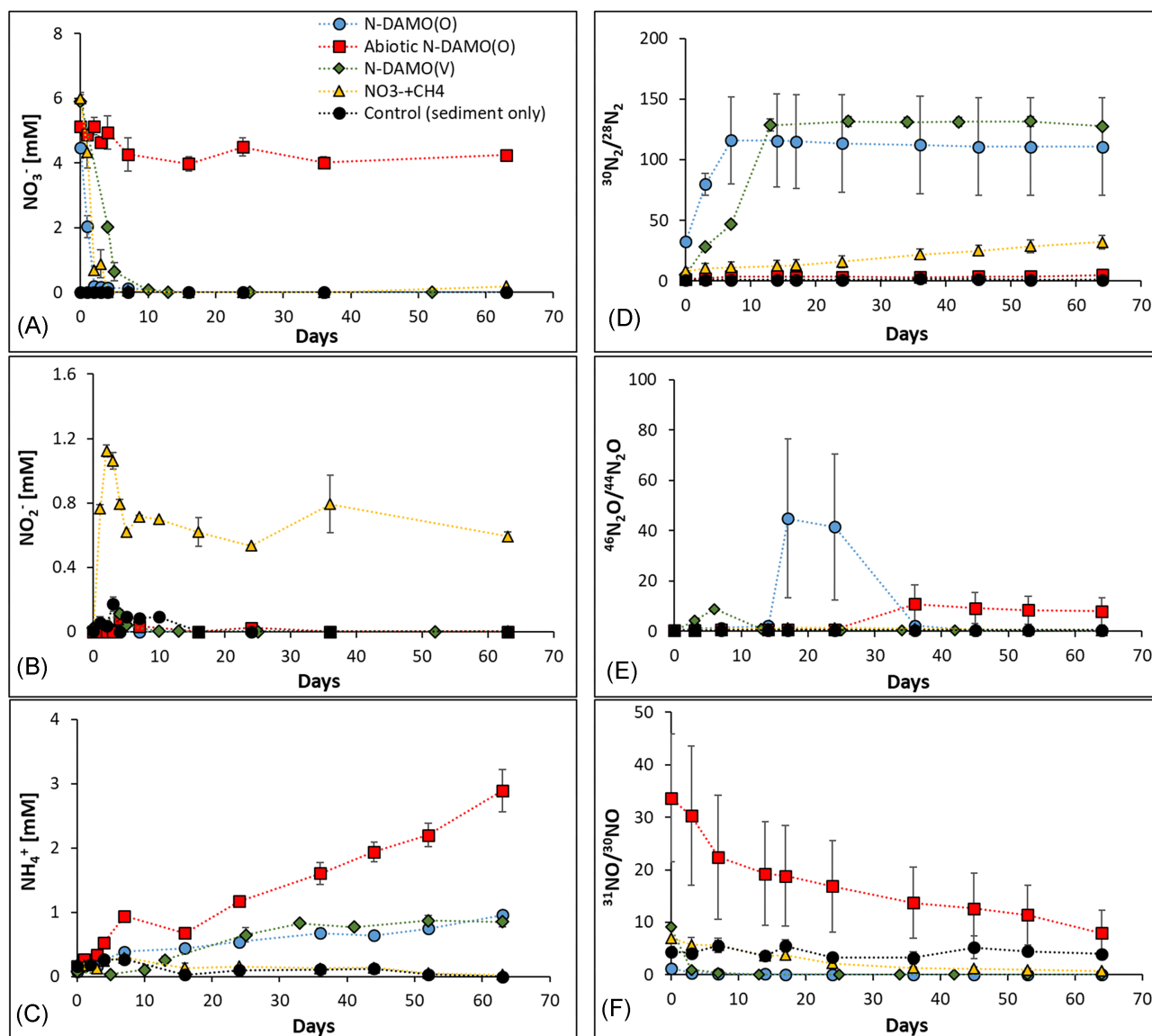


Figure 3. Evolution of N-compounds in microcosms within 65 days of aquifer sediment incubation. The concentration of **A)** NO₃⁻, **B)** NO₂⁻, and **C)** NH₄⁺; and the ratio of **D)** ³⁰N₂/²⁸N₂, **E)** ⁴⁶N₂O/⁴⁴N₂O, and **F)** ³¹NO/³⁰NO. Each microcosm was measured in technical triplicate, error bar stands for the standard deviation between biological triplicates of each treatment.

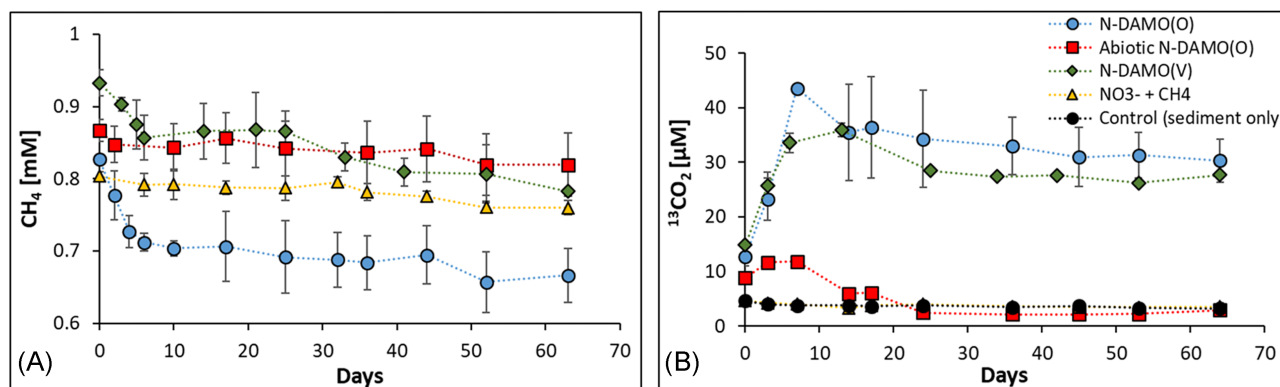


Figure 4. Methane oxidation and ¹³CO₂ production in the five different microcosm setups. The concentration of **(A)** CH₄ and **(B)** ¹³CO₂ formation over time. For a better presentation of the changes in CH₄ concentrations, the vertical axis starts at 0.6 mM as no CH₄ was added to the control group. Only negligible methanogenesis was observed in the control microcosms (Fig. S6). The error bar represents the standard deviation between biological triplicates of each treatment.

The CH₄ oxidation was most pronounced in the N-DAMO(O) treatment which consumed 0.16 mM CH₄ within 64 days (19.5%). The increase of the ⁴⁵CO₂/⁴⁴CO₂ ratio in this group was also the highest, reaching nearly 18% after 7 days of incubation (Fig. S5). Although the amount of generated ¹³CO₂ was similar between the two inoculated treatments (Fig. 4B), in the N-DAMO(V) inoculated microcosms the CH₄ consumption was lower (0.15 mmol by day 64), and the ratio of ⁴⁵CO₂/⁴⁴CO₂ was also relatively low compared to the N-DAMO(O) treatment (Fig. S5). We calculated that the N-DAMO(O) treatment converted 22% of the consumed CH₄ to CO₂, while the N-DAMO(V) converted 14%. Despite the strong ability of N-DAMO enrichment cultures to couple CH₄ oxidation to NO₃⁻ reduction, less than 20% of NO₃⁻ reduction in both N-DAMO treatments was due to CH₄ oxidation. The vast majority of NO₃⁻ reduction is thus attributed to heterotrophic denitrification via oxidation of residual natural organic matter (NOM) still present within the sediment (Equations S3 and S4).

Although our previous study showed anaerobic CH₄ oxidation coupled with Fe(III) reduction in this aquifer (Glodowska 2020, Pienkowska et al. 2021), the CH₄ concentration in uninoculated microcosms in this experiment remained stable until the end of incubation (65 days). This discrepancy is likely due to the much shorter incubation period of this experiment compared to our previous study, where Fe-DAMO activity was observed only after 100 days of incubation. It is also possible that Fe-DAMO was inhibited due to the presence of alternative electron acceptors such as NO₃⁻. The Fe(III)-dependent CH₄ oxidation could however take place after a longer period of incubation. Only a very small amount of methanogenic activity was observed within the original sediment as the CH₄ concentration in the control incubations reached its maximum value of 2.13 μmol after 44 days, then dropped to ~1 μmol until the end of the incubation (Fig. S6).

Iron reduction and as/mn mobilization

All microcosms except the uninoculated NO₃⁻/CH₄ supplemented treatment showed various degrees of Fe(III) reducing abilities (Fig 5). The most prominent Fe(III) reduction capacity was observed in N-DAMO inoculated treatment, demonstrating that the N-DAMO enriched laboratory cultures have the potential to use Fe(III) as an electron acceptor. It has been shown previously that *Ca. Methanoperedens* species can indeed reduce Fe(III) (Ettinger et al. 2016, Cai et al. 2018) most likely due to the extraordinarily high number of *c*-type cytochromes (Kletzin et al. 2015, Leu et al. 2020). This implies that despite Fe(III) being a less favorable electron acceptor than NO₃⁻ (equations 2 and 3), it can still be used by the N-DAMO community members and/or the native microbial community with either CH₄ and/or NOM as electron donor.

In our experiment, Fe(III) reduction was linked to organic carbon (OC) degradation, as we did not observe further CH₄ oxidation and ¹³CO₂ formation after NO₃⁻ depletion. The native OM also stimulated Fe(III) reduction in uninoculated control microcosms as both dissolved and solid phase Fe(II) increased over time (Fig. 3A, B). The Fe(II) concentration in the abiotic control and in the NO₃⁻ and CH₄ supplemented microcosms remained stable.

Dissolved Fe²⁺ concentration in both N-DAMO inoculated microcosms started to rise after NO₃⁻ depletion and the reduction rate increased significantly after 20 days, eventually reaching the highest values of 0.19 mM in N-DAMO(O) and 0.35 mM in N-DAMO(V) on days 52 and 64, respectively (Fig. 5A). However, a con-

siderable amount of reduced Fe(III) remained as Fe(II) in the solid state (Fig. 5B). Fe(II) content in the sediment of the two N-DAMO inoculated treatments increased rapidly after depletion of NO₃⁻, reaching a concentration of nearly 10 mM (Fig. 5B). At the end of the experiment Fe(II) represented 39% and 45% of total Fe in N-DAMO(O) and (N-DAMO(V) inoculated microcosms, respectively (Fig. 5C).

The vast majority of Fe(II) in the two N-DAMO inoculated microcosms remained in the solid phase most likely because the aquifer's sediment is rich in poorly crystalline Fe(III) minerals that are known to have a strong adsorption capacity for Fe(II) (Jeon et al. 2003). Furthermore, the formation of OM-Fe(II) complexes can also retain the newly formed Fe(II) (Du et al. 2018). In addition, CH₄ oxidation produces a large amount of CO₂, and a part of Fe²⁺ could return to the solid phase in the form of ferrous carbonate (FeCO₃) (Appelo et al. 2002).

In the control microcosms, the native microbial community also showed a certain ability to reduce Fe(III) heterotrophically using native organic carbon as an electron donor. As there was no competition for electron acceptors due to the lack of NO₃⁻ addition, the aqueous Fe²⁺ concentration in the control began to rise earlier than in the two treatments that were inoculated with N-DAMO (Fig. 5A). Although some reduced Fe(II) was released as dissolved Fe²⁺, the majority of the Fe(II) remained in sediment (Fig. 5B). Overall, the ratio of Fe(II)/Fe_{tot} in control microcosms increased from 5 to 15.5% (Fig. 5C).

Many previous studies have demonstrated that the addition of 150 mM NaN₃ successfully inhibited the activity of the sedimentary microbial community (Cabrol et al. 2017, Glodowska et al. 2020), however, a minor increase in Fe(II) concentration in our experiments indicated that it may have failed to completely inhibit the ability of N-DAMO to reduce Fe(III). According to previous studies, the bactericidal effectiveness of NaN₃ is mainly due to inhibiting oxidative phosphorylation via inhibiting cytochrome oxidase (Harvey et al. 1999). However, it appears that under enhanced nutrient or anoxic conditions, the inhibitory effects of NaN₃ might be reduced (Cabrol et al. 2017).

In NO₃⁻/CH₄ amended microcosms, no solid or aqueous Fe(II) was formed during the whole incubation period. This might be because either NO₃⁻ was reduced first as the preferred electron acceptor exhausting bioavailable OC, and/or because of NO₂⁻ accumulation potentially suppressed the metabolic activity of the native microbial community. Fang et al. investigated the effect of NO₃⁻ on Fe(III) reduction and As release in the Datong Basin (Fang et al. 2021). They also found that the presence of NO₃⁻ significantly inhibits the reduction of Fe(III) and thus decreases the release of As into groundwater. In contrast to our experiments, Fang et al. used a pure culture of Fe(III)- and NO₃⁻-reducing *Bacillus* D2201, whereas we focused on microbial communities (native and N-DAMO enriched laboratory cultures). In our experiment, the reaction sequence followed the thermodynamic hierarchy of electron acceptors, i.e. Fe(III) reduction did not take place until NO₃⁻ was depleted. However, in the Fang et al. experiments, the reduction of NO₃⁻ and Fe(III) were carried out simultaneously. The inhibitory effects of NO₃⁻ on dissimilatory Fe(III) reduction was also shown in a series of electron acceptor competition experiments with *Shewanella putrefaciens* (DiChristina 1992). Overall, based on our experiments and previous observations, the presence of NO₃⁻ clearly has an inhibitory effect on Fe(III) reduction.

The quantification of dissolved As suggested that Fe(III) reduction did not necessarily lead to As release (Fig. 6A). Only NO₃⁻/CH₄ amended microcosms showed no Fe(III) reduction and

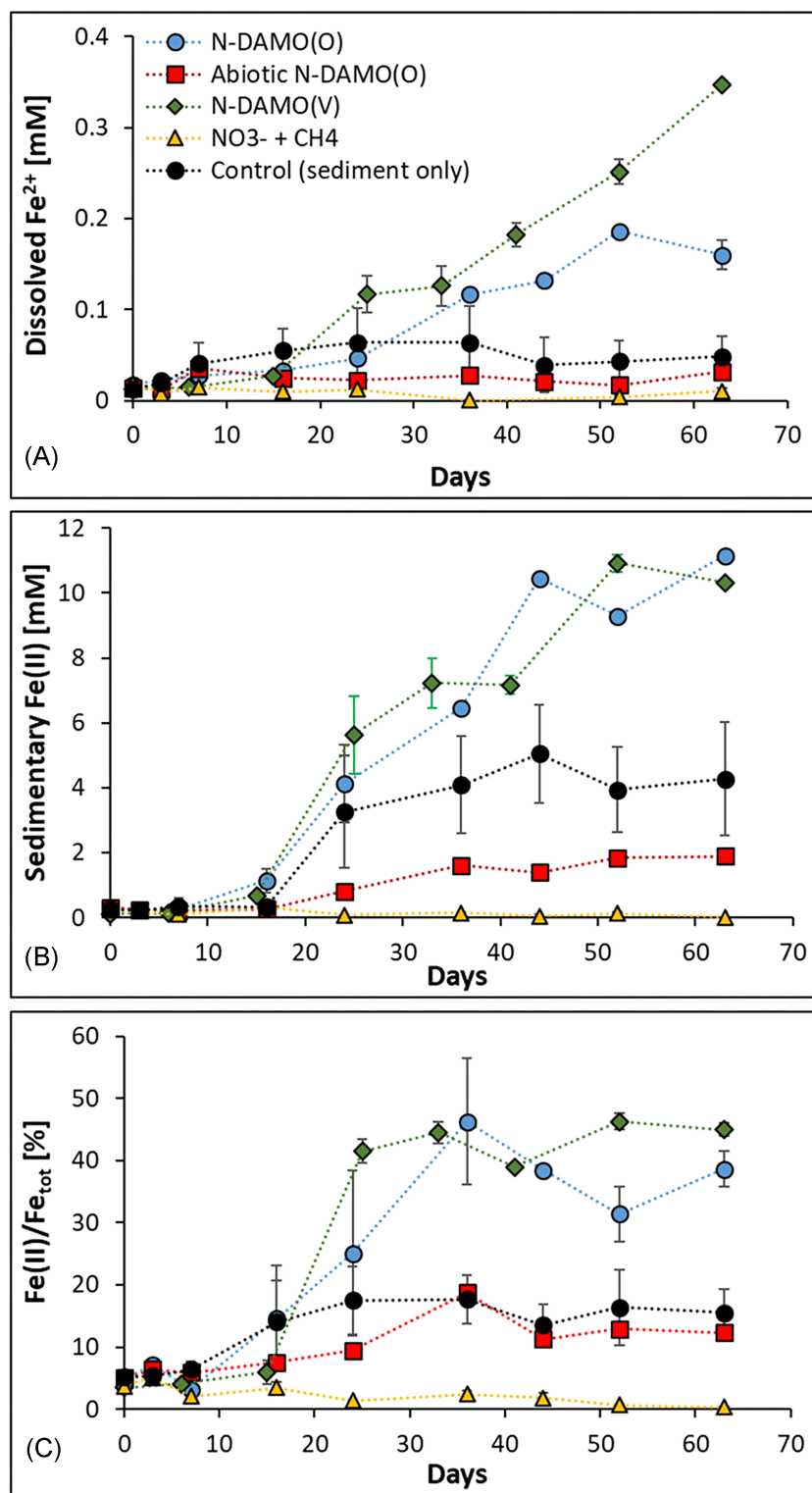


Figure 5. Changes in iron speciation. (A) Ferrous iron concentration in solution; (B) sedimentary Fe(II) concentration; and (C) Fe(II)/Fe_{tot} ratio. The error bar represents the standard deviation between biological triplicates of each treatment.

As mobilization, whereas Fe(II) concentration in other treatments increased considerably. Fe(III) reduction was highest in the N-DAMO(V) inoculated treatment where surprisingly nearly no As was released from the sediment. Instead, a strong mobilization of Mn was observed. The highest dissolved Mn concentration here

reached 33.5 mg/L after 25 days of incubation. Afterwards, the concentration of Mn started to decrease suggesting oversaturation and possible secondary mineral precipitation (Kawashima et al. 1988). The increasing dissolved Mn concentration was likely due to the reduction by the N-DAMO(V) enrichment culture of

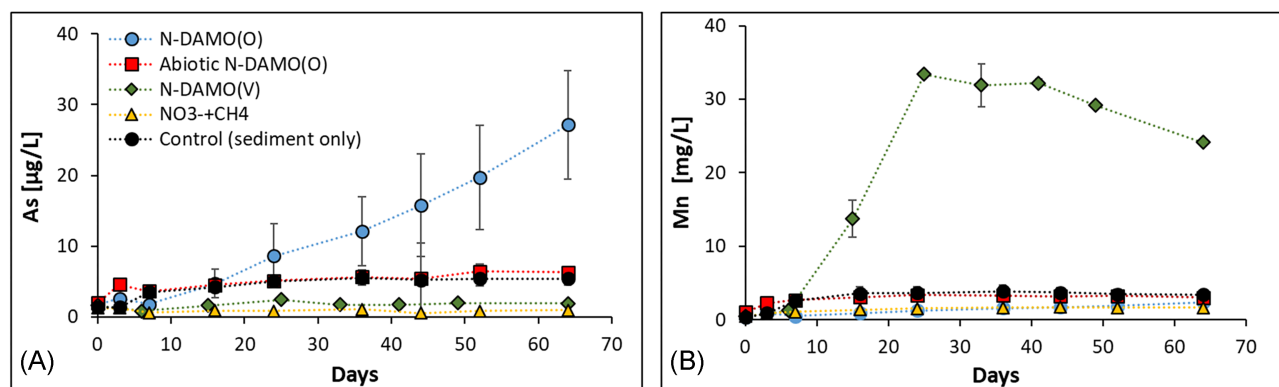
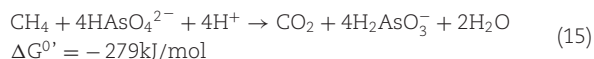
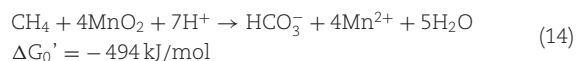


Figure 6. Change in concentration of (A) dissolved As and (B) Mn during 65 days of microcosm incubation. Please note that the concentration of Mn is in mg/l, and the concentration of As is in µg/l. The error bar represents the standard deviation between biological triplicates of each group.

Mn(IV) minerals, e.g. birnessite, present in the sediment. It was previously shown that some *Ca. Methanoperedens* species (i.e. *Ca. Methanoperedens manganicus* and *Ca. Methanoperedens manganireducens*) can reduce Mn(IV) to Mn(II) while oxidizing CH₄ according to equation 14 (Leu et al. 2020).



We suspect that *Ca. Methanoperedens* present in our N-DAMO(V) enrichment culture has a similar ability to use Mn(IV) as an electron acceptor.

Inoculation with N-DAMO(O) led to high As mobilization reaching 27 µg/l at the end of the experiment, even though Fe(III) reduction was lower than in the treatment with added N-DAMO(V) enriched laboratory culture. The variation in As concentration between triplicates was, however, relatively large, suggesting that the dissolution of As is susceptible to environmental/abiotic factors. It has been suggested that some *Ca. Methanoperedens* archaea are genetically equipped to use As(V) as an electron acceptor as many of the available genomes encode for arsenate reductase (*Arr*) (Leu 2020, Glodowska et al. 2022). However, until now there is a lack of laboratory studies linking the presence of *arr* genes with actual As(V) reduction and CH₄ oxidation. Also, our data do not provide evidence to support this hypothesis. Shi et al. demonstrated the coupling of anaerobic oxidation of CH₄ with As(V) reduction in wetland soils (Shi et al. 2020). Metagenomic analysis in that study revealed, however, that the *arrA* gene was absent from ANME-2 metagenome-assembled genomes and instead found in non-methanotrophic *Sulfurospirillum* and *Geobacter*, therefore CH₄ oxidation and the reduction of As(V) was likely mediated via a crossfeeding or syntrophic relationship of methanotrophic ANME archaea and As(V)-reducing bacteria. Nevertheless, it was concluded that CH₄ oxidation coupled with As(V) reduction may contribute to up to 49% of As release in wetland soils. It is, therefore, possible that in our N-DAMO(O) inoculated microcosms some part of As was mobilized via direct enzymatic reduction of As(V) by *Ca. Methanoperedens*, following equation 15 (Caldwell et al. 2008) or via metabolic

collaboration between *Ca. Methanoperedens* and As(V) reducing bacteria.

Changes in the microbial community

The archaeal 16S rRNA sequence abundance showed that the sediment used in our experiment was dominated by *Ca. Methanoperedens* species (Fig. 7A). Our previous study also revealed high enrichment of this archaeon in the sediment (Glodowska et al. 2020). The native *Ca. Methanoperedens* however was likely very different from those present in N-DAMO enrichment cultures as it did neither show significant NO₃⁻ reduction nor CH₄ consumption in the uninoculated control. *Ca. Methanoperedens* represented nearly 100% of the archaeal community in the inoculated microcosms, while in NO₃⁻/CH₄ supplemented and control microcosms it accounted for 76 and 64%, respectively.

The composition of the bacterial community substantially changed over time (after 65 days of incubation) compared to the original sediment across all treatments. In the N-DAMO(O) inoculated microcosms *Ca. Methyloirabilis* was a dominant taxon (Fig. 7B). This is not surprising as *Ca. Methyloirabilis* represented nearly 30% of the N-DAMO(O) inoculum. In the abiotic N-DAMO(O) microcosms the relative abundance of *Ca. Methyloirabilis* was much lower, however, suggesting that introduced biomass was likely mineralized and their DNA degraded. In the N-DAMO(V) microcosms on the other hand bacteria from an uncharacterized phylum DTB120, became the dominant taxon representing 24% of relative 16S rRNA gene abundance. It was previously suggested that these microorganisms might be involved in NO₃⁻ reduction and Fe(II) oxidation (McAllister et al. 2021). Also, putative Fe(III)-reducers affiliating with *Geobacteraceae* increased in abundance, at the end representing over 18% of the bacterial community. Finally, *Denitratisoma*, a putative NO₃⁻ reducer, also represented a substantial part of the microbial community reaching 17% relative abundance at the end of the experiment. The bacterial consortium in the NO₃⁻/CH₄ supplemented microcosms mainly consisted of denitrifying bacteria such as *Vogesella*, *Stenotrophomonas*, and bacteria within the *Comamonadaceae* family. Also, *Azoarcus*, a known N₂-fixing bacterium was highly enriched at the end of the experiment (Zorraquino et al. 2018). Finally, the bacterial community in control microcosms was dominated by Fe(III)-reducing bacteria within the *Geobacteraceae* family and *Thermincola*, which is consistent with our previous observations (Glodowska et al. 2020).

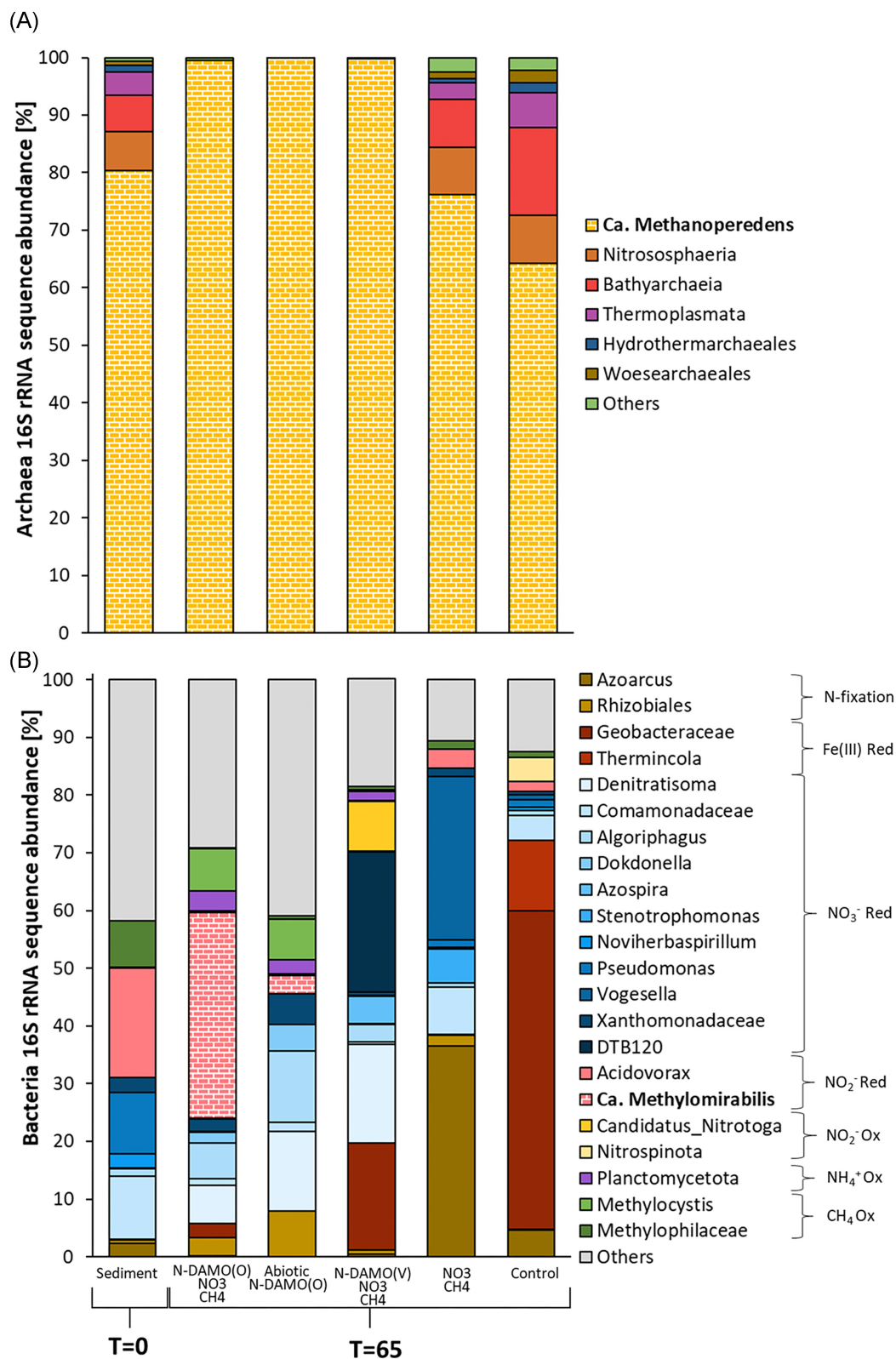


Figure 7. Relative abundance of archaeal (A) and bacterial 16S rRNA gene sequence amplicons (B) in microcosms at the end of the experiment (65 days). The highest taxonomic level is presented with its putative function. Sediment refers to the original sediment used in the experiment. Red = reduction, Ox = oxidation

Conclusions

Intensive use of N-fertilizers often comes with a risk of leaching and penetration of N compounds into aquifers thereby threatening groundwater quality. Moreover, the input of N compounds such as NO_3^- which is a favorable electron acceptor will likely trigger microbiological processes and subsequently affect the hydrochemistry of water. Our study suggests that counterintuitively, the input of NO_3^- to As-contaminated aquifer might be beneficial as NO_3^- inhibits Fe(III) reduction and subsequently prevents As mobilization to groundwater. It suggests that NO_3^- might be a more preferentially used electron acceptor than Fe(III) in Red River Delta sediment. This, however, might come with the risk of NO_2^- production, as the native microbial community in the Van Phuc aquifer was not capable of rapid NO_2^- reduction, leading to the accumulation of this toxic compound. Our study also demonstrated that although conditions appear suitable, the indigenous microbial community was not capable of N-DAMO yet input of NO_3^- stimulated the denitrifying community. Also, our geochemical data did not indicate potential Feammox activity. We did not observe a concomitant NH_4^+ decrease with the increase of Fe(II) concentration. It is however possible that the accumulation of NH_4^+ masked its oxidation via Feammox.

The microcosm approach is a convenient way to screen the metabolic potential of native microbial communities, it can however, not fully mimic the environmental conditions. For example, the leaching of fertilizers into groundwater is expected to be a slow and continuous supply of a rather low amount of NO_3^- . In our experiment, we supplied a single dose of 5 mM NO_3^- , which eventually led to the accumulation of NO_2^- in the $\text{CH}_4/\text{NO}_3^-$ amended treatment, inhibiting the activity of the native microbial community. To overcome this problem a dedicated bioreactor setup with a continuous supply of NO_3^- should be designed.

Supplementary data

Supplementary data is available at [FEMSEConline](https://www.femsec.org/).

Author contributions

Martyna Glodowska (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing), Yinxiao Ma (Investigation, Methodology, Visualization, Writing – original draft), Garrett Smith (Data curation, Software), Andreas Kappler (Funding acquisition, Project administration, Resources, Supervision), Mike Jetten (Funding acquisition, Project administration, Supervision, Writing – review & editing), and Cornelia U. Welte (Funding acquisition, Project administration, Resources, Writing – review & editing)

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Conflict of interest. None declared.

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