Nitrogen cycling and microbial cooperation in the terrestrial subsurface

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INTRODUCTION
Groundwater represents the largest accessible freshwater source on Earth and is stored in permeable geological units known as aquifers that are generally characterized by long water residence times, low organic matter, and slow water exchange rates [1–3]. Natural stores of inorganic nitrogen (nitrate, nitrite, ammonium) are typically present in low concentrations [4]. However, long residence times and close links to surface water (e.g., lakes, rivers, and wetlands) make groundwater susceptible to pollution from nitrogen-based fertilisers [5]. Nitrogen (N) contamination in the terrestrial subsurface has become a global problem [6–8], leading to the loss of fixed nitrogen.

Numerous aquifer organisms are equipped with genes encoding partial nitrogen cycle pathways, such as nitrite reduction [11, 16]. This has been shown to be a common feature of bacteria and archaea from other habitats [17], and suggests that cooperative interactions are commonly employed to complete individual nitrogen-cycling pathways.

The microbial nitrogen cycle comprises six distinct N-transformation processes, including ammonification, nitrogen fixation, nitrification, denitrification, anaerobic ammonium oxidation (anammox), and assimilation [18, 19]. Microorganisms that perform these processes can be sources and sinks of nitrate. Despite distinct requirements (e.g. for oxygen), many reactions in the nitrogen cycle tend to co-occur in the environment, leading to efficient nitrogen recycling [19], competition for the same resource (e.g. by respiratory ammonifiers and denitrifiers [20]), cooperative completion of the modular denitrification pathway [19], and coupled processes, such as nitrification—denitrification [21] or nitrification—anammox [22]. Accordingly, biological processes derived from networks of microorganisms in the terrestrial subsurface play a dominant role in N-transformations [23].

Denitrification is the most studied nitrogen cycling process in groundwater to-date, due to its importance for nitrogen pollution.
removal [5], although operation of the truncated pathway produces less desirable forms of inorganic nitrogen - NO₂⁻ due to its toxicity [24], and greenhouse gases NO and N₂O [5]. Microbial denitrification is typically linked to dissolved organic carbon concentrations in aquifers, but is also fuelled by inorganic electron donors, such as reduced forms of iron or sulfur [5, 25]. Inorganic donors may be the primary source of electrons for nitrogen-cycling taxa given widespread organic carbon-limitation in aquifers [26, 27]. Accordingly, nitrification and anammox appear to be typical features of shallowoxic or partially oxic aquifers [15, 26], and carbon-limitation can create an opportunity for anammox to outcompete denitrification [28]. However, the occurrence of, or capacity for, these processes may not be ubiquitous. A scarcity or lack of organisms capable of some processes, including nitrogen fixation, ammonia oxidation, and nitrous oxide reduction, has been reported from one low-oxygen aquifer [11]. Further work is needed to determine the distribution of nitrogen-cycling processes across different aquifers, including aquifers defined based on redox conditions and nutrient characteristics, such as pristine or N-contaminated.

This study investigates the microbial nitrogen cycle in aquifers traversing a wide range of nitrogen, organic carbon, and oxygen concentrations. We determined the metabolic capacity for each pathway in oxic and dysoxic groundwaters, and the transcriptional activity associated with these pathways (understudied in aquifers due to low cell densities) [29]. As aquifers comprise both suspended and attached communities, with distinct compositions and capacities for biogeochemical cycling [30], analyses included both groundwater (planktonic fraction) and groundwater enriched with the sediment-attached fraction. To further characterize reactions leading to nitrogen loss, we quantified ammonia monooxygenase (archaeal and bacterial ammonia oxidation), nitrous oxide reductase (final step in denitrification), and hydrazine synthase (anammox) genes in 64 groundwater samples (from 59 wells) and transcripts in 26, collected up to 860 km apart. Results give insights into environmental factors influencing the presence, co-occurrence, and transcriptional activity of nitrogen-cycling mechanisms, which determine the fate of nitrogen in aquifers.

MATERIALS AND METHODS

Study sites and sample collection

Eighty samples were collected from 59 wells, spanning 10 aquifers (mostly sandy-gravel) in the Auckland, Waikato, Wellington, and Canterbury regions, New Zealand (Fig. S1; Table S1). Wells were 4.5–114.6 m deep (18.9 m on average, Table S1). Wells were purged (∼3–5 borehole volumes). Then, 3–90 L of groundwater (67 samples) or 0.5–15 L of attached-fraction enriched groundwater (13 samples, Canterbury sites A–D) were collected and immediately filtered on-site. The biofilm or “attached” fraction enriched groundwater (i.e. combining planktonic and biofilm aquifer fractions) was collected directly following standard groundwater collection. Prior to collection of these samples, a low-frequency custom sonicator, as filtered on-site. The biofilm (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm stainless steel filter) using a 142 mm stainless steel filter (1.2 µm filter) or attached film (142 mm
Fig. 1  Geochemistry and protein-coding sequences (based on reads) involved in nitrogen cycling that are significantly different among sites used for metagenomics. A Bar plots showing geochemical data from groundwater samples, coloured according to site. Solid bar colour = groundwater samples. Grid lines = attached-fraction enriched groundwater. All samples from site D were characterized as dysoxic, although gwj15-16 contained 0.37 mg/L DO, which are near suboxic levels (i.e. <0.3 mg/L). For all samples shown, ammoniacal-N values were below detection. B, C Bar plots showing the abundance (average of four wells per site and standard deviation) of sequence reads encoding dissimilatory and assimilatory nitrogen-cycling proteins relative to all nitrogen-cycling processes. Predicted proteins that were statistically different between sites are bolded (y-axis). D Schematic of the nitrogen cycle displaying statistically significant differences between sites (LEfSe, Kruskal–Wallis test, \( p < 0.05 \)). Solid lines depict pathways that were significantly more abundant across the sites, whereas dashed lines indicate no significant difference. Arrows indicate the site with significantly more genes. Abbreviations: A-Amo Archeal Amo, B-Amo Bacterial Amo, Amo ammonia monooxygenase, Pmo Particulate methane monooxygenase, Hao hydroxylamine oxidoreductase, Nxr nitrite reductase (dissimilatory), Nas nitrate reductase (assimilatory), NirK copper-containing nitrite reductase, NirS cytochrome \( cd_{1} \)-containing nitrite reductase, Nor nitric oxide reductase, Nos nitrous oxide reductase, Nif nitrogenase (various), Hcp hydroxylamine reductase, Nir NADPH-nitrite reductase, Nirf nitrate reductase (associated with Nap), Hdh hydrazine hydrogenase, Hsz hydrazine synthase, Hzo hydrazine oxidoreductase.
Nitrogen cycle schematics display the average abundance of nitrogen-cycling transcripts (based on modified-TPM values) per site and sample type (relative to nitrogen-cycling pathways overall (as shown). The percentage of gene transcripts associated with each pathway component is shown in black font. Coloured arrows represent pathways (purple = nitrification, green = denitrification and red = anammox). Only NrfA and not NirBD are shown for the DNRA pathway. Heatmap shows nitrogen-cycling modified transcripts per million (modified-TPM) at each site (ordered gw9, gw11, gw13-gw16), scaled by row (Z-Score). Solid coloured blocks represent groundwater, black grid blocks represent the attached-fraction (or biomass) enriched groundwater. Stacked bar plots display four active nitrogen-cycling genomes and the relative abundance (modified-TPM normalized to genome coverage) of their nitrogen-cycling gene transcripts across each site. Abbreviations: amm ammonia monooxygenase, pmo particulate methane monooxygenase, nrf copper-containing membrane monooxygenase, nxr nitric oxide dismutase, nxr nitrite oxidoreductase, nar nitrate reductase (dissimilatory), nap periplasmic nitrate reductase, nirK copper-containing nitrite reductase, nirS cytochrome cd1-containing nitrite reductase, nos nitric oxide reductase, nrf nitric oxide reductase, hzo hydrazine oxidoreductase, hao hydroxylamine oxidoreductase.

A. Site C Oxic groundwater

B. Site D Dysoxic groundwater

C. Site D Dysoxic attached-fraction enriched groundwater

Fig. 2 Nitrogen-cycling gene transcription at site C groundwater and site D groundwater and attached-fraction enriched groundwater. A Nitrogen cycle schematics display the average abundance of nitrogen-cycling transcripts (based on modified-TPM values) per site and sample type (relative to nitrogen-cycling pathways overall (as shown). The percentage of gene transcripts associated with each pathway component is shown in black font. Coloured arrows represent pathways (purple = nitrification, green = denitrification and red = anammox). Only NrfA and not NirBD are shown for the DNRA pathway. B Heatmap shows nitrogen-cycling modified transcripts per million (modified-TPM) at each site (ordered gw9, gw11, gw13-gw16), scaled by row (Z-Score). Solid coloured blocks represent groundwater, black grid blocks represent the attached-fraction (or biomass) enriched groundwater. C Stacked bar plots display four active nitrogen-cycling genomes and the relative abundance (modified-TPM normalized to genome coverage) of their nitrogen-cycling gene transcripts across each site. Abbreviations: amm ammonia monooxygenase, pmo particulate methane monooxygenase, nrf copper-containing membrane monooxygenase, nxr nitric oxide dismutase, nxr nitrite oxidoreductase, nar nitrate reductase (dissimilatory), nap periplasmic nitrate reductase, nirK copper-containing nitrite reductase, nirS cytochrome cd1-containing nitrite reductase, nos nitric oxide reductase, nrf nitric oxide reductase, hzo hydrazine oxidoreductase, hao hydroxylamine oxidoreductase.
organisms we analyzed 396 non-redundant medium-high to high-quality MAGs. We reconstructed 7695 metagenome-assembled genomes (MAGs) of which 626 were non-redundant (ANI threshold of 99%), and 396 of these were estimated to be 70–100% complete, with 0–5% contamination. Nitrogen-cycling genes, specifically those involved in non-assimilatory redox reactions, such as nitrification, anammox, and complete or incomplete DNRA and denitrification (starting from nitrate reduction), were present in 40% of MAGs (Figs. 3, 4A, and Table S7). The capacity for diverse nitrogen-cycling processes was again observed to be pervasive across sites, and the overall richness of taxa capable of nitrogen cycling remained comparably diverse over most sites (Fig. 5A), regardless of large differences in measured inorganic N contents (>10-fold difference in nitrate-N, site averages 0–0.007 gm⁻³ nitrite-N and 0–0.018 gm⁻³ ammoniacal-N). Analysis of 16S rRNA gene amplicons across 59 groundwater wells likewise shows that taxa linked through phylogenetic affiliation to nitrogen-cycling processes comprise a notable fraction (0.3–26.3%) of complex oxic to anoxic groundwater communities (Fig. S2). Functional redundancy was common among N-cycling microorganisms. Multiple MAGs recovered from each sample had the collective capacity for DNRA and actively expressed genes associated with each of the major steps of nitrification and denitrification (Fig. 4B, C).

Results suggest that nitrogen cycling is a core function of aquifer microbiomes, despite typically low levels of inorganic N in groundwater [4]. This conjecture is supported by prior evidence from ammonium- or nitrate-poor groundwater of microorganisms capable of, or actively engaged in, nitrification, anammox, or denitrification [25, 26, 28, 53, 54]. Microbial nitrogen cycling is likely to be a significant factor governing nitrogen availability in typically oligotrophic habitats, such as groundwater, the open ocean, and lakes. Indeed, in oligotrophic ocean waters with low primary production, the turnover of the dissolved inorganic nitrogen pool via microbial ammonium regeneration and nitrification is rapid [55]. Moreover, numerous microorganisms have evolved high affinities for nitrogen compounds, conferring them with competitive advantages under N-limited conditions [47, 56–59].

Analysis of the spatial distributions of MAGs showed distinct site-specific compositions of bacteria and archaea capable of nitrogen cycling (Fig. 3), a feature also observed in groundwater...

Fig. 3  Heatmap showing 159 MAGs, coloured according to phylum, that contain nitrogen-cycling genes involved in non-assimilatory reduction and oxidation of N species in groundwater. Purple gradient (right) represents genome coverage scaled by row (Z-Score) across sites A–D (ordered gwj01-16). Rows = MAGs; columns = samples per site (groundwater and attached-fraction enriched groundwater). Orange gradient (right) represents number of nitrogen-cycling gene copies per genome. Microorganisms are ordered based on hierarchical clustering of abundance based Bray-Curtis dissimilarity matrix with ward.D2 clustering method. Final column (labelled with asterisk) indicates genomes that were significantly more abundant at a particular site (coloured rectangle) based on LEfSe analysis.
microbial communities as a whole [13]. Bray–Curtis dissimilarities, calculated based on relative abundance for MAGs and metagenomic reads encoding nitrogen-cycling proteins, revealed that spatial differences significantly influenced community composition ($r^2 = 0.49$ for MAGs, $r^2 = 0.42$ for metagenomic reads), more than DO, DOC, nitrate, and sample type (groundwater or attached-fraction enriched) (Fig. 5A, B; Table S8). Most MAGs inferred to undertake nitrogen cycling (87.4%) were significantly more abundant at a specific site (22–57 MAGs/site; LEfSe, Kruskal–Wallis test). Results therefore show site-specific environmental conditions drive species selection, and the capacity for certain reactions, such as ammonia oxidation (greater in oxic groundwater) or complete denitrification (greater in dysoxic groundwater) (Fig. S3).

Nitrifier diversity and activity in groundwater, and habitat specificity

Archaeal and bacterial ammonia-oxidizers exhibited distinct niche preferences, but higher similarity in transcriptional activity. Ammonia-oxidizing archaea (AOA) and bacteria (AOB) convert ammonia to nitrite using ammonia monooxygenase (Amo) and hydroxylamine oxidoreductase (Hao), and perform the rate-limiting step in nitrification [60]. Niche differentiation between the two domains is not clearly defined [57]. However, AOA usually have a higher affinity for ammonia than AOB [47, 49, 56], and typically outnumber AOB in oxicotropic environments with low ammonia concentrations and salinity, consistent with groundwater in this study (mean ammoniacal-N 0.36 g/m³ ± 1.4 SD; conductivity 220 µS/cm ± 142 SD). We found AOA and AOB exhibited distinct spatial trends in relative and absolute abundance, and activity related to various geochemical parameters in groundwater (e.g. ammonia, oxygen availability, and conductivity) (Fig. 6).

Based on metagenomic reads and MAGs, protein-coding sequences for ammonia monooxygenase (Amo) subunits were most abundant at oxic site A (Figs. 1d, 6A; LEfSe, Kruskal–Wallis test). This trend was mostly driven by AOA, as there were significantly more sequences encoding AOA-AmoA than AOB-AmoA overall at oxic sites (Wilcoxon rank, $p < 0.05$), presumably due to lower ammonia regeneration (measured ammonia/ammonium concentrations were low across all sites regardless of oxygen content, $>1$ gm$^{-3}$ in only 6% of wells, and most below detection, Table S1). While not all ammonia oxidizer diversity may have been captured by the ddPCR primer sets used (e.g. the AOA primer set has a known bias against some ammonia-oxidizing Thermoproteota/Thaumarchaeota) [61], quantification of amoA genes and transcripts demonstrated a similar relationship with oxygen across a wider set of groundwater sites (Fig. 6C). Archaeal/bacterial amoA gene ratios were significantly and positively correlated with ORP (Spearman’s $r = 0.39$), and negatively correlated with borehole depth (Spearman’s $r = -0.35$; Table S9), which is expected to become increasingly oxygen-depleted with depth [62]. These gene ratios were also significantly and negatively associated with ammonia concentrations, conductivity, TDS, and pH (Spearman’s $r = -0.34$ to 52). Transcript ratios exhibited similar trends, albeit significant only for pH.

Taken together, results indicate that AOA and AOB abundance and activity are governed by distinct environmental niches in groundwater, as found in soils [63]. However, while AOA amoA gene concentrations were on average 40x higher than AOB genes, this difference was ten-fold less for transcripts (Table S10). Moreover, the deficit in significant correlations between AOA/ AOB amoA transcript ratios and geochemical/physical groundwater parameters (Fig. 6), suggests comparatively little difference in AOA and AOB activity overall.

Ammonia-oxidizers constituted several major lineages, including a single comammox bacterium. Commensurate with a greater abundance of AOA, we reconstructed seven AOA MAGs, along with one Nitrospiraceae MAG capable of complete ammonia oxidation (comammox). All contained at least one ammonia monooxygenase gene and six contained genes encoding all AmoABC subunits (Table S7). AOA genomes (and their amoA genes, Figs. S4–S6) were phylogenetically diverse, with the MAGs comprising five different genera (Fig. 6a). Of these, nzgw14 (UBA8516) was the most abundant nitrogen cycling MAG overall (Fig. S7), and was most abundant at oxic sites, along with other...
AOA MAGs. However, AOA MAG relative abundances did not reflect their transcriptional activity (Fig. 6B).

Recently characterized comammox bacteria (*Nitrospira*, phylum *Nitrospirae*) [64] oxidize ammonia to nitrate in three steps (ammonia → hydroxylamine → nitrite → nitrate). *Nitrospiraceae* MAG (nzgw279) possesses genes for ammonia oxidation (amoABC), hydroxylamine oxidation (haoAB), and nitrite oxidoreductase (nixAB), consistent with comammox. It also possesses a dissimilatory nitrite reductase (nirK) present in comammox bacteria elsewhere [65, 66]. Based on 120 concatenated bacterial marker genes (GTDB-Tk) and the AOA subunit, nzgw279 is closely related to clade B sublineage II comammox *Nitrospira*, and is most similar to *Nitrospira* sp. RCB obtained from an aquifer in Colorado (USA) [66] (Fig. S5), indicating strong habitat driven selection, independent of geographical distance. As expected for comammox, nzgw279 relative abundance was positively correlated with ORP and DO (Spearman’s $r = 0.87$ and 0.63; respectively). Although comammox bacteria can be the most abundant ammonia-oxidizers in some settings (e.g. groundwater-fed sand filters, forest soils and biofilters [67–69]), AOA genomes were more abundant overall here (Figs. 6, S7). Nevertheless, nzgw279 was highly active in terms of nix (although not amo) gene expression (Fig. 2C).

Diverse taxa with nitrite oxidoreductase homologues, including *Nitrososphaerales*. In addition to comammox bacterium nzgw279, we recovered the genomes of two *Nitrospiraceae* that we predict are canonical nitrite oxidizers, nzgw274 (no genus designation; nixA gene present), and nzgw276 (genus: *40CM-3-62-11*; nixAB present) (Table S7). Neither are affiliated with known comammox bacteria (*Nitrospira* spp. [66]). The nixA from nzgw274 was expressed highest at dysoxic site D in attached-fraction enriched groundwater (nixA, planktonic-fraction = 0.36, attached-fraction = 8.97 TPM). MAG nzgw276 transcripts were also present, but at a much lower level. Results suggest that the well (E1) had sufficient oxygen for NOB to exist. NOB can be active at nanomolar-to-micromolar concentrations of DO [70], and thereby compete for nitrite alongside anammox and denitrifying bacteria.

Known NXR are reported to be genetically diverse [71, 72]. The known diversity of nitrifiers continues to grow [73] and NXR has alternative uses, for example, as a nitrate reductase [71, 72]. Here, homologues were present in a diverse range of other MAGs, including several anammox bacteria (*Planctomycetota*, class Ca. Brocadiae, Table S7) [15], which typically use NXR to oxidize a small amount of nitrite during anammox [74], an archaean *Nitrososphaerales* (nzgw5; nixABC present), which belongs to a taxonomic group more typically associated with ammonia oxidation (this dataset, Table S7) [74], and various other bacterial phyla, which represent a pool of potentially novel nitrifiers (Fig. S8a).

To further explore NXR in the archaean *Nitrososphaerales* MAG (nzgw5), we evaluated the 14,591 bp long contig (3135) on which the genes were found. The contig primarily comprises protein-coding genes with closest homology to archaea (based on the NCBI nr database) that are located on either side of a syntenous bacterial-like nixABC gene cluster (Fig. S8b; Table S11), including chaperone-encoding *topD* directly adjacent to *nixC*, as found in *Nitrospina gracilis*, which is thought to facilitate Mo cofactor maturation and insertion into NxrA [73]. To determine whether nix genes were reproducibly present in other closely related *Nitrososphaerales* genomes, we searched for dereplicated MAGs sharing >99% ANI with nzgw5. We found one "replicate" *Nitrososphaerales* MAG (nzgw5-b) sharing 99.2% ANI, which was recovered from site B (nzgw5 derived from a co-assembly of site B samples), and that possessed a similar nixABC gene cluster (Table S11).

The MAG nzgw5 shares NxrA protein sequence similarity with bacteria as phylogenetically diverse as *Nitrospira defluvii*, *Nitrospina gracilis*, and *Candidatus Brocadia* (Fig. S8a), but the highest
NCBI nr database matches were to other aquifer bacteria, Candidate division Zixibacteria bacterium RBG-1 (58.56% identity) and Planctomycetes bacterium RIFCSPHIGHO2_02_FULL_S2_S8 (56.7% identity)—both originally recovered genomically from an aquifer in Rifle, CO, USA [11, 76]. NxrB similarly had the highest match with other aquifer-derived genomes (also U.S.A.), including one Nitrosopinae bacterium (65.97% identity), and most notably, two archaea affiliated with the Thermoproteota (previously Thaumarchaeota) phylum—another Nitrososphaerales, and Thermoproteota bacterium (68.71–71.87% identity) [13]. While the gene cluster appears to have been horizontally acquired, we found no identifiable genomic island associated with contig 3135. However, lateral gene transfer occurs more frequently between organisms, including unrelated taxa, that share a habitat [77]. Results suggest aquifer-adapted Nitrososphaerales acquired nxr genes, and potentially also the ability for nitrite oxidation/reduction, from a co-occurring bacterium.

Nitrite-dependent methanotroph (Ca. Methylomirabilis)

Nitrite-dependent methanotrophs were prevalent. Analysis of metagenomic reads revealed that copper-containing, membrane-associated particulate methane monoxygenase subunit A (PmoA) sequences (a closely related AmoA homologue [78]) were ubiquitous, but significantly more abundant at oxic site A (Fig. 1C). This protein is associated with aerobic and anaerobic nitrite-dependent methanotrophs that are able to oxidize methane to CO₂, using methane as a sole carbon and energy source [79]. Methanotrophic bacteria can also oxidize ammonia to nitrite using particulate methane monoxygenase (pMMO) and a unique hydroxylamine oxidoreductase, HAO [80]. Interactions between methanotrophs and ammonia-oxidizers in aquifers are poorly understood. They are associated with opposite gradients of ammonia and methane, as ammonia inhibits the activity of methanotrophs and methane acts as a competitive inhibitor for ammonia oxidizers [81, 82]. Analysis of 16S rRNA gene amplicons across a wide distribution of aquifer samples (n = 80) showed that Ca. Methylomirabilis relative abundance was positively correlated with ammonia-oxidizers Nitrosotaleaeae, Nitrosomonadaceae, and Nitrospumilaceae (Spearman’s r = 0.42, r = 0.33 and r = 0.37, respectively). Aerobic ammonia-oxidizers consume O₂ and may provide a habitable environment for Ca. Methylomirabilis at oxic/anoxic interfaces and produce nitrite, which could potentially be directly used by these nitrite-dependent methanotrophs [83].

We recovered one nitrogen cycling methanotrophic MAG (nzgw240), related to the anaerobic methane-oxidizing genus Ca. Methylomirabilis (Figs. 6, 56) [84]. Members of Ca. Methylomirabilis perform NO₂⁻ dependent anaerobic methane oxidation through an intra-aerobic pathway involving the dismutation of NO into O₂ and N₂ [79, 84], and play an important role in controlling N₂O and methane emissions from natural ecosystems [79]. All pmoABC subunits for methane oxidation [79] were present in nzgw240, and were closely related to those from Ca. Methylomirabilis lanthanidiphila (74.80–95.5% amino acid identity, Fig S6), a methanotroph that dominated an enrichment...
culture after addition of rare-earth metal cerium [85]. Several
genomes involved in nitrogen cycling, such as nitrate reductase 
(nrrAB), nitrite reductase (nirS), putative NO dismutase (nod) 
and nitric oxide reductase (norZ) were also identified in MAG 
nzwg240, and were expressed at the dysoxic site alongside 
*pmoA* (Figs. 2c, 6b), where dissolved methane was also detected
[86]. The first of two nitric oxide-like reductases shares 95.6%
amino acid identity with *Nod* (DAMO_2434) in *Ca. Methylomir-
abils* oxyfera [87]. This enzyme is homologous to the quinol-
dependent NO reductases (qNOR) [87], however experimental
validation is still required to prove nitric oxide disproportionation.
The second is a NO reductase sharing 88.34% amino acid identity
with *NorZ* (DAMO_1889).

**Final step of denitrification**

*Atypical NosZ was more common than typical NosZ.* Thirty MAGs,
spanning 10 bacterial phyla, contained *nosZ* genes (Fig. 3). The
*NosZ* protein catalyzes the conversion of green-house gas *N2O* to
*N2* in the last step of denitrification. Typical *NosZ* proteins (clade I)
contain a twin-arginine translocation (Tat) signal peptide, and to
date are affiliated exclusively with *Proteobacteria*, which usually
perform complete denitrification [88]. A maximum-likelihood tree
revealed that *NosZ* predicted protein sequences comprised both
typical clade I twin-arginine (Tat) dependant *N2O* reductase with
*Proteobacteria* (2 MAGs) and atypical clade II secretory (Sec)
dependent *N2O* reductase proteins (23 MAGs) (Fig. S9) [88]. The
tree also shows a novel clade of *Nitrospira* and *Nitrospinae NosZ*
Sec-dependant sequences, including four *NosZ* sequences from
this study (Fig. S9) that were transcriptionally active (Fig. 7).
Members of clade II are considered non-denitrifiers, typically
performing just the final step of denitrification [89]. However, 4/7
MAGs capable of complete denitrification encode clade II *NosZ*,
demonstrating complete denitrifiers are present across both
classes. Sec-dependant protein translocation is considered more
energetically favourable than Tat, requiring between 700 and
5,000 molecules (or equivalent) of ATP per protein translocation
across the membrane, whereas Tat requires the equivalent of
~10,000 molecules of ATP [90]. A greater proportion of Sec signal
pathways in low nutrient groundwater would be favourable for
energy conservation.

**Nitrous oxide reductase gene expression was strongly associated
with oxygen availability and not limited by pathway fragmentation.**
Based on ddPCR, *nosZ* clade I genes (3 × 10−6−6 × 10^6 copies/L) and
transcripts (1 × 10−7–7 × 10^4 copies/L) were detected across most
aquifer samples tested (Table S10). Expression was significantly 
and negatively correlated with ORP (Fig. 6), reflecting observations 
elsewhere that the rate of denitrification decreases linearly with
increasing ORP [91]. Accordingly, at the dysoxic site there was also 
a proportionally higher abundance of MAGs with *nosZ* genes of
any type (Fig. 3), and of complete denitrifier MAGs, which
comprised up to 42% of the nitrogen cycling community (4–31×
more on average than oxic sites) (Fig. S3).

*N2O* generation due to incomplete denitrification has been shown
to be highest under oxic conditions in groundwater [92],
comparable to sites A–C here. A fragmented denitrification
pathway may explain higher *N2O* concentrations in some oxic
groundwaters. Fragmented genetic potential for biogeochemical 
cycling processes, such as denitrification, appears to be a common
trait in aquifer bacteria (Fig. 4) [11], necessitating metabolic 
handoffs among individuals to complete pathways. Our transcrip-
tomic data points to active collaboration among incomplete
denitrifiers for generation and removal of *N2O* (although in situ
measurements would be required to determine the presence or
absence of NO or *N2O* emissions). Transcriptional activity
associated with *N2O* reduction was at least equivalent to that
for generation, regardless of groundwater oxygen-content or the
portion of pathway fragmentation (Fig. 2A).

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**Fig. 7 Nitrous-oxide reductase (*nosZ*) gene transcripts in sites C and D groundwater and attached-fraction enriched groundwater.**

A Stacked barplot shows modified-TPM of Sec- and Tat- dependent *nosZ* genes at each site. B Stacked barplot shows modified-TPM normalized to genome coverage of Sec- and Tat-dependent *nosZ* genes at each site. While complete denitrifier, *Sulfuricella* MAG 
nzwg577, contributed the most transcripts, after normalizing to MAG relative abundance, *nosZ* genes from two novel *Nitrospira* MAGs (nzwg266–267, class UBA7883 [104]) were transcriptionally most active. *c = MAGs which contain genes for the complete denitrification pathway (nzwg numbers: 271, 530, 549, 554, 561, 566, and 577).*

**Transcriptional activity of co-occurring aerobic and anaerobic
nitrogen-cycling pathways in oxic versus dysoxic groundwater**

Based on transcripts mapped to MAGs, there was less nitrogen-
cycling transcriptional activity at the pristine oxic site compared to
the dysoxic site (average modified-TPM 69 ± 13 site C versus
359 ± 362 site D) (Fig. 2B). This is consistent with quantification of
8x more nitrogen-cycling transcripts by ddPCR in dysoxic versus
oxic groundwater, across a wider set of groundwaters (on average
7 × 10^5 transcripts/L in dysoxic and 8 × 10^4 transcripts/L in oxic
groundwater; Table S11). The greatest proportion of mapped
transcripts at the oxic site was associated with ammonia oxidation
to nitrate and re-reduction to nitrite, based on ammonia
monooxygenase (*amo*), nitrite oxidoreductase (*nxr*), and periplas-
mic nitrate reductase (*nap*) gene transcripts (Fig. 2A). Expression of
*nap* genes is suggestive of aerobic denitrification at this site, as
nitrate reduction in the periplasm is not inhibited by oxygen [93].

At the dysoxic site, the greatest portion of gene expression in
groundwater and attached-fraction enriched groundwater, based
on mapped transcripts was associated with, again, nitrite
oxidation (*nxr*), but also anammox (*hzo, hzs*), and denitrification
(*nor* and *nos*) (Fig. 2A). These genes each contributed up to
8–66% of nitrogen-cycling transcripts at site D. Hydrazine
synthase *hhs*8 transcripts (quantified by ddPCR) were also highest
at the dysoxic site (April 2018), one of only seven sites in the

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The ISME Journal (2022) 16:2561 – 2573
study with detectable nitrite concentrations. Contemporaneous measurements of excess N\textsubscript{2} indicated active N\textsubscript{2}\textsubscript{O} generation in dysoxic groundwater from this site (wells E1 and N3; Table S12) due denitrification and/or anammox [15]—the technique cannot distinguish between N\textsubscript{2} produced by these processes. Nitrate-based \(\delta^{18}\text{O}\) against \(\delta^{15}\text{N}\) measurements from groundwater in well N3 also indicated the occurrence of denitrification [86]. The majority of hzoA and hzsB genes were expressed by just two Planctomycetes MAGs (nzwg511–512) at this site (37% and 58%, respectively). However, when considering hzsB transcript concentrations in the wider aquifer dataset, we observed no relationship with DOC or oxygen availability (DO, ORP or borehole depth) (Fig. 6), indicating these bacteria are active under a wide variety of groundwater conditions, including those considered unfavourable for anammox (i.e. high DOC and DO). Ca. Brocadia genomes recovered from Sites A–D were previously found to have a broad range of ABC transport systems and variations in substrate importation such as phosphate, cobalt, nickel, iron(III), zinc, sulfate, molybdate, lipoproteins, ribose, rhamnose, poly-saccharides, and oligopeptides, suggesting that they may not just be autotrophic specialists [15]. We found evidence for greater competition among N\textsubscript{2}O reducers (Fig. 2B), although most nosZ transcripts overall (81%) were expressed by a single complete denitrifier, MAG nzwg577 (genus Sulfiticella; Fig. 7) at the dysoxic site (Fig. 2C), despite it being only the third most active in terms of nosZ expression after normalizing to MAG coverage (Fig. 7). Members of this genus are reported to perform autotrophic denitrification coupled with the oxidation of reduced sulfur compounds [94].

Several aerobic ammonia-oxidizers, for which we recovered MAGs, were also active at the dysoxic site, contributing 5-6% of nitrogen-cycling transcripts mapped (Fig. 2B). This suggests the potential for simultaneous nitrification and denitrification, and partial coupling of these pathways (overall modified-TPM: 1.27 amoA/nosZ, 1.23 nosZ/nrxA). Most (88%) amoA transcriptional activity at this dysoxic site was attributed to four AOA MAGs affiliated with Nitrosopumilaceae. The greatest proportion of ammonia monooxygenase transcription was associated with the attached-fraction enriched groundwater from this site (Fig. 6B), consistent with preferences previously reported for the ammonia-oxidizing genera, Nitrososphaera and Nitrosopumilus, in groundwater [30]. Prior findings from karstic aquifers also suggest that soil-derived ammonia oxidizers may be imported into groundwater [26], which may be important for shallow aquifers that directly receive leachate from the soil zone, along with any microorganisms it carries.

The most transcribed nitrogen-cycling gene among all MAGs was nrxB from comammox Nitrosospira nzwg279, which showed the highest expression in attached-fraction enriched groundwater at site D. This groundwater contained more total suspended solids (Table S1), and therefore more sediment particles coated in biofilms [95]. Comammox Nitrosospira populations have previously been found to dominate biofilms in wastewater, outnumbering all other nitrifiers [96]. As nzwg279 was associated with fewer amoA transcripts than other ammonia-oxidizers (average modified-TPM 0.77 ± 1.02 SD vs 2.01 ± 3.22 SD), and appeared to act largely as a canonical nitrifier [97], it potentially received nitrite as a by-product from the several active AOA.

Results show methanotrop methane monooxygenase gene transcription occurred alongside gene expression associated with nitrification, anammox, and denitrification at the dysoxic site. Methanotrophs and ammonia-oxidizers share many metabolic similarities based on a common evolutionary history, and supported by the structural similarities of ammonia and methane monooxygenases [81], methanotrophs have been implicated in both methane and ammonia oxidation in groundw\textsubscript{a}ter [98]. In this study, Ca. Methylophilus (nzwg240) expressed genes, associated with concurrent methane oxidation (pamoA) and nitric oxide dismutation (NO\textsubscript{\textsuperscript{2}} reductase nirS and NO dismutase nod) to N\textsubscript{2} and O\textsubscript{2} (Fig. 2C) [84]. Co-occurring gene expression reveals a potential interaction among AOA, Ca. Methylophilus, and anammox bacteria, whereby nitrite produced from aerobic ammonia oxidation by AOA, drives anammox and nitrite-dependent anaerobic methane oxidation by Ca. Methylophilus. Ca. Methylophilus produces oxygen which could create an interface whereby AOA and comammox can co-exist. Oxygen consumption and nitrite provisioning by AOA could represent synergism with anammox in the terrestrial subsurface, as previously predicted to occur in unconfined aquifer soils [99]. Indeed, ammonia oxidizer and anammox activities, based on transcript copy numbers, were found to be tightly linked across distinct groundwater chemistries in the wider set of samples [15]. These heterogeneous reactions at the dysoxic site indicate that it likely contained mixed redox conditions in situ. This could be due to oxygen penetration from above [100], and vertical stratification of electron donors [101], geochemical gradients created by biofilm formation [102], or oxygen produced by the intra-aerobic pathway of Ca. Methylophilus species [84].

CONCLUSION

Results show that the capacity for non-assimilatory nitrogen-cycling reactions, such as ammonia oxidation and denitrification, was prevalent in groundwater regardless of site-specific physicochemistry, although the relative abundance of each pathway differed. Phylogenetically diverse AOA and AOB were associated with distinct environmental niches in groundwater, and AOA-amoa genes and transcripts were more abundant overall. While incomplete denitrifiers were numerous, complete denitrifiers contributed to a substantial fraction of transcriptional activity under dysoxic conditions, where activity associated with denitrification, and N-cycling transcripts was greatest. Gene expression associated with nitrification, denitrification, nitrite-dependent methane oxidation, and anammox occurred simultaneously in dysoxic groundwater, such that nitrite (or nitrate) produced by AOA or comammox could fuel anammox, denitrification, and methanotrophy by Ca. Methylophilus. Results provide insights into microbial N-transformations in groundwater with distinct chemical characteristics (such as oxygen availability and DOC), and potential metabolic “handoffs” among nitrogen-cycling organisms.

DATA AVAILABILITY

Sequences are deposited with NCBI under BioProject PRJNA699054.

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AUTHOR CONTRIBUTIONS
KMH and CD conceived the study, and KMH, CD, LW, and MC obtained funding. OEM, EG and KMH collected samples. MC contributed technical expertise for in situ sonication. OEM and EG performed laboratory procedures and bioinformatic analyses. OEM led the analysis of data and writing of the manuscript with KMH. All authors reviewed the manuscript.

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