Environment International 178 (2023) 108124

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Full length article

Performance, microbial growth and community interactions of iron-dependent denitrification in freshwaters

Shengjie Li [a,b,c], Muhe Diao [b], Yinhao Liao [a], Guodong Ji [a,*]

[a] Key Laboratory of Water and Sediment Sciences, Ministry of Education, Department of Environmental Engineering, Peking University, Beijing 100871, China
[b] Department of Geoscience, University of Calgary, Calgary, AB T2N 1N4, Canada
[c] Department of Biogeochemistry, Max Planck Institute for Marine Microbiology, Bremen 28359, Germany

ARTICLE INFO

Handling Editor: Hefa Cheng

Keywords:
Iron-dependent denitrification
Freshwater
Metagenomics
Microbial growth
Syntrophy

ABSTRACT

Iron-dependent denitrification is a safe and promising technology for nitrogen removal in freshwaters. However, the understanding of microbial physiology and interactions during the process was limited. Denitrifying systems inoculated with freshwater samples were operated with and without iron(II) at a low C/N ratio for 54 days. Iron addition improved nitrogen removal. Batch experiments confirmed that microbially mediated reaction rather than abiotic reaction dominated during the process. Metagenomics recovered genomes of the five most abundant microorganisms, which accounted for over 99% of the community in every triplicate of the iron-based system. Based on codon usage bias, all of them were fast-growing organisms. The total abundance of fast-growing organisms was 38% higher in the system with iron than in the system without iron. Notably, the most abundant organism *Diaphorobacter* did not have enzymes for asparagine and aspartate biosynthesis, whereas *Rhodanobacter* could not produce serine and cobalamin. *Algoriphagus* and *Areminomonas* lost synthesis enzymes for more amino acids and vitamins. However, they could always obtain these growth-required substances from another microorganism in the community. The two-partner relationship minimized the limitation on microbial reproduction and increased community stability. Our results indicated that iron addition improved nitrogen removal by supplying electron donors, promoting microbial growth, and building up syntrophic interactions among microorganisms with timely communications. The findings provided new insights into the process, with implications for freshwater remediation.

1. Introduction

Nitrogen is increasingly being released into aquatic environments due to domestic sewage and farmland runoff (Seitzinger, 2008). Denitrification removes dissolved nitrogen to the atmosphere by transforming nitrate to nitrogen gases. However, the C/N ratio is relatively low in most freshwater systems and heterotrophic denitrification is limited owing to the lack of the electron donor (Di Capua et al., 2019; Newcomer et al., 2012). The role of iron in the nitrogen cycle has gradually been discovered in the last two decades (Michiels et al., 2017; Weber et al., 2006). One of the promising approaches to enhance nitrogen removal efficiency is adding iron(II) to conduct iron-dependent denitrification through Eq.(1),

$$Fe(II) + 2\ NO_3^- + 12H^+ \rightarrow 10\ Fe(III) + N_2 + 6\ H_2O \quad (1)$$

This process takes advantage of using inorganic electron donors, which reduces CO_2 emissions and operating costs compared to heterotrophic denitrification. As for other autotrophic nitrogen removal processes, sulfur-dependent denitrification produces sulfate as another contaminant (Qiu et al., 2020). In comparison, oxidant Fe(III) resulted from iron-dependent denitrification precipitates in the form of minerals, producing no secondary pollution and even adding to commercial benefits (Tian et al., 2020). Therefore, it has the great potential to be applied as a safe and cost-saving technology for the remediation of nitrate-contaminated freshwaters (Deng et al., 2022; Kiskira et al., 2017; Tian et al., 2020; Zhu et al., 2019).

Previous studies proposed three mechanisms explaining how iron-driven denitrification happens. Firstly, autotrophic bacteria couple denitrification to iron oxidation while fixing carbon dioxide, as observed in an enrichment culture (Straub et al., 1996). However, all isolated strains of iron-dependent denitrifying microorganisms could not grow with only iron - they still require organic compounds as an extra energy

* Corresponding author.
E-mail address: jiguodong@pku.edu.cn (G. Ji).

https://doi.org/10.1016/j.envint.2023.108124
Received 14 May 2023; Received in revised form 21 July 2023; Accepted 30 July 2023
Available online 31 July 2023

source (Bryce et al., 2018). This indicates a higher possibility of the second mechanism - mixotrophic denitrifying bacteria oxidize iron while still obtain energy from organic carbon. Though committing to autotrophy or heterotrophy increases proteome efficiency and enables individuals to focus on a specific metabolism (Johnson et al., 2012), the energy output from iron oxidation is relatively poor (Emerson et al., 2010). To overcome it, microorganisms instead use a combined energy from iron and organic carbon oxidation. The third mechanism is intermediate nitrogen metabolites from heterotrophic denitrification, such as nitrite and nitric oxide, abiotically interact with iron(II) extracellularly, which is the so-called chemodenitrification (Carlson et al., 2013).

In whichever process happens, microorganisms play a central role in mediating iron-dependent denitrification. A study revealed microbial community structure with 16S rRNA gene amplicon sequencing in an iron(II)-dependent denitrifying bioreactor, where the enriched organisms mainly included *Thiobacillus* and *Ferritrophicum* (Deng et al., 2022). Another research incubated freshwater sediments with iron(II) and indicated *Paracoccus* and *Thiobacillus* were potential iron-dependent denitrifiers (Pang et al., 2021). However, what is more important than the taxonomic distribution is the microbial metabolic function. The exact functional role of individual microorganisms during iron-dependent denitrification was still unknown. If iron-dependent denitrification is applied to aquatic ecosystems, how microorganisms deal with the presence of both organic and inorganic electron donors awaits to be answered. In addition, growth is a basic parameter of microbial lifestyle. If the first mechanism dominates, the growth of denitrifiers might be limited due to poor energy obtained from iron oxidation (Emerson et al., 2010), which further impacts nitrogen removal efficiency. Currently, we do not know whether microorganisms involved in iron-dependent denitrification are fast- or slow-growing in ecosystems, which is essential for the application and optimization of this potential remediation technology (Garrido-Amador et al., 2021).

Microbial interactions represent another crucial area of knowledge that is currently lacking for iron-dependent denitrification. Microorganisms exist in a community rather than grow in isolation. Community interactions are pervasive in ecosystems and paramount for the survival of individuals (Faust and Raes, 2012; Proulx et al., 2005). Especially, the cooperation of microorganisms through the exchange of amino acids and vitamins (termed as syntrophic relationship in the following text) is an important factor in structuring microbial community (Zengler and Zaramela, 2018). Many organisms are unable to synthesize all these growth-required substances and need to take up metabolites released by other community members (D'Souza et al., 2014; Mee et al., 2014). Previous research has discovered that less abundant heterotrophic denitrifiers supplied essential growth-required substances to the most abundant heterotrophic denitrifier in a partial-nitritation anammox reactor (Wang et al., 2019). Such cooperation lowers the energy burden of individuals and improves metabolic efficiency, but it relies on the contact area between the supplier and the acceptor. A previous study showed that cells only interact with other cells in their immediate neighbourhood (Dal Co et al., 2020). If substances are not delivered timely, microbial growth would be limited and the metabolic process would be adversely affected. Considering both aspects, syntrophic relationships may contribute to overall community robustness but may also reduce efficiency (Embree et al., 2015). Though syntrophy is important in controlling energy flux through a system, we do not know how such a relationship exists in an iron-dependent denitrifying system and if it could be used to promote nitrogen removal efficiency.

To solve the above problems, we ran two sets of triplicated denitrifying systems inoculated with freshwater samples with and without iron(II) addition. The performance of iron-dependent denitrification was evaluated. Contributions of biotic and abiotic processes were analyzed. 16S rRNA gene amplicon sequencing monitored microbial community assembly for 54 days. Metagenomes of the final community were obtained and sequenced to investigate microbial functions. Especially, genomes were assembled based on metagenomes and used to examine the physiology of individual organisms. The abundant populations (totally accounting for greater than 99% of the whole community) in the iron-based denitrifying system had recovered genomes with high completeness (greater than 95%) and low contamination (less than 4%). Growth parameters of the organisms were evaluated. Syntrophic relationships among community members were explored with specific enzymes and transporters. The findings are fundamental to understand the microbial world of iron-dependent denitrifying systems and shed light on the improvement of nitrogen removal performance during freshwater remediation.

2. Materials and methods

2.1. Experimental set-up

400 mL of denitrifying systems were each inoculated with 20 g of water–sediment samples collected from Guangfu River, China. The experiments included two treatments - both were set up in 500 mL serum bottles and operated for six experimental cycles. The first treatment included triplicated denitrifying systems with acetate as the only organic source. The second treatment included triplicated denitrifying systems with both acetate and iron. In both treatments, 5 mM nitrate and 1.5 mM acetate were added at the start of each experimental cycle, while in the second system 24 mM iron(II) was added. The first treatment had no iron (II) addition. Other basic medium components included 2.0 mM $MgCl_2{\cdot}6H_2O$, 0.90 mM $CaCl_2$, 11 mM KH_2PO_4, 0.50 mM NH_4Cl, 10 mM $NaHCO_3$, 1.0 mL trace element solution and 1.0 mL vitamin solution (Li et al., 2022a). The systems were flushed with nitrogen at the beginning of each cycle and operated in anoxic environments in the dark at room temperature in a shaker. At the end of each cycle, 200 mL homogenized culture was replaced with 200 mL basic medium during nitrogen purging.

2.2. Batch experiments for abiotic reactions

To evaluate biotic and abiotic reactions during iron-dependent denitrification, two treatments of abiotic batch experiments were conducted. The inoculum, basic medium and operation conditions for abiotic experiments were set to be the same as the iron-based denitrifying system mentioned above, but the abiotic systems were immediately sterilized with the ^{60}Co irradiation method after inoculation (Li et al., 2022d). 5 mM nitrate and 24 mM iron(II) were added to the first treatment at the beginning. 5 mM nitrite and 24 mM iron(II) were added to the second treatment at the beginning. Three replicates were included in each treatment. They were operated for eight days.

2.3. Sampling and chemical analysis

Slurry samples were collected with syringes from denitrifying systems and centrifuged at 4000 rpm for 10 min. Supernatants were filtered and used for chemical measurements. Pellets were used for DNA extraction. Nitrate, nitrite and iron(II) concentrations were measured according to previously described methods (Li et al., 2022d). Total organic carbon (TOC) content was measured with a TOC analyzer (TOC-L CPH, Shimadzu, Japan). Ammonia was measured using the indophenol reaction (McCullough, 1967).

2.4. DNA extraction, 16S rRNA amplicon sequencing and analysis

DNA was extracted with the FastDNA Spin Kit for Soil (MP Biomedicals, USA). DNA samples were quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA samples collected at the beginning of the experimental operation and at the end of each experimental cycle in the two treatments were used for amplicon sequencing. Bacterial 16S rRNA gene fragments were amplified from the extracted DNA using the primers 338F (5′-

ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR system and process were set with the previously described parameters (Li et al., 2022a). Amplicons were quality checked with agarose gel electrophoresis. Sequencing was performed with the Illumina MiSeq sequencing platform using PE300 chemicals at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Raw reads were quality filtered with Fastp v0.19.6 (Chen et al., 2018). Filtered reads were de-noised using DADA2 plugin in QIIME2 v2020.2 (Callahan et al., 2016). Taxonomic assignment was performed using the Naive bayes consensus taxonomy classifier implemented in QIIME2 and the SILVA 16S rRNA database v138. The relative abundance of each taxon was calculated by dividing the sequences belonging to the taxon by all sequences obtained from an amplicon sample. Non-metric multidimensional scaling (NMDS) analysis and analysis of similarity (ANOSIM) of the amplicon sequencing results were performed with the 'vegan' package in R v4.0.3. A p value less than 0.05 was considered to be statistically significant. Different treatments and replicates were grouped with the 'ordiellipse' function.

2.5. Metagenomic sequencing, assembly, binning and annotation

DNA samples collected at the end of the last experimental cycle in the two treatments were subjected to metagenomic sequencing. Metagenomic sequencing was performed as previously reported (Li et al., 2022b). Briefly, DNA samples were first fragmented to similar lengths (about 400 bp). The library was constructed with NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). Adapters were ligated to the blunt-end of fragments. Paired-end sequencing was performed with the Illumina NovaSeq 6000 platform using NovaSeq Reagent Kits at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The final output was about 40 M to 46 M sequences (151 bp) per sample.

Raw reads were quality filtered and checked with FastQC v0.11.9. Quality-checked reads were assembled with MEGAHIT v1.2.9 (Li et al., 2015). Sequencing depth for each contig was evaluated with BBMap v38.18 with over 99% identification. Metagenome-assembled-genomes (MAGs) were obtained with MetaBat v2:2.15 based on the tetranucleotide frequency of the contigs and the sequencing depth information (Kang et al., 2019). Only MAGs with completeness higher than 70% and contamination less than 10% were kept for further analysis. Marker genes in the recovered MAGs and reference genomes which were related to the MAGs were obtained with Phylosift v1.0.1 (Darling et al., 2014). The genes were aligned with MAFFT v7.490 using default parameters (Katoh et al., 2002). A phylogenetic tree including the MAGs and the reference genomes was built with RAxML v8.2.12 based on the alignments with "-m PROTGAMMAAUTO -p 13 -x 123 -# 100" (Stamatakis, 2014). MAGs were annotated with Prokka v1.13 (Seemann, 2014) and METABOLIC v4.0 (Zhou et al., 2022). Genes for iron oxidation were recognized with FeGenie (Garber et al., 2020). Genes for nitrogen cycling were identified with Hidden Markov Models (HMMs) v3.3.2 (Eddy and Rost, 2008) following a previously described method (Li et al., 2021). The peptide sequences of MAGs in FAA format output by Prokka were further annotated by the KEGG database using the Blast-KOALA method (https://www.kegg.jp/blastkoala/) and the TransAAP (https://www.membranetransport.org/transaap/) to respectively identify proteins for biosynthesis and transport of amino acids and vitamins. The relative abundance of each gene was normalized by the total abundance of all genes in a metagenome. The minimum doubling time of a microbial population was predicted with gRodon based on codon usage bias of ribosomal proteins (Weissman et al., 2021). Microorganisms with predicted minimum doubling times less than 5 h were considered copiotrophs and those with predicted minimum doubling times longer than 5 h were considered oligotrophs (Weissman et al., 2021). The in-situ replication rate of a population was estimated with iRep based on the sequencing coverage trend that results from bi-directional genome replication from a single origin of replication (Brown et al., 2016).

3. Results and discussion

3.1. Denitrification performance with and without iron

The experiment with iron(II) almost consumed all the nitrate at the end of each experimental cycle, while the experiment with only acetate only reduced half of the added nitrate during each cycle (Fig. 1a). Nitrite gradually accumulated and was above 3 mM at the end of the experiments in the acetate-based system, while nitrite accumulated to a much less extent in the iron-amended system (Fig. S1a). For ammonium, no obvious ammonium production was observed in both treatments, while ammonium was consumed likely due to nitrogen assimilation during each experimental cycle (Fig. S1b). TOC decreased to a similar extent in the two experiments, indicating acetate was intensively used in both treatments (Fig. 1b). In parallel, about half of the iron(II) added was oxidized during each cycle in the iron-based system (Fig. 1c). More iron (II) than acetate remained at the end of each cycle in the iron-based system, indicating that the organic compound was still a preferable

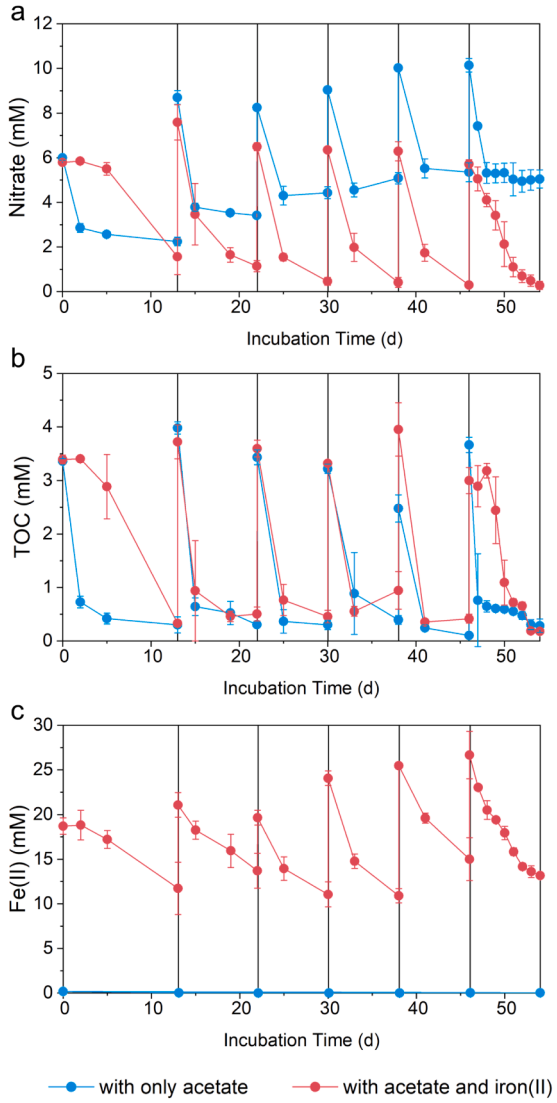

Fig. 1. Changes in (a) nitrate, (b) total organic carbon (TOC) and (c) iron(II) concentrations in the system with only acetate and the system with both acetate and iron(II). Black vertical lines indicted the start/end of an experimental cycle. Each treatment included three replicated systems.

electron donor. Overall, the results of nutrient concentration change demonstrated that the acetate-based denitrifying system actively used acetate, while the iron-amended denitrifying system consumed both acetate and iron(II). In a system lack of organic carbon compared to nitrogen pollutants, iron(II) addition could increase nitrogen removal efficiency. Such a strategy could be considered and applied in freshwaters, which typically have this problem.

3.2. Biotic and abiotic contributions of iron to denitrification

According to previous findings, iron-dependent denitrification process can occur abiotically or mediated by microorganisms (Melton et al., 2014). Nitrate and iron(II) hardly react in most systems, but the reaction between nitrite and iron(II) may happen to a significant level (Li et al., 2022d; Pang et al., 2021; Picardal, 2012). Abiotic systems were built with sterilized freshwater samples. For the treatment with nitrate and iron, no nitrate reduction or nitrite production was observed in all triplicates (Fig. 2a and b). For the treatment with nitrite and iron, about 1 mM nitrite was reduced in the first two days but there was almost no nitrite reduced afterwards (Fig. 2b). Both treatments showed a decline in iron(II) concentration in the first two days, while the treatment with nitrite had more iron(II) oxidized than the treatment with nitrate (Fig. 2c). The decrease of iron(II) content in the treatment with nitrate and extra decrease of iron(II) in the treatment with nitrite might result from the mineralization of dissolved iron(II) to bound iron(II) and adsorption of iron(II) to precipitates, which could not be detected with the current method. However, the results of Section 3.1 showed there was much less nitrate left at the end of each experimental cycle, indicating that microorganisms contributed more than the abiotic reaction to denitrification in the system with iron.

3.3. Microbial community assembly

As microorganisms play the most important role in iron-dependent denitrification, amplicon sequencing was applied to monitor the change in microbial community composition during the incubations (Fig. 3, Table S1). In the iron-based system, *Pseudomonas* was enriched to over 75% at the end of the third to the fifth cycles in the three replicates, but it decreased to 26%±3.6% at the end of the sixth cycle (Fig. 3a). *Diaphorobacter* accounted for 70%±3.0% of the community at the end, while *Rhodanobacter* accounted for 1.5%±0.63%. The fourth and the fifth most abundant organisms were *Algoriphagus* (0.35% ±0.31%) and *Arenimonas* (0.24%±0.34%). The final enriched community mainly consisted of these five abundant organisms.

In comparison, we did not observe a highly abundant microorganism in the system with only acetate. The final community consisted of populations such as *Thauera* (17%±0.62%), Comamonadaceae (13% ±2.3%), *Azoarcus* (12%±3.4%) and *Alicycliphilus* (11%±7.7%). There was not an abundant population common in the two treatments, suggesting that bacteria able to use both acetate and iron(II) were not the same bacteria only dependent on acetate during denitrification. NMDS analysis also indicated that the communities enriched from the same inoculum were very different in the two systems (p=0.0001 in ANOSIM, Fig. 3b). Based on the results, the abundant organisms in the system with iron (i.e. *Pseudomonas*, *Diaphorobacter*, *Rhodanobacter*, *Algoriphagus* and *Arenimonas*) might have the expertise to gain energy from both acetate and iron oxidation, which was not special for enriched microorganisms in the system with only acetate (e.g. *Thauera* and *Azoarcus*).

3.4. Physiology and functions of the enriched microorganisms

We obtained 39 metagenome-assembled-genomes (MAGs) in the two systems (Table S2), with 5 of them more abundant in the system with iron and 34 of them more abundant in the system with only acetate (Table S3). The 5 abundant members in the iron-based system included 4 Proteobacteria (Bur1, Pse, Xan1 and Xan2) and 1 Bacteroidota (Cyt) (Fig. 4, Table S2, S3). Bur1 (*Diaphorobacter*) was the most abundant population, accounting for 76%±8.9% of the community (Fig. 5, Table S3). Pse (*Pseudomonas*) was the second most abundant population, with an average abundance of 20%±10%. Xan1 (*Rhodanobacter*) made up 2.3%±0.84% of the community, while Xan2 (*Arenimonas*) made up 0.58%±0.83%. Cyt (*Algoriphagus*) averagely accounted for 0.30% ±0.23% of the community. Altogether, the five populations accounted for more than 99% of the whole community in every replicate of the iron-amended systems. The results were consistent with what was obtained by amplicon sequencing analysis and again indicated that iron-dependent denitrification was conducted by a few abundant organisms, which were not the same bacteria enriched in the system with only acetate.

Based on codon usage bias, the predicted minimal doubling times of the five abundant populations in the iron-amended system were less than 5 h (Table S4) (Weissman et al., 2021). According to the previous study, microorganisms with minimal doubling times less than 5 h are copiotrophs (fast-growing organisms, *r*-strategists), while oligotrophs (slow-growing organisms, *k*-strategists) refer to microorganisms with minimal doubling time over 5 h (Weissman et al., 2021). The results suggested that microorganisms in the system with iron were growing fast and preferred to live in regimes rich in nutrients (Fig. 4). Particularly, the minimum doubling times of Pse, Cyt and Bur1 were 1.5 h, 2.3 h and 2.5 h, respectively, less than the minimum doubling times of most microbial populations (85%) in the system. In comparison, the other 34 populations more abundant in the system with only acetate included 23 copiotrophs and 11 oligotrophs (Fig. 4, Table S4). The total abundance of copiotrophs and oligotrophs was calculated in the two treatments.

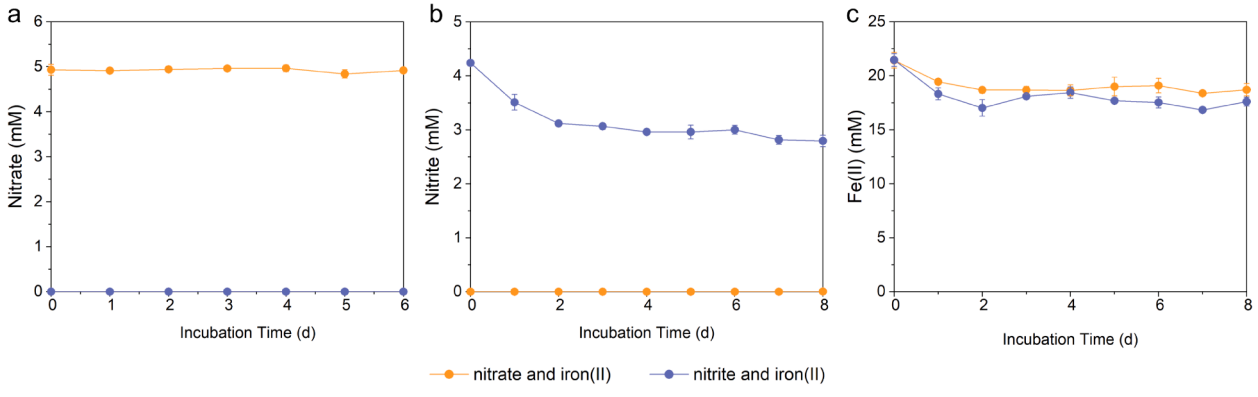

Fig. 2. Changes in (a) nitrate, (b) nitrite and (c) iron(II) concentrations in the abiotic system with nitrate and iron(II) and the abiotic system with nitrite and iron(II). Each treatment included three replicated systems.

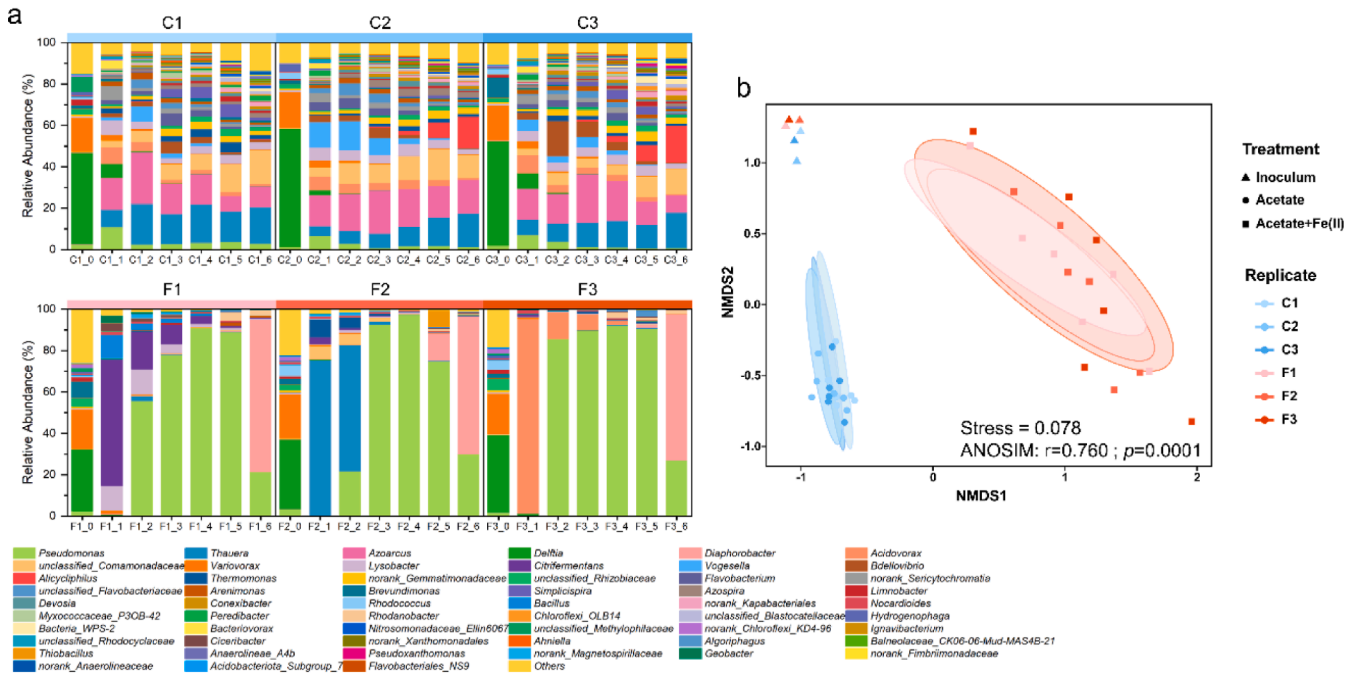

Fig. 3. (a) Change in relative abundance of microorganisms on the genus level. (b) Non-metric multidimensional scaling (NMDS) analysis of the samples. The three replicates of the system with only acetate are indicated as C1, C2 and C3. The three replicates of the system with both acetate and iron(II) are indicated as F1, F2 and F3. Analysis of similarity (ANOSIM) was performed between samples of the two systems. Samples from the same replicate were grouped using the 'ordiellipse' function from the 'vegan' package in R.

Oligotrophs accounted for 38%±5.4% in the system with only acetate but only 0.00081%±0.00032% in the system with iron. The two treatments both had a higher abundance of copiotrophs, but the proportion of copiotrophs was much higher in the system with iron (38% more abundant). The iron-based system mainly enriched *r*-strategists which were specialized for rapid growth. To confirm whether the organisms were actually growing fast in the iron-amended system, a software designed to evaluate *in-situ* bacterial replication rates, iRep, was further applied to the five populations. Compared to reported organisms residing in human guts or acetate-amended aquifers whose iRep values were around 1.3 (Brown et al., 2016), the five populations had higher iRep values in the iron-based system (ranging from 1.5 to 3.9) (Table S5). Especially for Bur1 and Xan1, their iRep values were no less than 3.6. The results indicated they had higher replication rates and were growing rapidly with iron.

In any of the five populations, we did not observe the presence of genes involved in carbon fixation pathways, including the Calvin cycle, the reverse tricarboxylic acid cycle, the 3-hydroxypropionate bi-cycle, the 4-hydroxybutyrate/3-hydroxypropionate cycle, the dicarboxylate/4-hydroxybutyrate cycle and the Wood Ljungdahl pathway (Table S6). According to previous studies, though several iron-dependent nitrate-reducing strains have been successfully isolated, none of them could survive merely with an inorganic carbon source (Bryce et al., 2018). They all needed external organic compounds as an extra energy source (Hu et al., 2017; Muehe et al., 2009). The results in our system also suggested that iron-dependent denitrifiers could not fix carbon and need organic compounds supplied. As for iron metabolism, Xan1 contained two sets of genes related to iron oxidation capability (Table S6). The first set was MtoA together with MtoB, which were decahaem c-type cytochromes discovered in the genome of a neutrophilic iron oxidizer, *Sideroxydans lithotrophicus* ES-1 (Liu et al., 2012). The other set of genes was Cyc1 and Cyc2, which were first identified from acidophilic aerobic iron oxidizers (Nicolle et al., 2009; Roger et al., 2012; Valdes et al., 2008) and later observed in microaerophilic iron oxidizers (Barco et al., 2015; Kato et al., 2015; Singer et al., 2011). Cyc1 and Cyc2 are both c-type cytochromes. Cyc2 is located at the outer membrane and directly receives electrons from iron(II), whereas Cyc1 is in the periplasm, receives electrons from Cyc2 and further transfers electrons to terminal electron acceptors (Roger et al., 2012). For Bur1, Pse and Xan2, we only observed the enzyme Cyc1 but not Cyc2. Nevertheless, enzymes responsible for iron oxidation typically had low sequence identity among different microbial taxa (Melton et al., 2014). Even though the Gallionellaceae family was best known for iron oxidation, a previous multi-omic study failed to elucidate genes responsible for iron oxidation in the genomes of several Gallionellaceae organisms (Jewell et al., 2016). We might neglect genes involved in iron oxidation as well - other abundant populations (Bur1, Pse, Xan2 and Cyt) might also be able to oxidize iron. Another pathway to oxidize iron was the coupling of heterotrophic nitrate reduction and chemodenitrification (Carlson et al., 2013). With the results of Section 3.2, the microorganisms might release nitrite during denitrification and induce abiotic oxidation of iron(II).

All of the five genomes contained genes involved in denitrification (Table S6). Specifically, Bur1, Pse and Xan1 could reduce nitrate to N_2O (NarG and/or NapA, NirK or NirS, and NorB). Xan2 might scavenge nitrite released during nitrate reduction, as no nitrate reductase was observed in the genome. Cyt was the only organism able to reduce N_2O (NosZ), but it lacked nitrate reductase as well. All of them encoded at least one pathway for acetate transformation. Bur1 and Xan2 directly produced acetyl CoA from acetate with acetyl-CoA synthetase (Acs and/or AcdA), whereas the other three populations could produce acetyl CoA not only directly from acetate, but also through the transformation of acetyl phosphate with acetate kinase (AckA) and phosphate acetyltransferase (Pta). Pse additionally encoded methane monooxygenase regulatory protein MMO, suggesting it could also grow through methanotrophy. Though no reduced sulfur was added to the medium, sulfur oxidation was possible for all five abundant populations. Cyt could also oxidize hydrogen. These processes might act as auxiliary energy-gaining pathways when acetate and iron were lacking in environments and indicated the versatility of functional microorganisms in the iron-dependent denitrifying system.

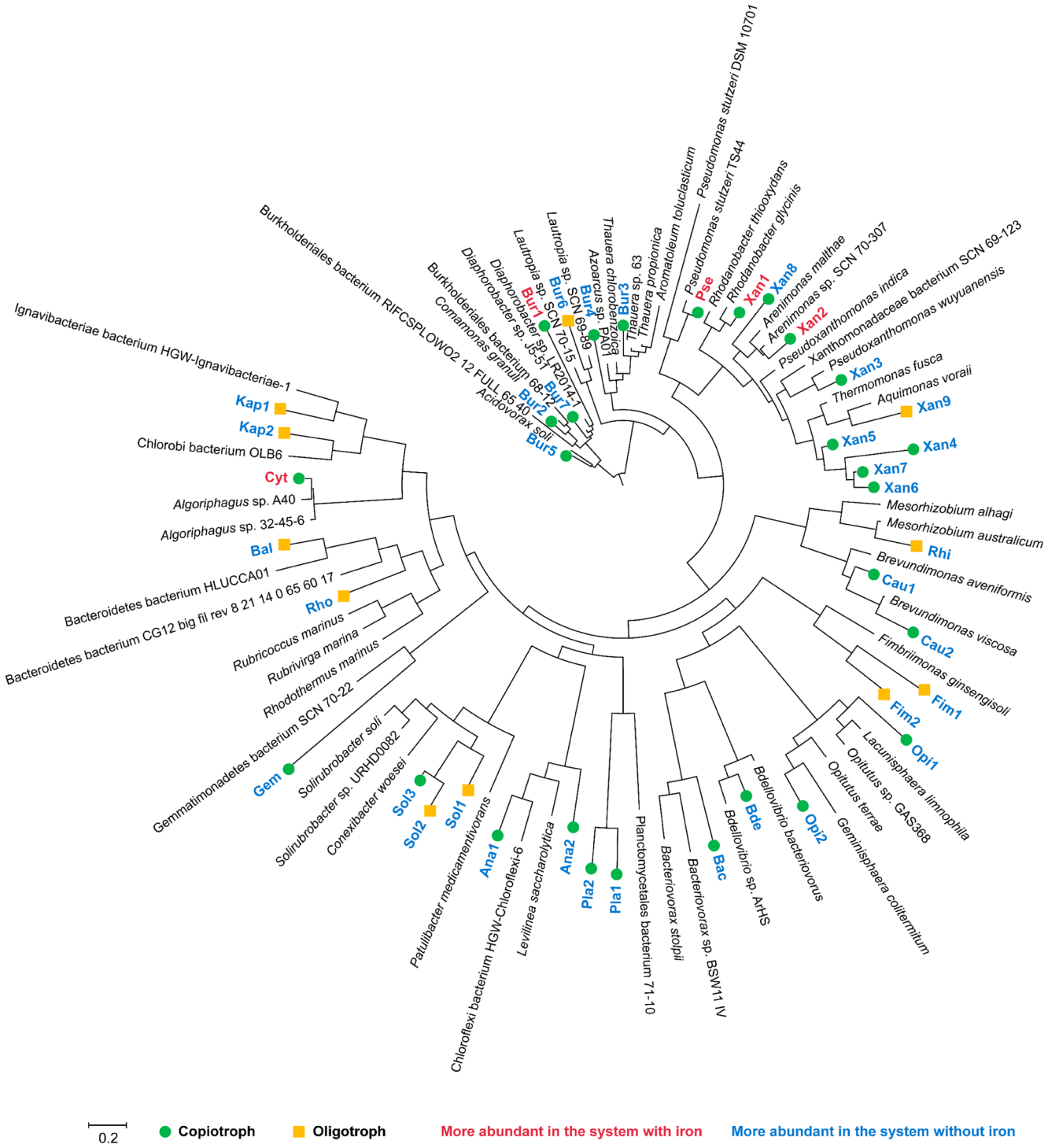

Fig. 4. Phylogenetic tree of the metagenome-assembled-genomes (MAGs) recovered from the incubations. 39 MAGs recovered from this study and 54 closely related genomes downloaded from the NCBI genome repository were included. Copiotrophs and oligotrophs evaluated by gRodon (Weissman et al., 2021) were indicated with different patterns. Populations more abundant in the systems with and without iron were indicated with different colors.

3.5. Syntrophic dependencies in the community

Microorganisms built up relationships with each other partly by the exchange of growth-required substances. This significantly influences the outcome of microbial community compositions (Zengler and Zaramela, 2018). The genomes of the 5 abundant populations all had a completeness higher than 95%, a contamination less than 4% and the highest coverage in the six samples higher than 10 (Table S2), indicating the opportunity to investigate their syntrophic relationship based on function annotations of genomes.

Genes involved in the biosynthesis of amino acids and vitamins which were essential for growth were included in the analyses (Table S7). Pse, the second most abundant organism in the community, was able to synthesize all kinds of amino acids and vitamins (Fig. 6a). In contrast, Bur1 lacked synthesis enzymes for aspartate (aspartate aminotransferase) and asparagine (asparagine synthase). Xan1 lacked

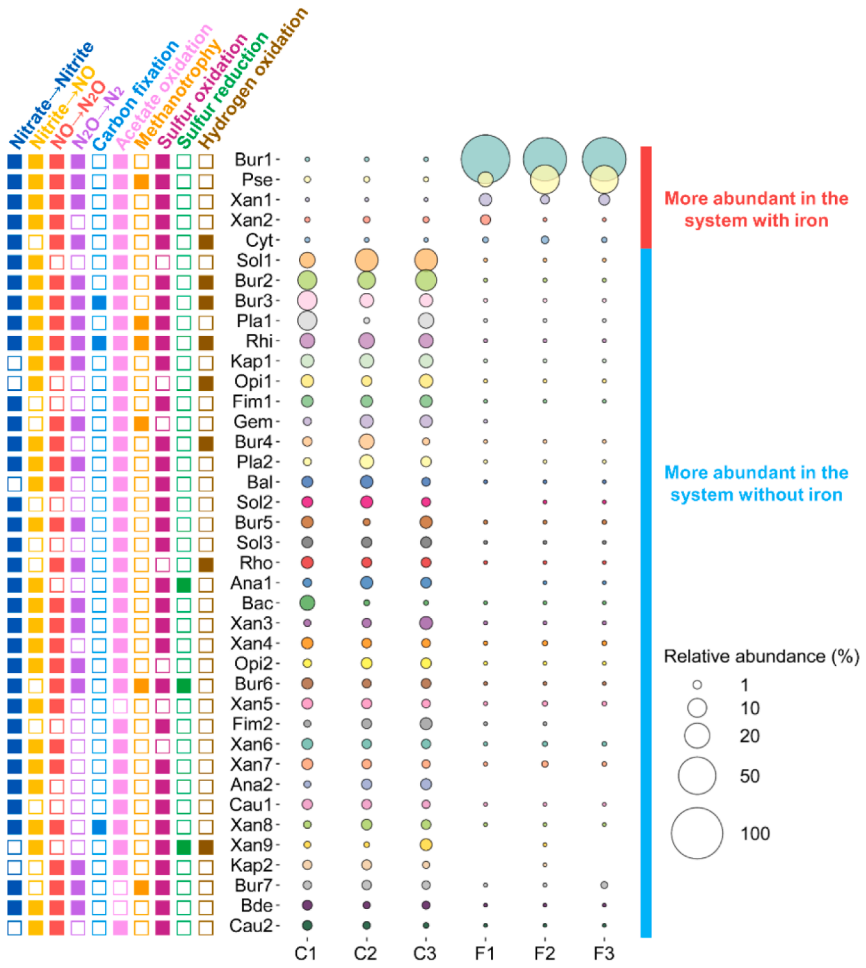

Fig. 5. Relative abundance and functional potentials of populations associated with MAGs.

synthesis enzymes for serine (phosphoserine phosphatase and phosphoserine/homoserine phosphotransferase) and cobalamin (adenosylcobinamide kinase, adenosylcobyric acid synthase, adenosylcobinamide-GDP ribazoletransferase and nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase). Xan2 and Cyt lacked even more genes. Apart from aspartate, asparagine, serine and cobalamin, Xan2 also lacked enzymes for leucine (isopropylmalate dehydrogenase), lysine (diaminopimelate decarboxylase, saccharopine dehydrogenase), niacin (ADP-ribosyl cyclase, NAD+ glycohydrolase, ADP-ribosyltransferase, NAD^+-dependent protein deacetylase, nicotinamide phosphoribosyltransferase, purine-nucleoside phosphorylase, purine nucleosidase and uridine nucleosidase) and folate (dihydrofolate reductase) biosynthesis. Cyt lost threonine synthase, biotin synthase, biotinidase and biotin/methionine sulfoxide reductase, which were needed for threonine and biotin synthesis. However, these amino acids and vitamins are essential for growth, so the lacking substances must be obtained by other means. For example, biosynthesis-related enzymes for asparagine were pretty abundant (more than 4 times of average abundance) in Xan1, which might satisfy the needs of Bur1 and Xan2. Requirements for serine and cobalamin by Xan1, Xan2 and Cyt might be solved through the transport of extracellular amino acids provided by Bur1 and Pse. This relied on the efficient translocation of amino acids and vitamins. Each of the five populations encoded at least four types of transporters of amino acids (Fig. 6b, Table S8). Xan1 and Cyt both lacked at least one type of vitamin, but we did not observe any vitamin transporter in their genomes, which might be due to a lack of known transporters in the database. In Bur1, Pse and Xan1, most of these genes were more abundant in the system with iron, demonstrating their metabolic activity was stimulated by iron addition.

Though several dependencies were identified in the system, it should be noted that the requirement of any organism could always be satisfied with one other organism in the community. Serine and cobalamin needed by Xan1 could be supplied by either Bur1 or Pse. This was the same for Bur1 - required asparagine and aspartate could be provided by either Xan1 or Pse. Similarly, the substances needed by Xan2 could all be supplied by Pse, whereas the requirement of Cyt could be satisfied with either Bur1 or Pse. Therefore, the cells could survive with the presence of cells from one population in the neighborhood and did not need cells from all other four populations. For example, a cell of Xan2 could survive as long as there was a cell of Pse around it (Dal Co et al., 2020). Whether Xan1, Bur1 and Cyt were present in its neighborhood did not influence Xan2′s growth. In general, the five populations were closely dependent on each other through the exchange of amino acids and vitamins, but the syntrophy could exist within two populations.

3.6. *Implications of this study*

The current study investigated iron-dependent denitrification as a remediation process for nitrogen-polluted freshwaters. A summary of biochemical pathways and microbial interactions among key populations in the iron-amended denitrifying system was proposed (Fig. 7). Our study provided important insights into this process from the following aspects.

Firstly, the performance of iron-dependent denitrification was

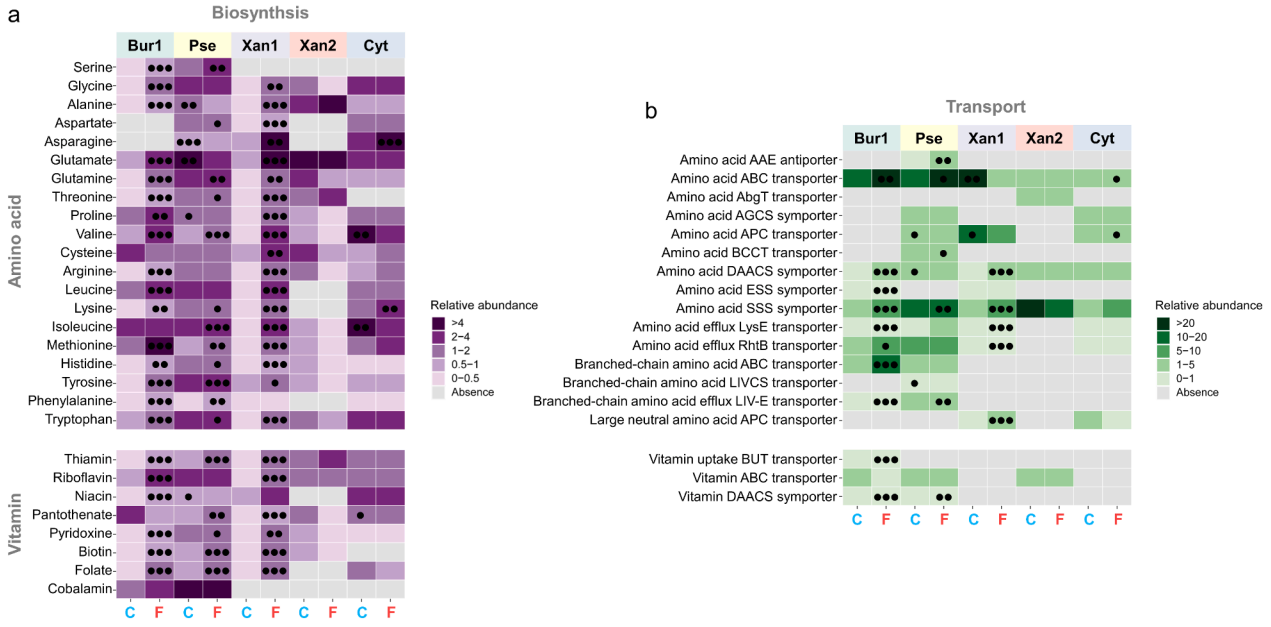

Fig. 6. Relative abundance of genes involved in (a) biosynthesis and (b) transport of amino acids and vitamins of the five most abundant populations in the system with iron. The relative abundance of each gene was normalized by the total abundance of all genes in a metagenome. The relative abundance of the genes between the two treatments (C and F) was compared by *t*-test. Solid circles in the cells indicate a significantly higher abundance in the treatment (*P* less than 0.05). One, two and three solid circles respectively represent *P* values less than 0.05, 0.01 and 0.001.

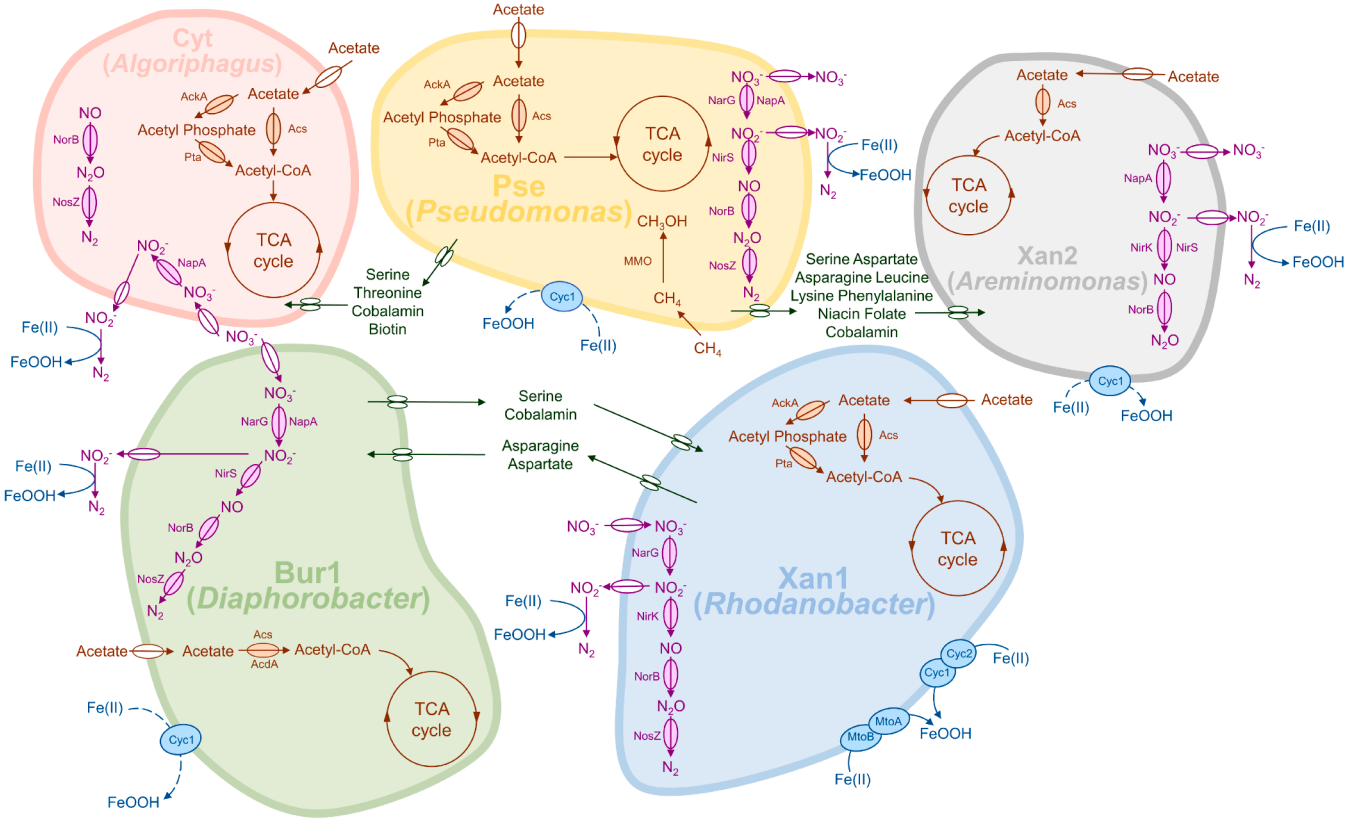

Fig. 7. Proposed functions and interactions of the five most abundant populations in the system with iron amendment.

evaluated in simulated systems of nitrate-polluted freshwaters for 54 days. Based on nutrient results, it performed well at the low C/N ratio and greatly improved nitrogen removal efficiency, suggesting the prospect of its application in freshwaters. The biotic reaction contributed as the largest part to nitrate removal during the process (Fig. 2), so the status of microorganisms is the core of the success of nitrogen

management. Five main organisms were found responsible for iron-dependent denitrification. Because poor energy could be gained from iron oxidation (Emerson et al., 2010), the five organisms were adopting a mixotrophic lifestyle - they coupled denitrification with both acetate and iron oxidation. Acetate was still a preferable electron donor for the microorganisms than iron(II), as acetate was mostly used up at the end of each experimental cycle but iron(II) remained to a certain amount (Fig. 1). This was consistent with a previous study which showed iron(II) acted as an auxiliary energy source when organic carbon was lacking (Chakraborty et al., 2011). The appropriate amount of iron(II) addition should be considered in the design of nitrogen removal technology, as the amount we added in this study extended beyond what microorganisms were able to consume (Fig. 1).

In addition, the study gave an understanding on the physiology of each enriched population with genome-centered analysis. The five main organisms were all fast-growing organisms (Fig. 4), which added to their compositions in the system with iron(II). Fast-growing organisms accounted for a higher proportion (about 38% more abundant) in the system with iron than the system without iron. This suggested mixotrophic iron-dependent denitrifiers were able to grow faster than some acetate-dependent denitrifiers in ecosystems. If the microbial community was considered as a whole, iron addition stimulated total microbial growth, which indirectly improved denitrification as more microorganisms were doing the job. Therefore, iron improved nitrogen removal not only by serving as an extra electron donor, but also by enriching more fast-growing organisms that were able to reduce nitrate more efficiently. Future research should use more accurate tools to analyze the growth of iron-dependent mixotrophic denitrifiers in an environmental setting (Nguyen et al., 2021).

Furthermore, microbial connections were analyzed with the presence and abundance of enzymes involved in the biosynthesis and transport of growth-required substances in each organism (Fig. 6). Some interdependencies among community members were demonstrated (Fig. 7). Bur1 (*Diaphorobacter*) was able to synthesize serine and cobalamin and supply them to Xan1 (*Rhodanobacter*) who could not generate them. On the flip side, Bur1 lacked asparagine and aspartate which Xan1 could provide. For Pse (*Pseudomonas*), it synthesized all kinds of amino acids and vitamins, so it could satisfy the need of Xan2 (*Areminomonas*) for some substances that Xan2 could not produce by itself. For Cyt (*Algoriphagus*), it needed serine, threonine, cobalamin and biotin, which could be provided by Bur1 or Pse. Therefore, different organisms supplied different amino acids and vitamins to others in the community. The division of metabolic labor could benefit individuals by reducing their energy burden (Pande et al., 2014). However, the more partners are involved in the transfer of amino acids and vitamins, the less reproductive success they would get (Dal Co et al., 2020). If there are more than three microorganisms participating in the cross-feeding, the relationship intensively limits microbial activity (Dal Co et al., 2020). In the iron-dependent denitrifying system, the need for growth-required substances in the five organisms could be satisfied with only one other community member (Fig. 7). Therefore, the syntrophic relationships increased the robustness of the community and meanwhile did not restrict microbial growth. The exchange of substances within the five organisms might explain the balance, stability and diversity of the community. Externally adding substances that could not be produced by some organisms, such as serine and cobalamin which was not self-sufficient with Xan1, could reduce the metabolic burden of Bur1 or Pse, and might promote overall nitrogen removal efficiency during remediation. Such a strategy should be evaluated further in engineering.

Taken together, the integrated analysis provided a holistic view of how iron(II) addition influenced denitrification performance in freshwaters, respectively by supplying electron donors, promoting microbial growth and building up syntrophic interactions with timely communications. These new observations would be crucial to predict and control microbial activity during iron-dependent denitrification. Future study could further examine optimized parameters for the operation of iron-dependent denitrification in real freshwater ecosystems.

4. Conclusions

Denitrifying systems with and without iron(II) addition were inoculated with freshwater samples and operated at a low C/N ratio. Microbially mediated reactions rather than abiotic reactions dominated iron-based denitrification and improved nitrogen removal performance. The five most abundant microbial populations were recognized in the system with iron(II). They could gain energy from both acetate and iron oxidation. All of them were fast-growing organisms in ecosystems, ensuring the capability of efficient denitrification. Particularly, they cooperated with each other by exchanging amino acids and vitamins to lower the energy burden of individuals. *Rhodanobacter* lost enzymes for synthesizing serine and cobalamin, which could be provided by *Diaphorobacter*. On the flip side, *Diaphorobacter* could not synthesize asparagine and aspartate and required them from *Rhodanobacter*. Notably, the syntrophic relationship only existed in two partners, which decreased reproduction limitation brought by spatial dependencies. The results added new and fundamental insights to iron-dependent denitrification and had implications for nitrogen management in freshwaters.

CRediT authorship contribution statement

Shengjie Li: Investigation, Conceptualization, Formal analysis, Writing – original draft, Visualization. **Muhe Diao:** Formal analysis, Writing – review & editing. **Yinhao Liao:** Methodology. **Guodong Ji:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

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

Data availability

All sequences of this study are available at NCBI (PRJNA848245). The Biosamples for the 16S rRNA sequences are SAMN28990678 - SAMN28990719. The Biosamples for the MAGs are SAMN28990789 - SAMN28990828. The Biosamples for the metagenome raw reads are SAMN28990741 - SAMN28990746.

Acknowledgements

We would like to thank Yuefei Ding and Shan Huang for help with iron characterization. This research was supported by the Key Project of the Joint Funds of the National Natural Science Foundation of China (No.U22A20557) and the Foundation for Innovative Research Groups of the National Natural Science Foundation of China (No.51721006). Sequence analysis was supported by High-performance Computing Platform of Peking University.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.108124.

References

Barco, R.A., Emerson, D., Sylvan, J.B., Orcutt, B.N., Jacobson Meyers, M.E., Ramírez, G. A., Zhong, J.D., Edwards, K.J., Voordouw, G., 2015. New insight into microbial iron oxidation as revealed by the proteomic profile of an obligate iron-oxidizing chemolithoautotroph. Appl. Environ. Microbiol. 81 (17), 5927–5937.

Brown, C.T., Olm, M.R., Thomas, B.C., Banfield, J.F., 2016. Measurement of bacterial replication rates in microbial communities. Nat. Biotechnol. 34 (12), 1256–1263. https://doi.org/10.1038/nbt.3704.

Bryce, C., Blackwell, N., Schmidt, C., Otte, J., Huang, Y.M., Kleindienst, S., Tomaszewski, E., Schad, M., Warter, V., Peng, C., Byrne, J.M., Kappler, A., 2018. Microbial anaerobic Fe(II) oxidation - Ecology, mechanisms and environmental implications. Environ. Microbiol. 20 (10), 3462–3483. https://doi.org/10.1111/1462-2920.14328.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13 (7), 581–583. https://doi.org/10.1038/nmeth.3869.

Carlson, H.K., Clark, I.C., Blazewicz, S.J., Iavarone, A.T., Coates, J.D., 2013. Fe(II) oxidation is an innate capability of nitrate-reducing bacteria that involves abiotic and biotic reactions. J. Bacteriol. 195 (14), 3260–3268. https://doi.org/10.1128/JB.00058-13.

Chakraborty, A., Roden, E.E., Schieber, J., Picardal, F., 2011. Enhanced growth of *Acidovorax* sp. strain 2AN during nitrate-dependent Fe(II) oxidation in batch and continuous-flow systems. Appl. Environ. Microbiol. 77 (24), 8548–8556. https://doi.org/10.1128/AEM.06214-11.

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34(17), i884-i890. 10.1093/bioinformatics/bty560.

Dal Co, A., van Vliet, S., Kiviet, D.J., Schlegel, S., Ackermann, M., 2020. Short-range interactions govern the dynamics and functions of microbial communities. Nat. Ecol. Evol. 4 (3), 366–375. https://doi.org/10.1038/s41559-019-1080-2.

Darling, A.E., Jospin, G., Lowe, E., Matsen, F.A., Bik, H.M., Eisen, J.A., 2014. PhyloSift: phylogenetic analysis of genomes and metagenomes. PeerJ 2, e243.

Deng, S., Peng, S., Ngo, H.H., Oh, S.-A., Hu, Z., Yao, H., Li, D., 2022. Characterization of nitrous oxide and nitrite accumulation during iron (Fe(0))- and ferrous iron (Fe(II))-driven autotrophic denitrification: mechanisms, environmental impact factors and molecular microbial characterization. Chem. Eng. J. 438, 135627.

Di Capua, F., Pirozzi, F., Lens, P.N.L., Esposito, G., 2019. Electron donors for autotrophic denitrification. Chem. Eng. J. 362, 922–937. https://doi.org/10.1016/j.cej.2019.01.069.

D'Souza, G., Waschina, S., Pande, S., Bohl, K., Kaleta, C., Kost, C., 2014. Less is more: selective advantages can explain the prevalent loss of biosynthetic genes in bacteria. Evolution 68 (9), 2559–2570. https://doi.org/10.1111/evo.12468.

Eddy, S.R., Rost, B., 2008. A probabilistic model of local sequence alignment that simplifies statistical significance estimation. PLoS Comput. Biol. 4 (5), e1000069.

Embree, M., Liu, J.K., Al-Bassam, M.M., Zengler, K., 2015. Networks of energetic and metabolic interactions define dynamics in microbial communities. PNAS 112 (50), 15450–15455. https://doi.org/10.1073/pnas.1506034112.

Emerson, D., Fleming, E.J., McBeth, J.M., 2010. Iron-oxidizing bacteria: an environmental and genomic perspective. Annu. Rev. Microbiol. 64, 561–583. https://doi.org/10.1146/annurev.micro.112408.134208.

Faust, K., Raes, J., 2012. Microbial interactions: from networks to models. Nat. Rev. Microbiol. 10 (8), 538–550. https://doi.org/10.1038/nrmicro2832.

Garber, A.I., Nealson, K.H., Okamoto, A., McAllister, S.M., Chan, C.S., Barco, R.A., Merino, N., 2020. FeGenie: A comprehensive tool for the identification of iron genes and iron gene neighborhoods in genome and metagenome assemblies. Front. Microbiol. 11, 37. https://doi.org/10.3389/fmicb.2020.00037.

Garrido-Amador, P., Kniaziuk, M., Vekeman, B., Kartal, B., 2021. Learning from microorganisms: using new insights in microbial physiology for sustainable nitrogen management. Curr. Opin. Biotechnol. 67, 42–48. https://doi.org/10.1016/j.copbio.2020.12.017.

Hu, M., Chen, P., Sun, W., Li, F., Cui, J., 2017. A novel organotrophic nitrate-reducing Fe (II)-oxidizing bacterium isolated from paddy soil and draft genome sequencing indicate its metabolic versatility. RSC Adv. 7 (89), 56611–56620. https://doi.org/10.1039/c7ra09328d.

Jewell, T.N., Karaoz, U., Brodie, E.L., Williams, K.H., Beller, H.R., 2016. Metatranscriptomic evidence of pervasive and diverse chemolithoautotrophy relevant to C, S, N and Fe cycling in a shallow alluvial aquifer. ISME J. 10 (9), 2106–2117. https://doi.org/10.1038/ismej.2016.25.

Johnson, D.R., Goldschmidt, F., Lilja, E.E., Ackermann, M., 2012. Metabolic specialization and the assembly of microbial communities. ISME J. 6 (11), 1985–1991. https://doi.org/10.1038/ismej.2012.46.

Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., Wang, Z., 2019. MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. PeerJ 7, e7359.

Kato, S., Ohkuma, M., Powell, D.H., Krepski, S.T., Oshima, K., Hattori, M., Shapiro, N., Woyke, T., Chan, C.S., 2015. Comparative genomic insights into ecophysiology of neutrophilic, microaerophilic iron oxidizing bacteria. Front. Microbiol. 6, 1265. https://doi.org/10.3389/fmicb.2015.01265.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30 (14), 3059–3066. https://doi.org/10.1093/nar/gkf436.

Kiskira, K., Papirio, S., van Hullebusch, E.D., Esposito, G., 2017. Fe(II)-mediated autotrophic denitrification: A new bioprocess for iron bioprecipitation/biorecovery and simultaneous treatment of nitrate-containing wastewaters. Int. Biodeter. Biodegr. 119, 631–648. https://doi.org/10.1016/j.ibiod.2016.09.020.

Li, D., Liu, C.M., Luo, R., Sadakane, K., Lam, T.W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 31(10), 1674-1676. 10.1093/bioinformatics/btv033.

Li, S., Pang, Y., Ji, G., 2021. Increase of N_2O production during nitrate reduction after long-term sulfide addition in lake sediment microcosms. Environ. Pollut. 291, 118231 https://doi.org/10.1016/j.envpol.2021.118231.

Li, S., Jiang, Z., Ji, G., 2022a. Effect of sulfur sources on the competition between denitrification and DNRA. Environ. Pollut. 305, 119322 https://doi.org/10.1016/j.envpol.2022.119322.

Li, S., Liao, Y., Pang, Y., Dong, X., Strous, M., Ji, G., 2022b. Denitrification and dissimilatory nitrate reduction to ammonia in long-term lake sediment microcosms with iron(II). Sci. Total Environ. 807 (1), 150835 https://doi.org/10.1016/j.scitotenv.2021.150835.

Li, S., Wang, S., Pang, Y., Ji, G., 2022d. Influence of electron donors (Fe, C, S) on N_2O production during nitrate reduction in lake sediments: Evidence from isotopes and functional genes. ACS ES&T Water 2 (7), 1254–1264. https://doi.org/10.1021/acsestwater.2c00104.

Liu, J., Wang, Z., Belchik, S.M., Edwards, M.J., Liu, C., Kennedy, D.W., Merkley, E.D., Lipton, M.S., Butt, J.N., Richardson, D.J., Zachara, J.M., Fredrickson, J.K., Rosso, K. M., Shi, L., 2012. Identification and characterization of MtoA: A decaheme c-type cytochrome of the neutrophilic Fe(II)-oxidizing bacterium *Sideroxydans lithotrophicus* ES-1. Front. Microbiol. 3, 37. https://doi.org/10.3389/fmicb.2012.00037.

McCullough, H., 1967. The determination of ammonia in whole blood by a direct colorimetric method. Clin. Chim. Acta 17 (2), 297–304. https://doi.org/10.1016/0009-8981(67)90133-7.

Mee, M.T., Collins, J.J., Church, G.M., Wang, H.H., 2014. Syntrophic exchange in synthetic microbial communities. PNAS 111 (20), E2149–E2156. https://doi.org/10.1073/pnas.1405641111.

Melton, E.D., Swanner, E.D., Behrens, S., Schmidt, C., Kappler, A., 2014. The interplay of microbially mediated and abiotic reactions in the biogeochemical Fe cycle. Nat. Rev. Microbiol. 12 (12), 797–808. https://doi.org/10.1038/nrmicro3347.

Michiels, C.C., Darchambeau, F., Roland, F.A.E., Morana, C., Llirós, M., García-Armisen, T., Thamdrup, B., Borges, A.V., Canfield, D.E., Servais, P., Descy, J.-P., Crowe, S.A., 2017. Iron-dependent nitrogen cycling in a ferruginous lake and the nutrient status of Proterozoic oceans. Nat. Geosci. 10 (3), 217–221. https://doi.org/10.1038/ngeo2886.

Muehe, E.M., Gerhardt, S., Schink, B., Kappler, A., 2009. Ecophysiology and the energetic benefit of mixotrophic Fe(II) oxidation by various strains of nitrate-reducing bacteria. FEMS Microbiol. Ecol. 70 (3), 335–343. https://doi.org/10.1111/j.1574-6941.2009.00755.x.

Newcomer, T.A., Kaushal, S.S., Mayer, P.M., Shields, A.R., Canuel, E.A., Groffman, P.M., Gold, A.J., 2012. Influence of natural and novel organic carbon sources on denitrification in forest, degraded urban, and restored streams. Ecol. Monogr. 82 (4), 449–466. https://doi.org/10.1890/12-0458.1.

Nguyen, J., Fernandez, V., Pontrelli, S., Sauer, U., Ackermann, M., Stocker, R., 2021. A distinct growth physiology enhances bacterial growth under rapid nutrient fluctuations. Nat. Commun. 12 (1), 3662. https://doi.org/10.1038/s41467-021-23439-8.

Nicolle, J.L.C., Simmons, S., Bathe, S., Norris, P.R., 2009. Ferrous iron oxidation and rusticyanin in halotolerant, acidophilic *'Thiobacillus prosperus'*. Microbiology 155 (4), 1302–1309. https://doi.org/10.1099/mic.0.023192-0.

Pande, S., Merker, H., Bohl, K., Reichelt, M., Schuster, S., de Figueiredo, L.F., Kaleta, C., Kost, C., 2014. Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria. ISME J. 8 (5), 953–962. https://doi.org/10.1038/ismej.2013.211.

Pang, Y.M., Wang, J.L., Li, S.J., Ji, G.D., 2021. Activity of autotrophic Fe(II)-oxidizing denitrifiers in freshwater lake sediments. ACS ES&T Water 1 (7), 1566–1576. https://doi.org/10.1021/acsestwater.1c00075.

Picardal, F., 2012. Abiotic and microbial interactions during anaerobic transformations of Fe(II) and NOx-. Front. Microbiol. 3, 112. https://doi.org/10.3389/fmicb.2012.00112.

Proulx, S.R., Promislow, D.E., Phillips, P.C., 2005. Network thinking in ecology and evolution. Trends Ecol. Evol. 20 (6), 345–353. https://doi.org/10.1016/j.tree.2005.04.004.

Qiu, Y.Y., Zhang, L., Mu, X., Li, G., Guan, X., Hong, J., Jiang, F., 2020. Overlooked pathways of denitrification in a sulfur-based denitrification system with organic supplementation. Water Res. 169, 115084 https://doi.org/10.1016/j.watres.2019.115084.

Roger, M., Castelle, C., Guiral, M., Infossi, P., Lojou, E., Giudici-Orticoni, M.T., Ilbert, M., 2012. Mineral respiration under extreme acidic conditions: from a supramolecular organization to a molecular adaptation in Acidithiobacillus ferrooxidans. Biochem. Soc. Trans. 40 (6), 1324–1329. https://doi.org/10.1042/BST20120141.

Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30 (14), 2068-2069. 10.1093/bioinformatics/btu153.

Seitzinger, S., 2008. Nitrogen cycle: out of reach. Nature 452 (7184), 162–163. https://doi.org/10.1038/452162a.

Singer, E., Emerson, D., Webb, E.A., Barco, R.A., Kuenen, J.G., Nelson, W.C., Chan, C.S., Comolli, L.R., Ferriera, S., Johnson, J., Heidelberg, J.F., Edwards, K.J., Khodursky, A.B., 2011. *Mariprofundus ferrooxydans* PV-1 the first genome of a marine Fe(II) oxidizing Zetaproteobacterium. PLoS One 6 (9), e25386.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9), 1312-1313. 10.1093/bioinformatics/btu033.

Straub, K.L., Benz, M., Schink, B., Widdel, F., 1996. Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. Appl. Environ. Microbiol. 4 (62), 1458–1460. https://doi.org/10.1016/0921-8777(95)00057-7.

Tian, T., Zhou, K., Xuan, L., Zhang, J.X., Li, Y.S., Liu, D.F., Yu, H.Q., 2020. Exclusive microbially driven autotrophic iron-dependent denitrification in a reactor inoculated with activated sludge. Water Res. 170, 115300 https://doi.org/10.1016/j.watres.2019.115300.

Valdes, J., Pedroso, I., Quatrini, R., Dodson, R.J., Tettelin, H., Blake 2nd, R., Eisen, J.A., Holmes, D.S., 2008. *Acidithiobacillus ferrooxidans* metabolism: from genome

sequence to industrial applications. BMC Genomics 9, 597. https://doi.org/10.1186/1471-2164-9-597.

Wang, Y., Niu, Q., Zhang, X., Liu, L., Wang, Y., Chen, Y., Negi, M., Figeys, D., Li, Y.Y., Zhang, T., 2019. Exploring the effects of operational mode and microbial interactions on bacterial community assembly in a one-stage partial-nitritation anammox reactor using integrated multi-omics. Microbiome 7 (1), 122. https://doi.org/10.1186/s40168-019-0730-6.

Weber, K.A., Achenbach, L.A., Coates, J.D., 2006. Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. Nat. Rev. Microbiol. 4 (10), 752–764. https://doi.org/10.1038/nrmicro1490.

Weissman, J.L., Hou, S., Fuhrman, J.A., 2021. Estimating maximal microbial growth rates from cultures, metagenomes, and single cells via codon usage patterns. PNAS 118 (12). https://doi.org/10.1073/pnas.2016810118.

Zengler, K., Zaramela, L.S., 2018. The social network of microorganisms - how auxotrophies shape complex communities. Nat. Rev. Microbiol. 16 (6), 383–390. https://doi.org/10.1038/s41579-018-0004-5.

Zhou, Z., Tran, P.Q., Breister, A.M., Liu, Y., Kieft, K., Cowley, E.S., Karaoz, U., Anantharaman, K., 2022. METABOLIC: high-throughput profiling of microbial genomes for functional traits, metabolism, biogeochemistry, and community-scale functional networks. Microbiome 10 (1), 33. https://doi.org/10.1186/s40168-021-01213-8.

Zhu, T.T., Cheng, H.Y., Yang, L.H., Su, S.G., Wang, H.C., Wang, S.S., Wang, A.J., 2019. Coupled sulfur and iron(II) carbonate-driven autotrophic denitrification for significantly enhanced nitrate removal. Environ. Sci. Tech. 53 (3), 1545–1554. https://doi.org/10.1021/acs.est.8b06865.