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Response of Fe(III)-reducing kinetics, microbial community structure and Fe(III)-related functional genes to Fe(III)-organic matter complexes and ferrihydrite in lake sediment

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Abstract Microbial Fe(III) reduction significantly influences the fate of various elements and contaminants. Previous research has employed different Fe(III)-OM complexes and ferrihydrite to study Fe(III)-reduction-related biogeochemistry processes. However, the effects of adding specific Fe(III)-OM complexes and ferrihydrite on the Fe(III)-reducing bacterial community, Fe(III)-reducing kinetics, and Fe(III)-related functional genes remain largely unexplored. This study applied microcosm experiments and metagenomic analysis of lake sediments with and without amendments of ferrihydrite, Fe(III)-citrate, or Fe(III)-EDTA. Results showed that sediments amended with Fe(III)-citrate and Fe(III)-EDTA exhibited faster Fe(III) reduction rates and more significant changes in bacterial community structures compared to those amended with ferrihydrite. *Geobacter* and *Clostridium* were enriched in the sediments amended with Fe(III)-EDTA and Fe(III)-citrate, respectively. Despite a slower reduction rate and lack of enrichment of specific Fe(III)-reducing bacteria, ferrihydrite still led to an increase in the copy numbers of genes related to Fe(III) reduction and iron assimilation in the metagenomes, suggesting an increase in these capacities. These results

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suggest that introducing various Fe(III)-OM complexes and ferrihydrite into the environment would result in differences in not only Fe(III) reduction rates and Fe(III)-reducing bacterial communities but also in iron-related functional genes. Meanwhile, variations in Fe(III) reduction rates and Fe(III)-reducing bacterial communities do not necessarily correlate with changes in the abundances of functional genes relevant to Fe(III) reduction and iron assimilation in the metagenomes. These results provide a better understanding of the adaptive mechanisms of Fe(III)-reducing bacteria in different environmental systems.

Keywords Fe(III) reduction · Ferrihydrite · Fe(III)-OM · Metagenomic · Microcosm

Introduction

Iron (Fe) is widely distributed across aquatic and terrestrial ecosystems (Raiswell and Canfield 2012). Fe(III) reduction not only impacts the migration and transformation of various environmental pollutants but is also associated with the biogeochemical cycling of multiple elements, including carbon, nitrogen, and phosphorus, under natural conditions (Hall et al. 2018; Kappler et al. 2021) and plays a significant role in controlling processes such as global warming, particularly through its impact on carbon cycling and the modulation of greenhouse gas emissions (Beal et al. 2009; Laufer-Meiser et al. 2021; Wang et al. 2022).

Under anoxic conditions, Fe(III) can be reduced to Fe(II) by Fe(III)-reducing bacteria. Among them, dissimilatory Fe(III)-reducing bacteria utilize Fe(III) as an electron acceptor during their energy metabolism. These Fe(III)-reducing bacteria are widely distributed across diverse genera and have been isolated from various organic-rich environments, including rivers, lakes, and wetlands (Lovley and Holmes 2021). To reduce solid Fe(III) minerals, dissimilatory Fe(III)-reducing bacteria have developed several different mechanisms (Shi et al. 2016). Some Fe(III)-reducing bacteria within the same genus may share a common type of mechanism (Shi et al. 2012), while some bacteria that utilize similar Fe(III)-reducing systems may employ different genes to reduce Fe(III) (Baker et al. 2022; Conley et al. 2018). Among these different genes, some are particularly important for the reduction of solid-phase Fe(III), for example, genes that encode outer membrane c-type cytochromes, while they appear less important for reducing dissolved Fe(III)-OM complexes such as Fe(III)-citrate (Aklujkar et al. 2013; Leang et al. 2005; Richter et al. 2012).

In addition to the microbes themselves, the organic matter present in the environment can also significantly influence the microbial Fe(III) reduction process (Dong et al. 2023). One class of organic compounds influencing Fe(III) reduction is those capable of forming dissolved Fe(III)-OM complexes. Such dissolved Fe(III)-OM complexes are abundant in environments rich in organic matter, including rivers, lakes, and wetlands (Lofts et al. 2008; Sundman et al. 2014). Previous studies utilizing bacterial pure cultures have demonstrated that probably due to their higher solubility and bioavailability, the reduction rates of dissolved Fe(III)-OM complexes are significantly higher than those observed for solid Fe(III) minerals (Bridge and Barrie Johnson 2000; Conley et al. 2018; Haas and Dichristina 2002; Vecchia et al. 2014). Microbial Fe(III) reduction rates also varied among different Fe(III)-OM complexes (Wang et al. 2008). In addition, the phenotypes of Fe(III)-reducing bacteria were also shown to be associated with the source of Fe(III), whether it was composed of Fe(III) minerals or Fe(III)-OM complexes. For example, the flagella and pili of *Geobacter metallireducens* are not expressed when cultured with Fe(III)-citrate (Lovley and Walker 2019). The *Acidimicrobiaceae* sp. A6 strain only performs anaerobic ammonia oxidation in the presence of solid Fe(III) minerals as an Fe(III) source but not with Fe(III)-citrate (Huang and Jaffé 2018).

Various dissolved Fe(III)-OM complexes are also widely used as substrates for the analysis of other processes related to Fe(III) reduction, especially anaerobic methane oxidation (AOM) (Ettwig et al. 2016; Hori et al. 2015; Scheller et al. 2016). Many widely studied Fe(III)-reducing bacteria belonging to the genus *Geobacter* were also isolated using dissolved Fe(III)-OM complexes instead of solid Fe(III) minerals (Coates et al. 1996). Furthermore, even though the concentration of dissolved Fe-OM may not be very high in some environments, the Fe(III)-OM that is reduced to Fe(II)-OM can be rapidly reoxidized to Fe(III)-OM by Fe(II)-oxidizing microbes, forming a cryptic iron cycle (Kügler et al. 2019; Peng et al. 2019). As a result, the

contribution of Fe(III)-OM to microbial Fe(III) reduction might still be significant (Dong et al. 2023).

In addition to different Fe(III)-OM complexes which show different reduction rates by the same Fe(III)-reducing enzymatic systems (Wang et al. 2008), different Fe(III)-reducing bacteria may also have different Fe(III)-reducing mechanisms (Lovley and Holmes 2021). These results suggest that different Fe(III)-reducing bacteria may respond differently to the presence of different Fe(III)-OM complexes. However, much less attention has been given to these effects in environmental samples such as lake sediments. Such environmental systems are much more complex and contain a variety of organic compounds, minerals, and different Fe(III)-reducing bacteria. Previous studies on the impact of Fe(III)-OM complexes in the environment have focused mainly on the effect of a single type of Fe(III)-OM on the community structure of Fe(III)-reducing bacteria (He et al. 2023; Wang et al. 2009). However, not all Fe(III)-reducing microbes within the same genus have the same genes for Fe(III) reduction (Baker et al. 2022); therefore, variations in microbial community structure and Fe(III)-reducing capacity may not be consistent.

To understand the influence of adding different Fe(III)-OM complexes and ferrihydrite on the community structure, kinetics, and genes relevant to microbial Fe(III) reduction in environments, we performed microcosm experiments using lake sediments. We compared the Fe(III) reduction kinetics, bacterial structure, and iron-related genes in the metagenomes of sediments without any Fe(III) addition or amended with either one of the Fe(III)-OM complexes (Fe(III)-citrate, Fe(III)-EDTA) or ferrihydrite. The objective of this study was to answer the following questions: (i) do variations in Fe(III) source lead to differential impacts on Fe(III)-reducing bacterial community composition and iron-related genes, and (ii) which Fe(III) source—ferrihydrite minerals or Fe(III)-OM complexes—is more effective in enriching certain genera of Fe(III)-reducing bacteria with specific genes for Fe(III) reduction?

Materials and methods

Sediment and water sampling

Sediment and lake water samples were collected in April 2023 from a lake within the campus of China West Normal University in Nanchong city, Sichuan Province, China (25°48′ N, 113°02′ E). The sediment samples were placed in sterile polyethylene bags, sealed, and stored within ice blocks, and the lake water samples were stored in polyethylene bottles. All the samples were quickly transported to the laboratory and stored at 4 °C. To remove oxygen, the water samples were first centrifuged (3020 g, 5 min), then filtered (0.22 μm), and finally purged with N_2 for 30 min.

Synthesis of ferrihydrite and Fe(III)-OM

Ferrihydrite was synthesized using $Fe(NO_3)_3{\cdot}9H_2O$ and KOH following a previously described protocol (Schwertmann and Cornell 2008). After synthesis, the ferrihydrite was suspended in anoxic H_2O in a glovebox (100% N_2). The ferrihydrite suspension was used for experiments within 4 weeks after its synthesis. An Fe(III)-EDTA stock solution (250 mM) was synthesized using $FeCl_3{\cdot}6H_2O$ and Na_2-EDTA, and the pH was adjusted to 7.0 with NaOH. An Fe(III)-citrate stock solution (500 mM) was prepared by dissolving the Fe(III)-citrate salt and adjusting the pH to 7.0 with NaOH. After synthesis, these solutions were boiled and cooled under N_2 to remove oxygen.

Setup of the microcosm experiment

To set up the microcosm experiments, 25 g of lake sediment and 25 mL of lake water sample were mixed in 100 mL serum bottles. After flushing the headspace with N_2 for 1 min, the serum bottles were sealed with butyl rubber stoppers and aluminum caps and then wrapped with aluminum foil.

Resazurin (final concentration: 5 mg/L) was added to three control serum bottles with sediment as an oxygen indicator. All sediment microcosms were preincubated at 25 °C in the dark for 8 days until the oxygen indicator shifted from pink to clear (indicating anoxic conditions) and the Fe(II) concentrations stabilized (Figs. S1–S3).

To avoid the lack of electron donors due to precultivation, 2 mM sodium acetate was added to all the serum bottles because it can be used for microbial Fe(III) respiration but not fermentation (Zhang et al. 2020). Afterwards, 20 mM Fe(III)-citrate, 20 mM Fe(III)-EDTA, or 20 mM ferrihydrite was added to individual serum bottles in triplicate for

each experimental setup and incubated at 25 °C in the dark. In total, there were 4 microcosm conditions: (1) sediment without Fe(III) addition, (2) sediment amended with ferrihydrite, (3) sediment amended with Fe(III)-citrate, and (4) sediment amended with Fe(III)-EDTA.

Sampling

Sediment slurry samples were collected during the incubation. For Fe(III)-citrate and Fe(III)-EDTA samples, we sampled every 1–2 days, while for the slower-reducing ferrihydrite samples, we sampled every 4–6 days. Prior to sampling, the soil slurry was homogenized by hand shaking. Each time, 2 mL of the soil slurry was removed using sterile syringes flushed with 100% N_2. Immediately after sampling, 100 µL of the collected samples were transferred to another Eppendorf tube and diluted with 1 M HCl for the extraction of Fe(II). The remaining soil slurries were centrifuged at 16,000 × g for 3 min. The pellets were stored at – 20 °C for subsequent DNA extraction.

Determination of Fe(III) reduction rates

HCl extracted sediment slurry samples for quantification of Fe(II) were kept in the dark for 12 h for Fe(II) leaching. Afterwards, the samples were centrifuged (12,000 g, 3 min), and the supernatant was used to quantify the concentration of HCl-extractable Fe(II) using the ferrozine assay (Gibbs 1976). The absorbance of the purple ferrozine-Fe(II) complex was quantified at 562 nm using a microtiter plate reader (Multiskan Go, Thermo Scientific). The reduction rates of Fe(III) were calculated by linear regression analysis of the Fe(II) concentrations.

DNA extraction and metagenomic sequencing

DNA was extracted for metagenomic sequencing from the samples at the time point when Fe(III) reduction reached equilibrium, i.e. no longer changed rapidly over time. For experiments with Fe(III)-citrate and Fe(III)-EDTA, samples were taken on day 4, following an 8-day preincubation and the introduction of Fe(III), and for the remaining experiments, samples were taken on day 59.

DNA was extracted from 0.5 g sediment samples using the Fast DNA spin kit for soil (MP Biomedicals, USA) according to the manufacturer's instructions. Purity and quantity were evaluated using a NanoDrop 2000 spectrophotometer. The quantified DNA (concentration > 136 ng/µL, mass > 9.5 µg) from triplicate samples was pooled and used for library construction and then sequenced by Majorbio Biopharm Technology Co., Ltd. (Shanghai, China) using the Illumina NovaSeq 6000 platform. Approximately 6 Gb of raw data were generated per sample, which were then quality controlled using fastp software (Chen et al. 2018) by removing low-quality reads (length < 50 bp or with a quality value < 20 or having N bases). Sequence data associated with this project have been deposited in the NCBI with accession number PRJNA1090275.

Sequencing data analysis and bioinformatics

The quality-controlled reads of each sample were further assembled to collect the contigs using MEGAHIT software (Li et al. 2015). Prodigal software was used to predict open read frames (ORFs) (Hyatt et al. 2010). A nonredundant gene catalog was constructed using CD-HIT (Fu et al. 2012) with 90% sequence identity and 90% coverage. High-quality reads were aligned to nonredundant gene catalogs to calculate gene abundance with 95% identity using SOAPaligner (Li et al. 2008). Representative sequences of the nonredundant gene catalog were aligned to the NR database with an e-value cutoff of $1e^{-5}$ using Diamond (Buchfink et al. 2015) for taxonomic annotations.

Previously reported genera of Fe(III)-reducing bacteria were selected to assess the impact of various enrichment conditions on the composition of the Fe(III)-reducing bacterial community (Fan et al. 2018; Ionescu et al. 2015; Lentini et al. 2012; Lovley and Holmes 2021; Yang et al. 2021). Cluster analysis was conducted based on the relative abundance of Fe(III)-reducing bacteria across 20 genera or gene quantities associated with iron assimilation processes in sediment under distinct culture conditions. A heatmap was generated using https://www.bioinformatics.com.cn (last accessed on February 20, 2024), an online platform dedicated to data analysis and visualization. To compare genes relevant to Fe(III) reduction and iron assimilation, FeGenie (Garber et al. 2020) was utilized. Genomes of strains belonging to the

genus *Clostridium* were downloaded from the Joint Genome Institute (JGI) and analyzed using FeGenie to test for the presence of genes encoding extracellular cytochrome c proteins in this genus.

Results

Rates and extent of Fe(III) reduction

Based on the changes in Fe(II) concentration over time, we found that the amount of Fe(III) added to the sediment decreased under all conditions; however, the rates of Fe(III) reduction varied across the different treatments (Fig. 1). First, in sediment amended with acetate only, there was only negligible Fe(III) reduction, with rates below 0.02 mmol L^{-1} day^{-1}. However, in sediment amended with acetate plus ferrihydrite, Fe(III)-EDTA, or Fe(III)-citrate, the Fe(III) reduction rates reached 0.27 mmol L^{-1} day^{-1}, 1.15 mmol L^{-1} day^{-1}, and 5.43 mmol L^{-1} day^{-1}, respectively. The reduction rates of Fe(III)-citrate and Fe(III)-EDTA were 20 and 4 times greater than that of ferrihydrite, respectively.

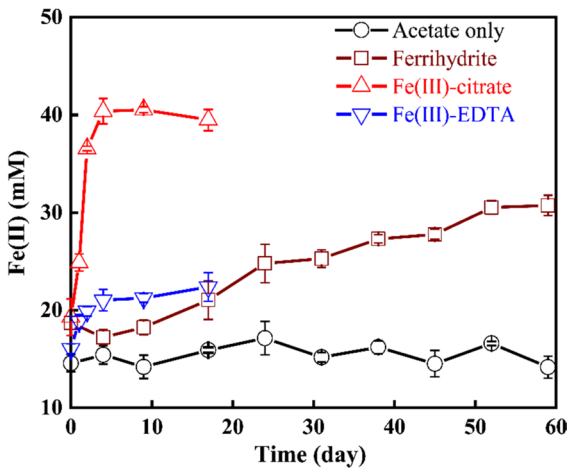

Fig. 1 Fe(II) production in microcosm experiments under various conditions. The horizontal axis indicates the incubation time of the microcosm experiment after preincubation. Day 0 corresponds to the time when Fe(III) was introduced after the 8-day preincubation period. After preincubation, all lake sediment samples were amended with 2 mM acetate. Some samples were further amended with 20 mM of a specific form of Fe(III), ferrihydrite, Fe(III)-citrate, or Fe(III)-EDTA. Error bars indicate standard errors derived from three biological replicates

The addition of different source of Fe(III) to the sediment also influenced the duration of incubation needed for the experiments and the extent of Fe(III) reduction achieved. In sediment samples amended with Fe(III)-citrate and Fe(III)-EDTA, maximum Fe(II) concentrations were achieved within approximately four days. In contrast, nearly 60 days were required for the maximum Fe(II) concentration in the sediment amended with ferrihydrite. The Fe(II) concentrations in sediment amended with 20 mM Fe(III)-citrate and Fe(III)-EDTA increased by approximately 20 mM and 6 mM Fe(II) compared to their initial concentrations right after preincubation when Fe(III) was amended (i.e., 100% of the Fe(III)-citrate but only 30% of the Fe(III)-EDTA was reduced). Sediment amended with 20 mM ferrihydrite exhibited an additional increase in Fe(II) production in the range of 12 mM (i.e., ca. 60% of the added ferrihydrite was reduced).

Community structure of Fe(III)-reducing bacteria

In addition to the varying rates of Fe(III) reduction, Fe(III)-reducing bacterial communities also varied in the sediment samples amended with different Fe(III) sources. The six most abundant genera of Fe(III)-reducing bacteria after preincubation when Fe(III) was amended (T0) were *Thiobacillus*, *Methanothrix*, *Syntrophus*, *Pseudomonas*, *Geobacter*, and *Rhodoferax*. After incubation for 59 days, the sediment amended with only acetate or with acetate plus ferrihydrite showed no significant shifts in the relative abundance of the dominant Fe(III)-reducing bacteria (Figs. 2, 3, S4 and S5). Only minor variations were evident among these samples. In these sediment setups, the changes in abundance for each of the six genera of Fe(III)-reducing bacteria remained within a 1.4-fold difference. In contrast, despite being cultivated for only four days, the relative abundance of Fe(III)-reducing bacteria in the sediment samples amended with acetate plus either Fe(III)-citrate or Fe(III)-EDTA significantly changed compared to that in the T0 sediment samples (Figs. 2 and 3).

In the sediment amended with Fe(III)-citrate (Figs. 2 and 3), the relative abundance of *Clostridium* increased from 0.2 to 5.1‰, indicating a remarkable 25.5-fold increase. *Clostridium* became the most abundant genus among the Fe(III)-reducing bacteria (Fig. 2). In addition, the relative abundances of

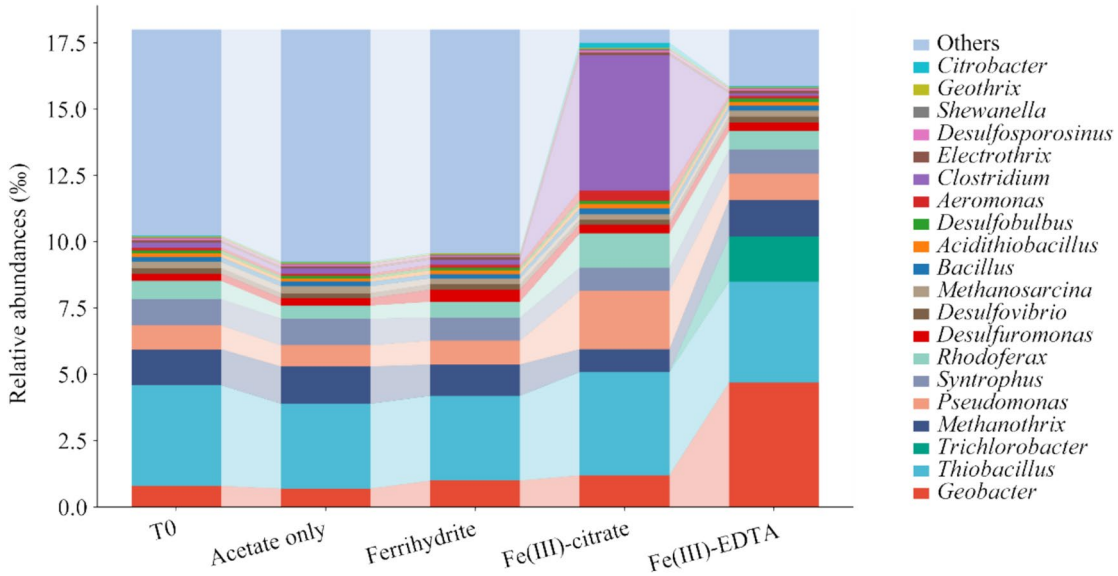

Fig. 2 Composition structure of the 20 most abundant Fe(III)-reducing bacterial genera in initial sediments (T0) and in the sediments incubated with different forms of Fe(III). Samples were taken at the time when the Fe(II) concentration reached equilibrium. In experiments with Fe(III)-citrate and Fe(III)-EDTA, samples were collected on day 4, and for the other experiments, samples were collected on day 59. The proportions are shown in permille (‰)

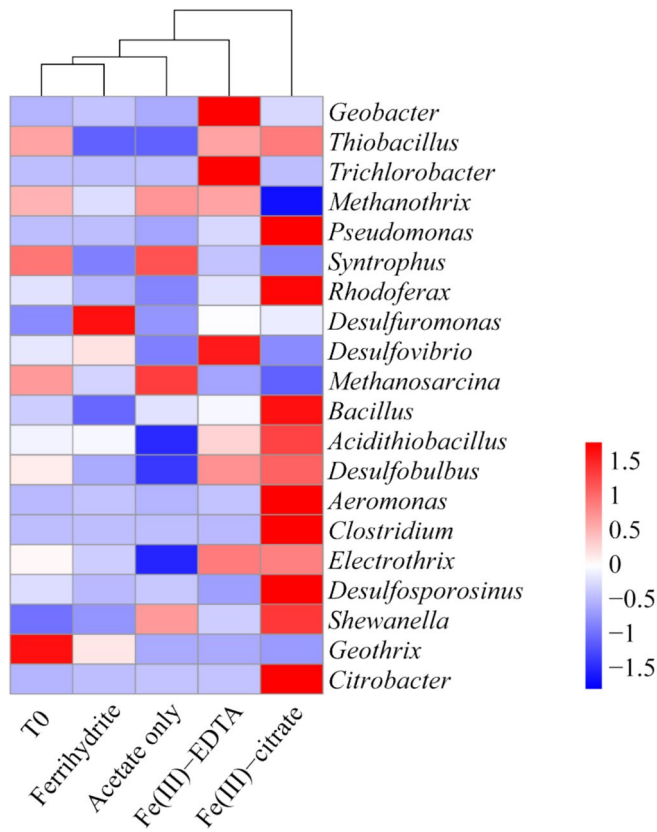

Fig. 3 Heatmap and cluster analysis of the relative abundance of the 20 most abundant Fe(III)-reducing bacterial genera in initial sediments (T0) and in sediment after incubation with different amounts of Fe(III) (ferrihydrite, Fe(III)-citrate or Fe(III)-EDTA) or without the addition of Fe(III) (acetate only). Samples were taken when the Fe(II) concentration reached equilibrium

Pseudomonas, *Rhodoferax*, and *Aeromonas* increased by 2.4, 1.9, and 3.9 times, respectively, increasing from 0.90‰, 0.70‰, and 0.098‰ to 2.2‰, 1.3‰, and 0.38‰, respectively (Fig. 2). Moreover, the relative abundances of *Geobacter* and *Shewanella* also increased by 1.5 times, from 0.80‰ and 0.36‰ to 1.2‰ and 0.55‰, respectively. The genus *Citrobacter*, which was present in very low amounts in the other samples, was also detected in the sediment amended with Fe(III)-citrate, exhibiting a noteworthy abundance 13.2 times greater than that at T0 (Fig. 2).

Compared to the sediment amended with acetate plus Fe(III)-citrate, the sediment with added acetate and Fe(III)-EDTA exhibited a relatively smaller change in the structure of the Fe(III)-reducing bacterial community (Fig. 3). In the sediment with Fe(III)-EDTA addition, the Fe(III)-reducing bacterial genera that were notably enriched were *Geobacter* and *Trichlorobacter*, with relative abundances surpassing even those in the samples with ferrihydrite (Fig. 2). In the Fe(III)-EDTA-amended sediment, the relative abundance of *Geobacter* increased from 0.80 to 4.7‰, representing a 6.7-fold increase. The relative abundance of *Trichlorobacter* increased from below the detection limit (< 0.001‰) to 1.7‰ after four days of incubation, making it the genus with the most significant relative change in abundance. However, in addition to *Geobacter* and *Trichlorobacter*, the increases in the relative abundances of other Fe(III)-reducing genera in the sediment amended with Fe(III)-EDTA were less than 1.4-fold. Additionally, although we identified another well-known genus of Fe(III)-reducing bacteria, *Shewanella,* in all sediment incubations, the relative abundance of *Shewanella* remained relatively low (< 0.054‰) with no significant change after incubation for four days. In addition, the relative abundance of *Geobacter* was generally much greater (> 0.7‰) than that of *Shewanella* in all the sediment incubations (Fig. 2).

Genes involved in Fe(III) reduction

The diversity and quantity of genes involved in Fe(III) reduction also varied in the metagenomes of sediment amended with different Fe(III) sources (Fig. 4). In general, the overall copy numbers of Fe(III) reduction functional genes increased only in microcosms amended with iron. However, the overall copy numbers of these genes varied depending on the type of Fe(III) used (ferrihydrite, Fe(III)-EDTA, or Fe(III)-citrate). In sediments amended with Fe(III)-EDTA and ferrihydrite, the copy numbers of Fe(III) reduction functional genes nearly doubled, increasing from 9 copies at T0 to 17 copies. In contrast, sediments amended with Fe(III)-citrate showed only a slight increase to 10 copies after incubation.

In addition to the overall copy numbers of Fe(III) reduction functional genes, the copy numbers of individual Fe(III) reduction genes also varied across different samples. The copy number of the gene encoding an outer membrane cytochrome c protein (*omcS*) differed among sediments subjected to different amendments. In the metagenomes of the sediment amended with ferrihydrite, 12 copies were detected at the end of the incubation, whereas at T0 and in the sediment amended with Fe(III)-citrate, Fe(III)-EDTA only 4, 6 and 6 copies of this gene were detected. Moreover, genes encoding another type of outer membrane cytochrome c protein (*mtrC*) were detected only in sediments with added ferrihydrite (Fig. 4). Additionally, several hypothetical Fe(III) reduction genes encoding porins or c-type cytochrome proteins with potential Fe(III)-reducing functions were detected only before Fe(III)-amendment or in sediments amended with ferrihydrite or Fe(III)-EDTA, while some genes with potential Fe(III) reduction functions were no longer detectable after the Fe(III) amendments (Fig. 4). Notably, some of these genes were consistently found together.

Genes relevant to iron assimilation

In addition to c-type cytochrome genes, which are relevant to Fe(III) reduction, genes associated with iron assimilation processes also demonstrated variability across sediments subjected to different treatments (Fig. 5). Notably, in the sediments amended with Fe(III)-citrate, although the copy number of Fe(III) reduction genes did not increase significantly, metagenomic analyses revealed a significant increase in the abundance of genes associated with the transportation of iron, heme and siderophore, and iron storage. In sediments amended with ferrihydrite, not only were the same numbers of gene copies detected as in sediments amended with Fe(III)-EDTA, but there was also a significant increase in the abundance of genes regulating iron and siderophore transport (Fig. 5). Cluster analysis utilizing the quantities of these genes

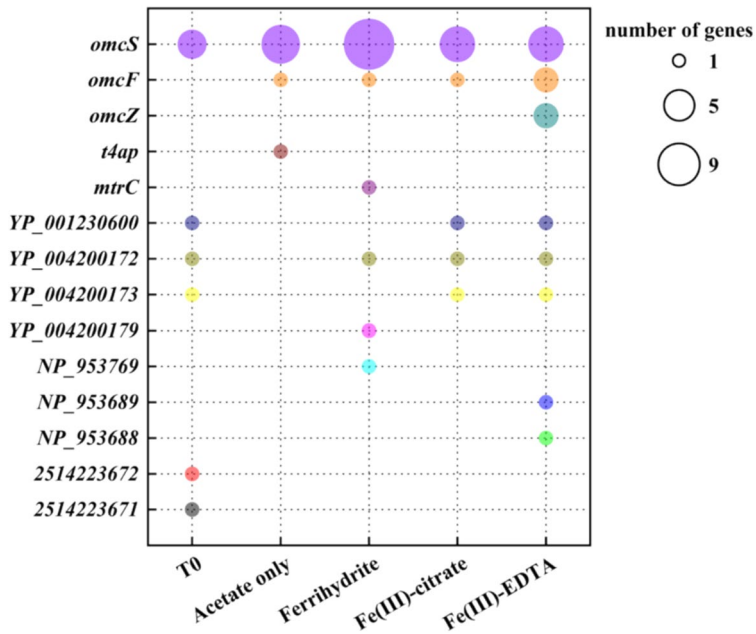

Fig. 4 Quantities of genes for Fe(III) reduction in initial sediments (T0) and in sediments after incubation with different amounts of Fe(III) (ferrihydrite, Fe(III)-citrate or Fe(III)-EDTA) or without the addition of Fe(III) (acetate only). These genes were identified, and their functions were predicted using FeGenie (Garber et al. 2020). Among these genes, the *T4ap* encodes a type of bacterial pilus, 2514223672, NP_953689, *YP_004200173* and *YP_004200179* encode hypothetical porins, and the other genes depicted in this graph all encode c-type cytochromes. The gene copy numbers indicate how many times each gene was detected in the entire metagenome of each sample

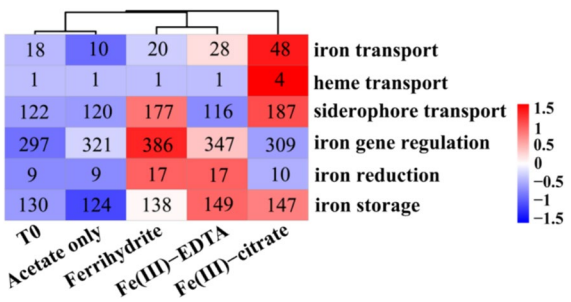

Fig. 5 Heatmap and cluster analysis of gene quantities associated with iron assimilation processes in sediments right after preincubation (T0), and after incubation with either no added Fe(III) (acetate only) or with the addition of 20 mM ferrihydrite, Fe(III)-citrate or Fe(III)-EDTA. These genes were identified, and their functions were predicted using FeGenie (Garber et al. 2020). The copy numbers of each type of genes identified in each sample are displayed within the color-coded cell of the heatmap

revealed that sediments incubated with Fe(III)-EDTA and with ferrihydrite clustered more closely together than those incubated with Fe(III)-citrate (Fig. 5). Genes related to heme oxygenase, siderophore synthesis, Fe(II) oxidation, and magnetosome formation were also searched within the metagenomic datasets; however, no genes related to these functions were detected.

Discussion

Different Fe(III)-OM complexes have different impacts on the kinetics of microbial Fe(III) reduction in sediments

The results of this study revealed that the reduction rates of Fe(III)-citrate and Fe(III)-EDTA in lake sediment far exceeded those of ferrihydrite. This finding is consistent with previous findings based on pure culture experiments, while extending these findings to more complex environmental samples, demonstrate that the observed differences in reduction rates between Fe(III)-OM complexes and ferrihydrite are consistent across both controlled pure culture

conditions and natural, diverse microbial communities. Although different Fe(III)-reducing bacteria were employed in prior studies, the reduction rates of Fe(III)-citrate and Fe(III)-EDTA by bacterial pure cultures were approximately 5–24 times and 2–6 times greater, respectively, than those of ferrihydrite (Bridge and Barrie Johnson 2000; Conley et al. 2018; Haas and Dichristina 2002; Vecchia et al. 2014). This finding also aligns with theoretical predictions based on redox potentials (redox potential Fe(III)-citrate > Fe(III)-EDTA > ferrihydrite) (Bird et al. 2011; Kappler et al. 2021). Similar observations have also been reported in previous studies involving paddy soils. For instance, in comparison to setups with ferrihydrite alone, one study revealed that the addition of Fe(III)-citrate to paddy soil stimulated the Fe(III) reduction rate by approximately 14 times (Wang et al. 2009). Another study revealed that the addition of Fe(III)-EDTA to different paddy soils increased the Fe(III) reduction rate by approximately 2.3 times (He et al. 2023). This indicates that the extent of differences in reduction rates between Fe(III)-OM and ferrihydrite is relatively consistent across different environments, such as paddy soils and lake sediments.

The analysis of microbial community structures and Fe(III)-reduction functional genes also supported our findings on Fe(III) reduction kinetics. Specifically, compared to those in the initial sediments (T0) and sediments with only acetate added, the relative abundance of Fe(III)-reducing bacteria did not significantly increase in the sediments amended with ferrihydrite (Fig. 2). The slower reduction of Fe(III) in sediments amended with ferrihydrite, coupled with a relatively stable community composition, indicates that the addition of 20 mM ferrihydrite might not be as effective as the addition of Fe(III)-OM in stimulating microbial Fe(III) reduction.

Different Fe(III)-reducing bacteria show different preferences for utilizing Fe(III)-citrate, Fe(III)-EDTA, and ferrihydrite

In sediment amended with different source of Fe(III) notable variations were observed in the diversity and abundance of Fe(III)-reducing bacteria, along with the copy numbers of genes for Fe(III) reduction. This indicates that certain species of Fe(III)-reducing bacteria and Fe(III) reduction pathways may exhibit preferences toward specific sources of Fe(III).

In the sediment amended with Fe(III)-EDTA, two closely related Fe(III)-reducing genera, *Geobacter* and *Trichlorobacter* (Nevin et al. 2007; Snoeyenbos West et al. 2001), were significantly enriched (Fig. 2). The relative abundances of *Geobacter* and *Trichlorobacter* in sediment amended with Fe(III)-EDTA were also the highest among all microcosms, even surpassing those in microcosms with ferrihydrite. The finding that *Geobacter* strains are more enriched when Fe(III)-EDTA instead of ferrihydrite was used as the iron source aligns with prior research, which also utilized Fe(III)-EDTA for isolating strains of this genus (Coates et al. 1996). This preference of enrichment of *Geobacter* and *Trichlorobacter* is possibly due to the limited permeability of these compounds through the cell's outer membrane (Madigan et al. 2017), similar to previous observations with Fe(II)-EDTA and Fe(II)-oxidizing bacteria that oxidize Fe(II) in the periplasm (Peng et al. 2018). This indicates that in addition to solid-state iron minerals (Zhang et al. 2020), Fe(III)-EDTA may also favor the activity of *Geobacter* and *Trichlorobacter*. Moreover, since the reduction of Fe(III)-OM was also faster than that of ferrihydrite, this finding also implies that utilizing Fe(III)-EDTA instead of solid iron minerals could be an effective strategy for enriching and isolating Fe(III)-reducing bacteria of the genera *Geobacter* and *Trichlorobacter*.

However, in microcosms amended with Fe(III)-citrate, *Geobacter* and *Trichlorobacter* were not significantly enriched. In these samples, the most notably enriched Fe(III)-reducing bacteria were *Clostridium* (Fig. 2). After searching the genomes in the JGI database, we found that no *Clostridium* species has genes encoding extracellular cytochrome c proteins, confirming that it is a fermentative Fe(III) reducer (List et al. 2019). Apart from the genus *Clostridium*, the microcosm with Fe(III)-citrate was also enriched in the potential Fe(III)-reducing bacterial genera *Pseudomonas*, *Rhodoferax*, and *Aeromonas*, some strains that have genes encoding extracellular cytochrome c proteins (Ambler 1963; Conley et al. 2018; Ellfolk et al. 1983). Interestingly, previous studies revealed a significant increase in the relative abundance of the genus *Clostridium* in paddy soils even when no Fe(III) and only citrate was added (Liu et al. 2022). This implies that citrate in the Fe(III)-citrate complex might be utilized as an electron donor, producing fermentation products that lead to abiotic Fe(III)

reduction. The decrease in the number and variety of functional genes related to Fe(III) reduction in our study also confirms this hypothesis (Figs. 4 and 5).

Given the variation in the content and types of Fe(III)-organic matter complexes across different environments (Sundman et al. 2014), the findings of this study suggest that the type and abundance of Fe(III)-OM may be factors influencing the distribution of Fe(III)-reducing bacteria.

The impact of Fe(III)-OM and ferrihydrite on genes involved in Fe(III)-reduction and iron assimilation

Result of this study revealed that variations in the abundance of certain Fe(III)-reducing bacteria were not correlated with the overall copy number of Fe(III)-reducing functional genes in the metagenomes (Figs. 3, 4 and 5). This could be explained by previous studies which have shown that not all strains within the same genus possess Fe(III)-reducing ability or functional genes (Baker et al. 2022). Additionally, some bacteria, such as the enriched *Clostridium*, which could produce fermentation products capable of reducing Fe(III) abiotically, may lack Fe(III)-reducing functional genes, such as those encoding outer membrane c-type cytochromes. Beside the quantities of genes relevant to Fe(III) reduction, incubation with different Fe(III) sources not only influenced the Fe(III)-reducing microbial community structure but also affected the quantities of genes relevant to iron assimilation processes. For example, the metagenomic results indicated that ferrihydrite, which did not significantly affect the diversity and abundance of Fe(III)-reducing bacteria (Figs. 2 and 3), may have a potential long-term impact on the capacity of Fe(III) reduction and iron assimilation in the sediment, as evidenced by a significant increase in the copy number of these functional genes (Fig. 5). This result corroborates the finding that diverse types of Fe(III)-reducing genes were identified in the metagenomes of different environments (Garber et al. 2021) and suggest that the type of Fe(III) added plays a crucial role not only in Fe(III) reduction but also in other processes related to iron.

Furthermore, the abundance of the *omcS* and *omcF* genes, which are thought to be related to *Geobacter* (Kim et al. 2005; Lovley and Walker 2019; Reguera et al. 2005), increased in the sediments amended with ferrihydrite; however, the abundance of *Geobacter* did not increase. This result suggests that *omcS* may be crucial for the reduction of solid Fe(III) minerals, but not Fe(III)-OM, and bacteria from genera other than *Geobacter* may also possess this gene. Overall, the inconsistency between the abundances of Fe(III)-reducing bacteria and the copy numbers of correlated functional genes observed in this study is also supported by the variations in Fe(III)-reducing gene copies in bacterial pure strains. Some Fe(III)-reducing bacteria possess multiple copies of these genes, while others have fewer (Garber et al. 2020). The well-known Fe(III)-reducing bacteria *Geobacter metallireducens* GS-15 did not even contain *omcS* genes in its genome (Lovley and Walker 2019).

Another interesting finding is that in addition to the genes related to the genus *Geobacter*, the Fe(III) reduction gene *mtrC* related to *Shewanella* was exclusively detected when ferrihydrite was added, although the relative abundance of *Shewanella* did not change significantly (between 0.004 and 0.005%). This suggests that *Shewanella* might be sensitive to the addition of the solid type of Fe(III), even when the supplied electron donor is acetate instead of lactate, which *Shewanella* typically prefers (Nealson and Scott 2006). Additionally, in sediments after incubation with Fe(III), some hypothetical Fe(III) reduction genes encoding c-type cytochrome proteins and porins were detected (Fig. 4), while others disappeared. This finding suggests the existence of a few yet-to-be-isolated strains with Fe(III) reduction capabilities in the environment. This also suggests that differences between the Fe(III) in the original environment and the laboratory conditions may have reduced the competitiveness of certain indigenous Fe(III)-reducing bacteria. Further exploration of their metabolic capabilities depends on future advances in pure culture isolation techniques.

Conclusions and implications

Results of this study revealed that ferrihydrite, Fe(III)-citrate, and Fe(III)-EDTA had variable influences on the kinetics of microbial Fe(III) reduction, the structure of the Fe(III)-reducing bacterial community, and the abundance of genes involved in Fe(III) reduction and iron assimilation. Compared with those amended with ferrihydrite, the sediments amended with Fe(III)-EDTA and Fe(III)-citrate accelerated

Fe(III) reduction. The addition of ferrihydrite had a minimal influence on the Fe(III)-reducing bacterial community, whereas the two Fe(III)-OM complexes favored specific genera. In particular, *Geobacter* and *Clostridium* were enriched in sediments amended with Fe(III)-EDTA and Fe(III)-citrate respectively, indicating that they may be the source of Fe(III) for the enrichment of these genera. Additionally, shifts in community structure did not parallel the changes in the quantities of genes involved in Fe(III) reduction and iron assimilation. Despite its gradual reduction rate and lesser impact on community structure, ferrihydrite significantly influenced not only the abundance of Fe(III) reduction genes, which was also observed with Fe(III)-EDTA, but also the abundances of genes for regulation and assimilation of iron. This suggests that adding ferrihydrite could have a potential long-term impact on the capacity of Fe(III) reduction and iron assimilation in the sediment. Our microcosm experiments demonstrate that these different Fe(III)-OM complexes found in the environment (Sundman et al. 2014) not only exhibit varying microbial Fe(III) reduction rates but also significantly influence the community structure of Fe(III)-reducing bacteria and the diversity of their functional genes involved in microbial Fe(III) reduction. These results enrich our theoretical understanding of the iron biogeochemical cycle in the environment and provide strategic approaches for studying and cultivating specific Fe(III)-reducing bacteria.

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Author contributions TS conducted the experiments, participated in data analysis and visualization, and co-wrote the initial draft of the manuscript together with CP. CP designed the experiments, participated in data analysis, wrote and revised the manuscript. LL supervised the entire project and provided feedback on the manuscript. ZY, YW, and ZW contributed to the revision of the manuscript. AK offered critical and constructive suggestions throughout the entire manuscript.

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Data availability Data can be accessed from the corresponding author upon request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

References

Aklujkar M, Coppi MV, Leang C et al (2013) Proteins involved in electron transfer to Fe(III) and Mn(IV) oxides by *Geobacter sulfurreducens* and *Geobacter uraniireducens*. Microbiology 159(3):515–535

Ambler RJBJ (1963) The purification and amino acid composition of *Pseudomonas* cytochrome c-551. Biochem J 89(2):341

Baker IR, Conley BE, Gralnick JA et al (2022) Evidence for horizontal and vertical transmission of mtr-mediated extracellular electron transfer among the *Bacteria*. mBio 13(1): e02904–02921

Beal EJ, House CH, Orphan VJ (2009) Manganese- and iron-dependent marine methane oxidation. Science 325(5937):184–187

Bird LJ, Bonnefoy V, Newman DK (2011) Bioenergetic challenges of microbial iron metabolisms. Trends Microbiol 19(7):330–340

Bridge TAM, Barrie Johnson D (2000) Reductive dissolution of ferric iron minerals by *Acidiphilium* SJH. Geomicrobiol J 17(3):193–206

Buchfink B, Xie C, Huson DH (2015) Fast and sensitive protein alignment using DIAMOND. Nat Methods 12(1):59–60

Chen S, Zhou Y, Chen Y et al (2018) fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34(17):i884–i890

Coates JD, Phillips E, Lonergan DJ et al (1996) Isolation of *Geobacter* species from diverse sedimentary environments. Appl Environ Microbiol 62(5):1531–1536

Conley BE, Intile PJ, Bond DR et al (2018) Divergent Nrf family proteins and MtrCAB homologs facilitate extracellular electron transfer in *Aeromonas hydrophila*. Appl Environ Microbiol 84(23):e02134-e12118

Dong H, Zeng Q, Sheng Y et al (2023) Coupled iron cycling and organic matter transformation across redox interfaces. Nat Rev Earth Env 4(9):659–673

Ellfolk N, Rönnberg M, Aasa R et al (1983) Properties and function of the two hemes in *Pseudomonas* cytochrome c peroxidase. Biochim Biophys Acta Bioenerg 743(1):23–30

Ettwig KF, Zhu B, Speth D et al (2016) Archaea catalyze iron-dependent anaerobic oxidation of methane. PNAS 113(45):12792–12796

Fan YY, Li BB, Yang ZC et al (2018) Abundance and diversity of iron reducing bacteria communities in the sediments of a heavily polluted freshwater lake. Appl Microbiol Biot 102(24):10791–10801

Fu L, Niu B, Zhu Z et al (2012) CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28(23):3150–3152

Garber AI, Nealson KH, Okamoto A et al (2020) FeGenie: a comprehensive tool for the identification of iron genes and iron gene neighborhoods in genome and metagenome assemblies. Front Microbiol 11:37

Garber AI, Cohen AB, Nealson KH et al (2021) Metagenomic insights into the microbial iron cycle of subseafloor habitats. Front Microbiol 12:667944

Gibbs CR (1976) Characterization and application of Ferro-Zine iron reagent as a ferrous iron indicator. Anal Chem 48(8):1197–1201

Haas JR, Dichristina TJ (2002) Effects of Fe (III) chemical speciation on dissimilatory Fe (III) reduction by *Shewanella putrefaciens*. Environ Sci Technol 36(3):373–380

Hall SJ, Berhe AA, Thompson A (2018) Order from disorder: do soil organic matter composition and turnover co-vary with iron phase crystallinity? Biogeochemistry 140(1):93–110

He Z, Xu Y, Zhu Y et al (2023) Long-term effects of soluble and insoluble ferric irons on anaerobic oxidation of methane in paddy soil. Chemosphere 317:137901

Hori T, Aoyagi T, Itoh H et al (2015) Isolation of microorganisms involved in reduction of crystalline iron(III) oxides in natural environments. Front Microbiol 6:386

Huang S, Jaffé PR (2018) Isolation and characterization of an ammonium-oxidizing iron reducer: *Acidimicrobiaceae* sp. A6. PLoS One 13(4): e0194007

Hyatt D, Chen GL, LoCascio PF et al (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinform 11:1–11

Ionescu D, Heim C, Polerecky L et al (2015) Diversity of iron oxidizing and reducing bacteria in flow reactors in the Äspö hard rock laboratory. Geomicrobiol J 32(3–4):207–220

Kappler A, Bryce C, Mansor M et al (2021) An evolving view on biogeochemical cycling of iron. Nat Rev Microbiol 19(6):360–374

Kim BC, Leang C, Ding YH et al (2005) OmcF, a putative c-Type monoheme outer membrane cytochrome required for the expression of other outer membrane cytochromes in *Geobacter sulfurreducens*. J Bacteriol 187(13):4505–4513

Kügler S, Cooper RE, Wegner CE et al (2019) Iron-organic matter complexes accelerate microbial iron cycling in an iron-rich fen. Sci Total Environ 646:972–988

Laufer Meiser K, Michaud AB, Maisch M et al (2021) Potentially bioavailable iron produced through benthic cycling in glaciated Arctic fjords of Svalbard. Nat Commun 12(1):1349

Leang C, Adams LA, Chin KJ et al (2005) Adaptation to disruption of the electron transfer pathway for Fe(III) reduction in *Geobacter sulfurreducens*. J Bacteriol 187(17):5918–5926

Lentini C, Wankel S, Hansel C (2012) Enriched iron(III)-reducing bacterial communities are shaped by carbon substrate and iron oxide mineralogy. Front Microbiol 3:404

Li R, Li Y, Kristiansen K et al (2008) SOAP: short oligonucleotide alignment program. Bioinformatics 24(5):713–714

Li D, Liu CM, Luo R et al (2015) MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 31(10):1674–1676

List C, Hosseini Z, Lederballe Meibom K et al (2019) Impact of iron reduction on the metabolism of *Clostridium acetobutylicum*. Environ Microbiol 21(10):3548–3563

Liu L, Yang YP, Duan GL et al (2022) The chemical-microbial release and transformation of arsenic induced by citric acid in paddy soil. J Hazard Mater 421:126731

Lofts S, Tipping E, Hamilton Taylor J (2008) The chemical speciation of Fe(III) in freshwaters. Aquat Geochem 14(4):337–358

Lovley DR, Holmes DE (2021) Electromicrobiology: the ecophysiology of phylogenetically diverse electroactive microorganisms. Nat Rev Microbiol 20(1):5–19

Lovley DR, Walker DJF (2019) Geobacter protein nanowires. Front Microbiol 10:2078

Madigan M, Bender K, Buckley D et al (2017) Brock biology of microorganisms 15 edn. Google Scholar, pp 1064

Nealson KH, Scott J (2006) Ecophysiology of the genus *Shewanella*. Prokaryotes 6:1133–1151

Nevin KP, Holmes DE, Woodard TL et al (2007) Reclassification of *Trichlorobacter thiogenes* as *Geobacter thiogenes* comb. nov. Int J Syst Evol Microbiol 57(3): 463–466

Peng C, Bryce C, Sundman A et al (2019) Organic matter complexation promotes Fe(II) oxidation by the photoautotrophic Fe(II)-oxidizer *Rhodopseudomonas palustris* TIE-1. ACS Earth Space Chem 3(4):531–536

Peng C, Sundman A, Bryce C et al (2018) Oxidation of Fe(II)–organic matter complexes in the presence of the mixotrophic nitrate-reducing Fe(II)-oxidizing bacterium *Acidovorax* sp. BoFeN1. Environ Sci Technol 52(10): 5753–5763

Raiswell R, Canfield DE (2012) The iron biogeochemical cycle past and present. Geochem Perspect 1(1):1–2

Reguera G, McCarthy KD, Mehta T et al (2005) Extracellular electron transfer via microbial nanowires. Nature 435(7045):1098–1101

Richter K, Schicklberger M, Gescher J (2012) Dissimilatory reduction of extracellular electron acceptors in anaerobic respiration. Appl Environ Microbiol 78(4):913–921

Scheller S, Yu H, Chadwick GL et al (2016) Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction. Science 351(6274):703–707

Schwertmann U, Cornell RM (2008) Iron oxides in the laboratory: preparation and characterization. John Wiley & Sons, Germany

Shi L, Rosso Kevin M, Zachara John M et al (2012) Mtr extracellular electron-transfer pathways in Fe(III)-reducing or Fe(II)-oxidizing bacteria: a genomic perspective. Biochem Soc Trans 40(6):1261–1267

Shi L, Dong H, Reguera G et al (2016) Extracellular electron transfer mechanisms between microorganisms and minerals. Nat Rev Microbiol 14:651

Snoeyenbos West O, Van Praagh CG, Lovley DR (2001) *Trichlorobacter thiogenes* should be renamed as a *Geobacter* species. Appl Environ Microbiol 67(2):1020–1022

Sundman A, Karlsson T, Laudon H et al (2014) XAS study of iron speciation in soils and waters from a boreal catchment. Chem Geol 364:93–102

Vecchia DE, Suvorova EI, Maillard J et al (2014) Fe(III) reduction during pyruvate fermentation by *Desulfotomaculum reducens* strain MI-1. Geobiology 12(1):48–61

Wang Z, Liu C, Wang X et al (2008) Kinetics of reduction of Fe(III) complexes by outer membrane cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1. Appl Environ Microbiol 74(21):6746–6755

Wang XJ, Yang J, Chen XP et al (2009) Phylogenetic diversity of dissimilatory ferric iron reducers in paddy soil of Hunan. South China J Soil Sediment 9(6):568–577

Wang M, Zhao Z, Li Y et al (2022) Control the greenhouse gas emission via mediating the dissimilatory iron reduction: Fulvic acid inhibit secondary mineralization of ferrihydrite. Water Res 218:118501

Yang L, Jiang M, Zou Y et al (2021) Geographical distribution of iron redox cycling bacterial community in peatlands: distinct assemble mechanism across environmental gradient. Front Microbiol 12:674411

Zhang F, Battaglia Brunet F, Hellal J et al (2020) Impact of Fe(III) (Oxyhydr)oxides mineralogy on iron solubilization and associated microbial communities. Front Microbiol 11:571244