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Microbe interactions drive the formation of floating iron films in circumneutral wetlands

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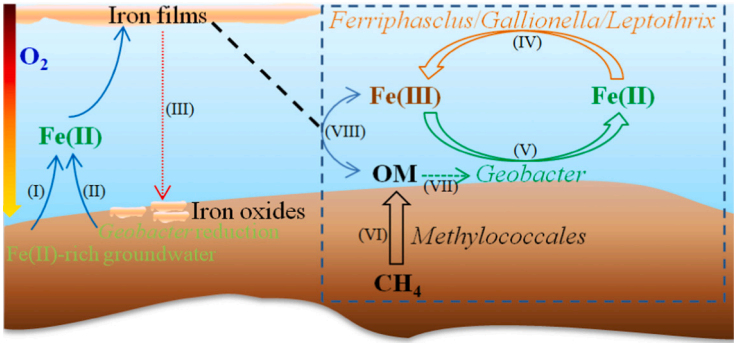
HIGHLIGHTS

- Floating Fe films contain mixed-valent Fe and can form biotically in wetlands.
- Ferrihydrite was the dominant Fe(III) phase, accompanied by carbon and silicon in films.
- Fe cycling bacteria and methanotrophs were the dominant species in floating Fe films.
- Fe(II)-oxidizing bacteria produced recognized morphotypes in floating Fe films.
- Microbial Fe and carbon cycling accelerated the floating Fe film formation in wetlands.

GRAPHICAL ABSTRACT



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ABSTRACT

Floating iron (Fe) films are widely found in wetlands that can form oxic-anoxic boundaries under circumneutral conditions. These films play a crucial role in the redox transformations and bioavailability of nutrients and trace metals. Current studies mainly focus on chemical oxidation during Fe film formation under circumneutral conditions. The functional microorganisms and associated microbial processes involved in Fe film formation have yet to be investigated in detail. Here, we investigated the microbial communities and involved microbial processes for the formation of floating Fe films in wetlands. Ferrihydrite was the dominant Fe(III) phase in films, accompanied by moderate levels of carbon and silicon. The Fe species and microbial analysis indicated that Fe films contain mixed-valent Fe and can form biotically. Microbial community analysis showed that the dominant genera in these Fe films were Fe-oxidizing and reducing bacteria and methanotrophs, including *Leptothrix*, *Ferriphasclus*, *Gallionella*, *Geobacter* and *Methylococcales*. *Leptothrix*, *Ferriphasclus* and *Gallionella*, as classical Fe (II)-oxidizing bacteria (FeOB), can oxidize Fe(II) with limited oxygen and form special structures that are consistent with Fe film morphology. *Geobacter* can provide a source of Fe(II) for FeOB growth, and *Methylococcales* can perform methane oxidation to provide energy for Fe cycling. The high ratios of *Gallionella*- and

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Geobacter-related microorganisms and carbon fixation genes proved the contribution of potential of Fe cycling and autotrophic microbial communities to the formation of Fe films. The diversity of microbial community suggested that Fe(II) oxidation could trigger carbon fixation, while Fe(III) reduction accelerated Fe and carbon cycling through anaerobic respiration and autotrophic chemosynthesis. These results highlight the contribution of these multiple microbial processes to Fe and carbon cycling during the formation of floating Fe films in wetlands. However, further studies are required to fully elucidate the interaction of functional microorganisms involved in floating film formation and their biogeochemical role in wetlands.

1. Introduction

Iron (Fe) is an abundant nutrient that is essential for many physiological processes in plants and microorganisms in wetlands (Wu et al., 2019). Generally, Fe(II) and Fe(III) are the most common forms of Fe found in natural wetlands. Under anoxic soil conditions, Fe(II), as a soluble form of Fe, is the dominant species utilized by plants and microorganisms. However, with the vertical diffusion of oxygen, the Fe(II) pumped from anaerobic soil to water surface is oxidized to Fe(III) at the oxic-anoxic interface, and then forms very low solubility Fe oxyhydroxides under circumneutral conditions (Reina et al., 2015). These Fe(III) precipitates affect the transformation and geochemistry of trace metals, nutrients, and inorganic and organic pollutants through adsorption and/or co-precipitation (Faivre, 2016). The change in Fe oxidation state, also known as Fe cycling, is believed to play a crucial role in many key environmental processes, such as photosynthesis, carbon and nitrogen fixation, soil formation, and pollutant transformation (Andrews et al., 2003; Wu et al., 2019). In wetlands, two main forms of Fe precipitates (Fe plaque and floating Fe films) are associated with Fe cycling. However, most research has focused on the rhizosphere environment, including the formation of Fe plaques and their effect on ecosystems (Weiss et al., 2003; Xiao et al., 2021; Yu et al., 2021). Floating Fe films are often mistaken for oil and biofilms due to their oily and silvery appearance. The occurrence of these films has attracted little attention from researchers, resulting in that these films are often overlooked and their formation mechanism and biogeochemical role are still not well understood.

In recent years, floating Fe films are frequently found and mentioned in acid mine drainage (AMD) affected streams, wetlands, small rivers, and lakes, particularly in Fe-rich environments (Grathoff et al., 2007; Reina et al., 2015; Sánchez-España et al., 2023). These films mainly consist of schwertmannite or jarosite under acidic conditions (Fernandez-Rojo et al., 2017; Sánchez-España et al., 2023), while ferrihydrite or a mixture of Fe(III)-organic complexes and hydroxides is dominant under circumneutral conditions (Grathoff et al., 2007; Kleja et al., 2012; Perkins et al., 2016). In AMD-affected streams, the rate of Fe(II) oxidation with oxygen is minimal below pH 3 (Davison and Seed, 1983). Nonetheless, the rapid chemical oxidation of Fe(II) is considered to be the main cause of Fe(III) hydroxide formation at the redox interface under circumneutral conditions (Emerson and Floyd, 2005; Emerson et al., 2010). Even in the presence of neutrophilic Fe(II)-oxidizing bacteria (FeOB) under microaerobic conditions, chemical oxidation can also contribute to observed Fe(II)-oxidation rates (Rentz et al., 2007). These phenomena lead to current research focusing on chemical oxidation for film formation and the effect of microbial oxidation is often overlooked under circumneutral conditions. However, morphological and microbiological observations have shown that floating Fe films contain a mass of stalk and sheath structures, indicating the involvement of neutrophilic, microaerophilic FeOB and their activities in the formation of floating Fe films (Grathoff et al., 2007; Chan et al., 2011). In addition, the incubation of wetland soil suggests that microaerophilic FeOB are the dominant bacteria in floating films and they play an important role in floating film formation (Reina et al., 2015).

Further analyses revealed that these films contain mixed-valent Fe with a high ratio of Fe(II):Fe(III) (Grathoff et al., 2007). There are two possible mechanisms for these high Fe(II) concentrations: i) the reductive dissolution of Fe(III)-oxyhydroxides by a photo-catalytic process, and ii) the microbial reduction by Fe(III)-reducing bacteria (FeRB) due to the lack of oxygen (Reina et al., 2015; ThomasArrigo et al., 2022). Previous reports have emphasized that the photo-redox system can allow Fe(II) to remain in solution and form mix-valent Fe minerals (Voelker et al., 1997; Perkins et al., 2016). However, oxygen depletion has been observed at the bottom of Fe floating films that form in the field or in experimental incubation (Fernandez-Rojo et al., 2017), suggesting that beneath the films, FeRB may be active, using Fe(III) oxyhydroxides as an electron acceptor. The roles of FeRB in the transformation of Fe floating films have been neglected in previous studies. Hence, more studies are needed to investigate the diversity of microbial communities and their potential functions in the formation of these floating films, particularly in their contribution to Fe cycling in wetlands.

Wetland ecosystems are sites of rapid biogeochemical cycling due to the interactions between the oxic water surface and anoxic soils (Weiss et al., 2003). The alternation of redox potential due to fluctuations in the water table produces a variety of oxic-anoxic interfaces on the water surface and sediment layers (Reina et al., 2015). In addition, the oxic-anoxic interface is further extended by the presence of wetland plants that leak oxygen from their roots via radial oxygen loss (Armstrong, 1964; Weiss et al., 2003). These special niches and the gradient of Fe(II) in wetlands can provide a suitable environment for the growth of microaerophilic FeOB, which may play a critical role in the formation of floating Fe films (Weiss et al., 2005; Grathoff et al., 2007). In this study, naturally occurring floating Fe films were collected from several wetlands to characterize their environmental, mineralogical, and microbiological features under circumneutral conditions. In addition, water column, sediment, and rhizosphere samples associated with the formation of floating films were obtained for microbial community analysis. The aims of the present work were to i) characterize the Fe floating films at a circumneutral pH in several wetlands, and ii) explore the microorganisms and biogeochemical processes involved in the formation of floating Fe films. Based on the chemical properties and potential functional microorganisms of floating films, a possible mechanism was drafted for the formation of floating Fe films in wetlands. The results would provide a better understanding of the Fe cycling in wetlands and their effects on other types of nutrient cycling and pollutant transformation.

2. Materials and methods

2.1. Collection of natural floating Fe films

Floating Fe films were collected from the pools along the Kongjiang reservoir in Nanxiong (25°17′3″N, 114°39′1″E) and the Nanhu reservoir in Ruyuan (24°43′58″N, 113°8′13″E and 24°44′32″N, 113°10′44″E) in Guangdong province, China (Fig. S1). The areas of pools ranged from 1 m^2 to 4 m^2. In these areas, floating Fe films, water column below the floating Fe films, sediment, and roots were collected for further research (Fig. S2). Floating Fe film samples for Fe species and microbial analysis were collected on glass slides by wiping slides across the Fe films to avoid collecting other debris and excess water (Grathoff et al., 2007). Then, a portion of collected glass slides with Fe films was placed into a centrifuge tube containing 0.5 M HCl to dissolve the Fe films and protect

Fe(II) from oxidation by oxygen, which were filtered through 0.22 μm filter. The other portion of collected glass slides with Fe films was stored in sterile centrifuge tube for microbial analysis. Water samples involved in the formation of Fe films were obtained below the Fe films with syringes and immediately filtered through a 0.22 μm filter into acid-washed centrifuge tubes containing 0.5 M HCl to avoid chemical oxidation. Sediments below the Fe films were collected and stored in serum bottles to analyze the microorganisms and the concentrations of amorphous Fe oxides. The serum bottle was flushed with nitrogen to minimize the effect of oxygen on sediments.

The collection methods of floating Fe film samples for scanning electron microscopy (SEM), high-resolution transmission electron microscope (HRTEM), and X-ray diffraction (XRD) were described previously (Grathoff et al., 2007; Perkins et al., 2016). SEM samples were collected on glass slides or carbon stubs and then stored in centrifuge tubes, which were filled with nitrogen to minimize further oxidation of Fe films. TEM samples were collected on 3 mm copper grids by gently guiding the Fe films toward the grid by eyelash probe. And then the grids were dried in air for 10 min to remove excess water around the Fe films. The samples for Mössbauer spectroscopy analysis were collected on glass sliders (at least four slides to ensure the sufficient Fe) and transferred into nitrogen-filled centrifuge tubes. Samples were immediately freeze-dried for Mössbauer spectroscopy after returning to the laboratory.

2.2. Sample analysis

The water pH, temperature, and the oxygen concentration below the Fe films and in the areas without Fe films were detected with a portable water quality meter (AZ-86031, China). The meter was slowly immersed in water to reduce human disturbance. The oxidation-reduction potential (Eh) was measured with a Eh meter (Seven2Go, Mettle Toledo, Switzerland). The Fe(II) and Fe(III) concentrations in Fe films (dissolved with 0.5 M HCl in section 2.1) and water were detected as described previously by the 1,10-phenanthroline colorimetric method (Tong et al., 2021). The pore water in sediment was obtained by centrifugation, and the supernatant was filtered and acidified with concentrated HCl for Fe analysis (Wang et al., 2011). The residue was used to determine Fe(II) and Fe(III) levels in amorphous Fe oxides in sediment (Wang et al., 2011). Approximately 0.5 g residue was extracted with 0.5 M HCl by shaking for 1 h in a glove box. Subsequently, the extracts were separated from the sediments by centrifugation. The Fe(II) concentrations in pore water and 0.5 M HCl extraction were determined with the 1,10-phenanthroline colorimetric method. The total Fe concentration was measured with a mixed solution of 0.25 mol/L hydroxylamine and 1,10-phenanthroline (Tong et al., 2021). The difference between the values of Fe (II) and total Fe concentrations represented the Fe(III) concentration in pore water and sediments. HRTEM (JEM-2100F, Japan) equipped with a Noran Energy Dispersive X-ray Spectrometer (EDS, Noran system 7, USA) was used to characterize the morphotypes and analyze the element composition of Fe films. The total silicon of Fe film was measured using inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer, CA, USA). The carbon content of Fe films was determined by total combustion using an elemental analyzer (2400 CHNS analyzer, Perkin Elmer, CA, USA). The Mössbauer spectroscopy was conducted at 12 K and 375 K using a WissEl Elektronik (Germany) instrument that included a closed-cycle cryostat SHI-850, a Sumitomo CKW-21 He compressor unit, and an Ar—Kr proportional counter detector. A ^{57}Co (~50-mCi) source was used as the gamma energy source. All samples were prepared following previously established methods (Easterly, 2005). Mössbauer spectroscopy data were fitted with MossWinn 4.0, as described in a previous study by this research group (Tong et al., 2019).

2.3. Enrichment of dominant FeOB from the rhizosphere

Oxygen availability in the rhizosphere varies both spatially and temporally along a root system, creating an anoxic-anoxic interface, which is similar to the microaerobic environment formed by floating Fe films (Weiss et al., 2003; Kleja et al., 2012). In addition, some isolated FeOB can produce special sheath-like or stalk-like structures, which are consistent with the special structures found in floating Fe films (Grathoff et al., 2007; Emerson et al., 2010; Chan et al., 2011). Therefore, we collected the root samples to characterize the FeOB in the rhizosphere for comparison with microbial communities in floating Fe films. In order to characterize the FeOB in the rhizosphere, the roots that contained Fe-plaques from the pools were used to incubate and enrich FeOB. The roots were washed three times in sterile water to remove soil particles around the roots, and then sliced into sections. The sliced root samples were transferred into gradient tubes for incubation. The details of the gradient tubes, gradient media, and carbon source with opposing gradients were described in previous reports (Emerson and Moyer, 1997; Tong et al., 2019). The enrichments were incubated with constant temperature 25 ± 1 °C in the dark. After 2 weeks of incubation, a stable rust-colored band, mainly consisting of Fe minerals (Emerson and Floyd, 2005), appeared around the oxic-anoxic interface. The colored band and media around the band were removed from the gradient tubes to extract DNA for further analysis.

2.4. DNA extraction, quantitative PCR and 16S rRNA high-throughput sequencing

The floating Fe films, bulk water, sediment, and rhizosphere are the major reservoirs for microbial communities within the wetlands. The samples collected for DNA extraction (floating Fe films, water column, and sediment) were shown in Fig. S2. DNA from approximately 0.3 g sediment was extracted by a DNeasy PowerSoil Pro kit (Qiagen, USA). Approximately 0.3 g floating Fe films and rhizosphere enrichment were collected for DNA extraction using a modified procedure described previously (Tong et al., 2021). A portable peristaltic sampler was applied to concentrate microorganisms in water below the floating Fe films (approximately 3 L) through 0.22 μm filters. DNA from these filters was extracted using modified steps described by Lambrecht et al. (2020). The extracted DNA was analyzed using a Qubit 2.0 Fluorometer DNA (Invitrogen, NY, USA) and polymerase chain reaction (PCR) amplification of the 16S rRNA gene fragments was conducted with Illumina-specific fusion primers F515 and R806 (Bates et al., 2011). The PCR products were purified and normalized in equimolar amounts for Miseq Illumina high-throughput sequencing by Magigen Biotechnology (Guangzhou, China). The bioinformatics analysis was performed using QIIME 2 (v2017.6.0) (Caporaso et al., 2010) and the details were described in our previous study by Tong et al. (2019). The microbial communities from floating Fe films, water column, sediment, and rhizosphere enrichment were designated iron film, water, sediment, and root in different figures, respectively. The copies of 16S rRNA genes of *Gallionella*-related FeOB and *Geobacter*-related FeRB, identified as two typical Fe cycling microorganisms, were quantified by quantitative PCR (qPCR) using the primers M122F (5′-TATCGGAACRTRTCCGGA-3′) and Beta3R (5′-ACGCATTTCACTGCTACACG-3′) and Geo494F (5'-AGGAAGCACCGGCTAACTCC-3′) and Geo825R (5′-TACCCGCRACACCTAGTTCT-3′), respectively (Tong et al., 2014; Watanabe et al., 2021). The copies of carbon fixation genes of *cbb*L for carbon dioxide (CO_2) fixation and *pmo*A for methane (CH_4) assimilation were quantified with primers cbbLK2F (5′-ACCAYCAAGCCSAAGCTSGG-3′) and cbbLV2R (5′-GCCTTCSAGCTTGCCSACCRC-3′) and A189f (5′-GGNGACTGGGACTTCTGG-3′) and mb661r (5′-CCGGMGCAACGTCYTTACC-3′), respectively (Berg, 2011; Ma et al., 2013). Total bacterial abundance was measured by qPCR on the 16S rRNA gene using universal bacterial primers 519F (5′-CAGCMGCCGCGGTAATWC-3′) and 1406R (5′-ACGGGCGGTGTGTRC-3′) (Tong et al., 2014). The qPCR calibration curves were generated with serial dilutions (ranging from 1 × 10^2 to 1 × 10^9 copies/μl) of plasmids containing the cloned target sequences. The plasmid DNA concentration was quantified by Qubit 2.0 Fluorometer (Invitrogen, NY, USA), and the corresponding gene copy

number was calculated relatively to the plasmid size, insert lengths and Avogadro number (Whelan et al., 2003). The linear discriminant analysis effect size (LEfSe) was carried out to identify the potential FeOB that reflected the difference between floating Fe films and other samples (Wang et al., 2020a, 2020b). LEfSe applied LDA to those different microorganisms identified as significantly different ($P < 0.05$ and LDA score > 3). Principal component analysis (PCA) was performed by the 'vegan' package of R (2.6–4) to detect the difference of microbial structure from different samples. The genomic datasets were deposited in the NCBI under BioProject ID PRJNA850878 and accession number SAMN29204362.

3. Results and discussion

3.1. Chemistry of waterand sediment samples from the floating film field

Three sampling sites were located in different natural wetland parks in Guangdong province, China (Fig. S1). Field measurements of water pools, including the temperature, pH, Eh, and dissolved oxygen, are presented in Table S1. The pH values of these water samples ranged from 6.3 to 7.0, which are the typical circumneutral conditions for the growth of neutrophilic, microaerophilic FeOB (Bryce et al., 2018; Gülay et al., 2018). These pH values were similar to those reported in previous studies of naturally occurring floating Fe films under circumneutral conditions, in which neutrophilic, microaerophilic FeOB played a critical role in the formation of Fe films (Portillo et al., 2008; Reina et al., 2015; Perkins et al., 2016).The Eh values of water bodies were determined to be intermediate redox conditions (from +200 mV to +350 mV) (Perkins et al., 2016). These transitional Eh-pH conditions were suitable for the formation of floating Fe films in circumneutral wetlands. The oxygen concentrations under neath the floating Fe films were much lower than those observed in water body without Fe films (Table S1). This may have occurred because the chemical or/and microbial Fe(II) oxidation consumed oxygenand the films prevented oxygen diffusion at some extent. The oxygen depletion could form anoxic conditions underneath the floating Fe films that could stimulate the growth of anaerobic microorganisms. The anaerobic metabolic processes conducted by these microorganisms might contribute to the pH decrease (Portillo et al., 2008), reflecting that the pH values may have a positive correlation with the dissolved oxygen concentrations as observed in Tables S1. Extractable Fe from poorly crystalline phases in sediments and Fe in pore water mainly existed as Fe(II) species (Table S1), indicating that the wetlands could pump Fe(II) from sediment to the water surface for Fe film formation (Wang et al., 2011). As a result, the redox interface of Fe(II) and oxygen gradients provided a suitable microenvironment for the growth of microaerophilic FeOB in the wetlands (Emerson and Floyd, 2005). The dissolved Fe species analysis in water showed that Fe(III) was the predominant species in the studied water environments (Table S1), suggesting the strong chemical and/or microbial Fe(II) oxidation during the flux of Fe(II) from the sediments.

3.2. Floating film analysis

The Fe species in the floating Fe films were determined by dissolving these films in HCl solution (Perkins et al., 2016). The ratio of Fe(II):Fe(III) ranged from 0.02 to 0.12, with an average of 0.08 (data not shown). These values were lower than those in the previous reports (Grathoff et al., 2007; Perkins et al., 2016), suggesting that most dissolved Fe(II) was oxidized by oxygen and/or FeOB. In order to characterize the structure of Fe minerals, Mössbauer spectroscopy was performed for all film samples at room temperature (298 K) and at low temperature (12 K) (Fig. 1). The corresponding fit parameters of Mössbauer spectroscopy are shown in Table S2. The center shift (CS) and quadrupole splitting (QS) values of floating films in this study were similar to those described in previous reports under circumneutral conditions (Stolyar et al., 2018; Knyazev et al., 2021), which were characterized as being typical of ferrihydrite. Generally, the oxidation of dissolved Fe(II) at circumneutral pH leads to the precipitation of ferrihydrites (Reina et al., 2015). Depending on changes in the environmental conditions, poor crystalline ferrihydrites could transform to goethite with time (Cornell and Schwertmann, 2003; Faivre, 2016). However, no additional crystallized Fe minerals were detected in the floating films. The relatively high organic matter contents (8.9–10.3 %, w/w, Table S1), as observed in the Fe films, may hinder the ferrihydrites transformation by blocking dissolution sites on the ferrihydrites or hampering nucleation of more stable Fe(III) mineral phases (Jones et al., 2009; Kleja et al., 2012). These results were in good accordance with previous reports that low-crystalline forms were the predominant crystal structures in these floating films under circumneutral conditions (Grathoff et al., 2007; Kleja et al., 2012). Although Fe species analysis revealed the presence of

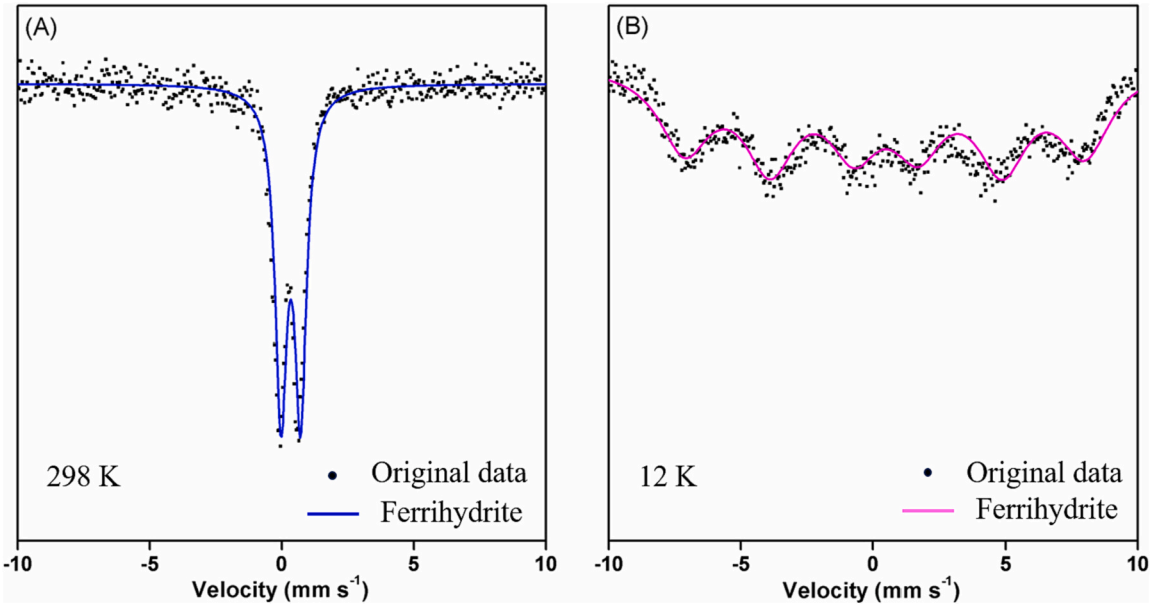

Fig. 1. Fitted Mössbauer spectra of floating Fe films at (A) 298 K and (B) 12 K. The circles represent the original data, and the solid lines represent the fitted curves. Corresponding fit parameters are summarized in Table S1.

Fe(II) in these films, there was no evidence for the formation of Fe(II) minerals. A possible reason for this finding was that some Fe(II) might be adsorbed on Fe(III) oxyhydroxide surfaces and/or be bound to organic matter by electrostatic interactions in these films (Kleja et al., 2012; Reina et al., 2015; ThomasArrigo et al., 2018). This Fe(II) would not be in a solid phase in the film structure, and thus would not be detected by Mössbauer spectroscopy.

The TEM image of floating Fe films, with corresponding EDS maps of Fe and oxygen, indicated that disorganized Fe(III) oxyhydroxide particulates were precipitated around the cell surface (Figs.2A, C, D). Based on the EDS results, carbon along with small amounts of silicon was also the dominant element in floating Fe films (Figs.2C and F), suggesting that biological activities were involved in the formation of floating Fe films (Grathoff et al., 2007; Fernandez-Rojo et al., 2017). The element analysis showed that the Fe films contained 2.5–3.1 % silicon (w/w) and 8.9–10.3 % carbon (w/w) (Table S1). In addition, TEM and EDS analysis revealed some filamentous stalks or sheath morphologies around the bacteria and these distinct morphologies were composed of Fe oxyhydroxide (Fig. 2B). Previous reports have pointed out that these typical morphologies associated with Fe(III) oxyhydroxide are produced by microaerophilic FeOB, such as *Leptothrix* sp. and *Gallionella* sp. (Chan et al., 2011; Chan et al., 2016). The dissolved oxygen concentration below the Fe films indicated that the formation of floating Fe films could develop an anoxic-oxic interface to promote the enrichment and growth of microaerophilic FeOB (Emerson et al., 2010; Fernandez-Rojo et al., 2017; Hädrich et al., 2018). A previous study has confirmed that microaerophilic FeOB that produce stalk or sheath morphologies are ubiquitous in wetlands (Emerson and Weiss, 2004).

3.3. Bacterial community composition of floating films and film-associated water and sediments

Because the physicochemical properties of the three water samples were similar, only one sample location as well as the aquatic plants was selected for microbial analysis. The DNA extracted from sediment, water column, and floating Fe films was used for high-throughput sequencing. In addition, the microbial community around the rhizosphere was analyzed because the oxic-anoxic interface was formed in the rhizosphere and rhizosphere microorganisms were closely related to the Fe (II) oxidation process (Weiss et al., 2005; Xiao et al., 2021). The dominant bacterial phyla were detected across all samples associated with the Fe films (Fig. 3A), and the most abundant phyla were Proteobacteria (42.5–79.4 %), followed by Bacteroidetes (3.6–14.2 %), Chloroflexi (0.2–12.7 %), Firmicutes (0.4–8.3 %), and Actinobacteria (0.3–6.1 %). The abundance of Proteobacteria in sediments was markedly lower than that in other samples, while other dominant phyla were higher in sediments. Previous reports showed that functional microorganisms associated with Fe cycling belonged to Proteobacteria (Weber et al., 2006; Melton et al., 2014; Kappler et al., 2021). Therefore, the high Fe redox activity in different niches, such as floating Fe films and rhizosphere, might contribute to the highly relative abundance of Proteobacteria. According to principal component analysis (PCA), the microbial communities were clearly divided into two groups (Fig. S3). The first group included the microbial communities of sediments, and the second group included the microbial communities of floating Fe films, water column, and the rhizosphere, which were associated with Fe(II) oxidation. The principal coordinate 1 (PC1) and PC2 represented 44.9 % and 17.6 % of the variation in the microbial community, respectively. Generally, the PC1 suggested the differences of the microbial community between the sediment samples and the other samples, while the PC2 highlighted the effect of Fe(II) oxidation on the microbial community. Additionally, the bacterial richness index in sediments was higher than that in floating Fe films and rhizosphere (Fig. 3B), indicating that special niches could enrich distinct microbial communities, thereby reducing the diversity of microorganisms.

At the genus level, the microbial community structures showed

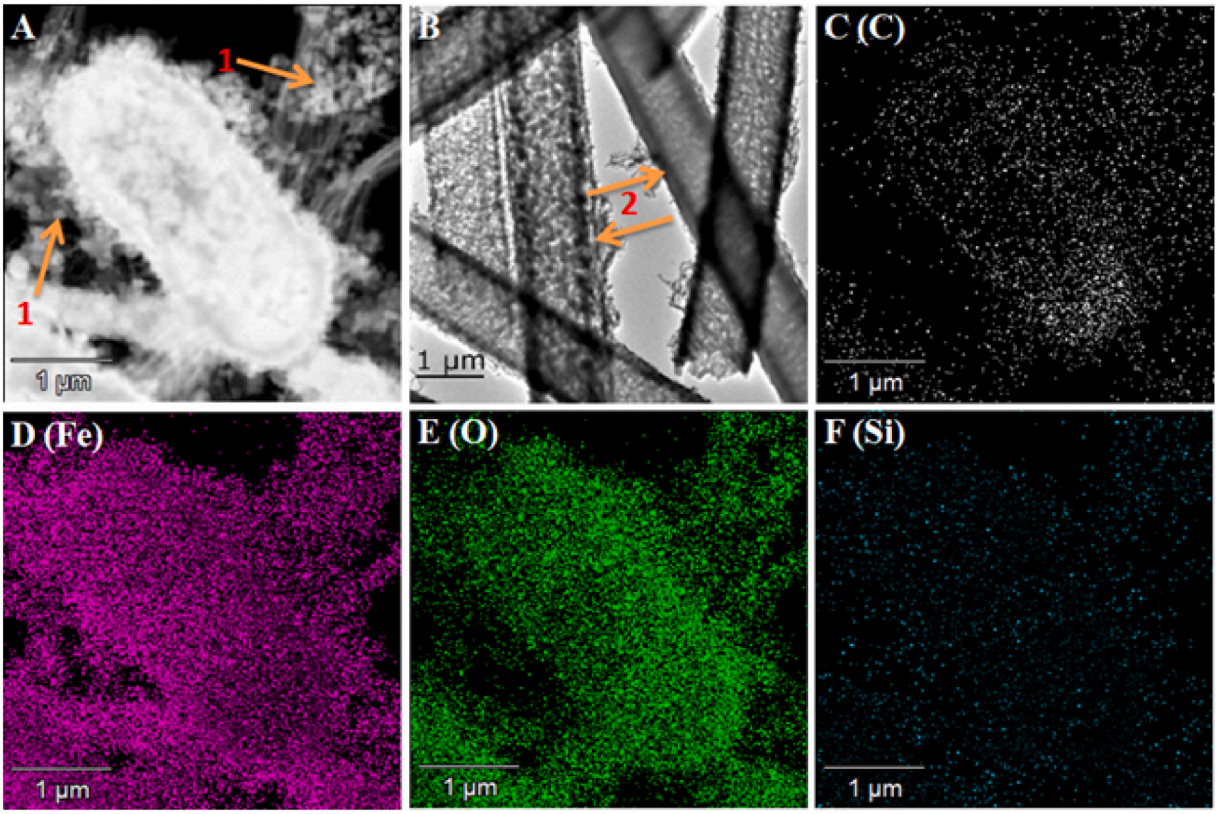

Fig. 2. The TEM image of floating Fe films (A-B) with disorganized Fe-oxyhydroxide particulates (1) and sheaths morphologies (2). The EDS mapping of floating Fe films shows carbon, Fe, oxygen, and silicon distributions in micrographs (C-F).

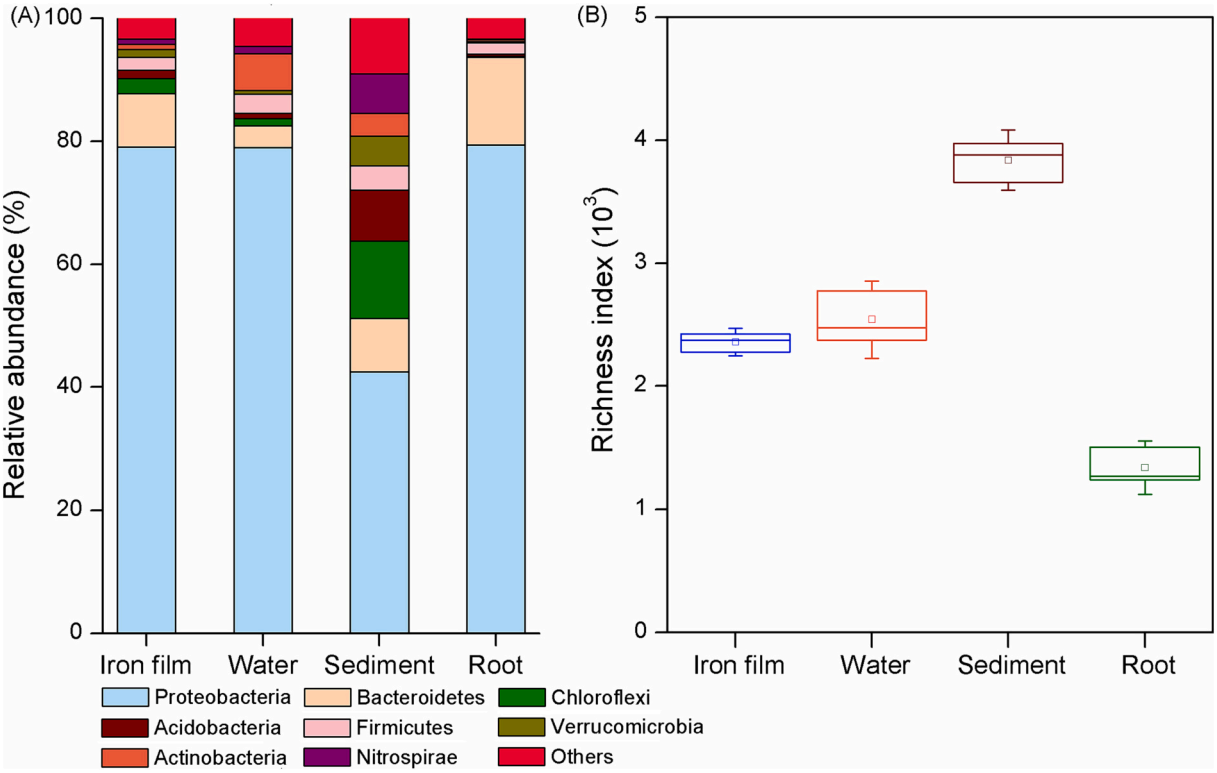

Fig. 3. The relative abundance of the microbial community at the phylum level (A) and Richness index (B). The iron film, water, sediment, and root represent the microbial communities from floating Fe films, water column, sediment, and rhizosphere, respectively.

significant differences among different samples, and the dominant genera were divided into three major clusters (Fig. 4). Cluster I included the dominant genera around the rhizosphere, including *Acinetobacter*, *Comamonas*, *Dyadobacter*, *Gallionella*, *Kaistia*, *Peredibacter*, *Magnetospirillum*, and unclassified *Betaproteobacteriales*. In the floating Fe films, the dominant genera were mainly affiliated to cluster II, such as *Acetobacteroides*, *Geobacter*, *Gallionella*, unclassified *Betaproteobacteriales*, and uncultured *Methylomonaceae*. Additionally, *Hydrogenophaga* and *Leptothrix* were other major representatives in the floating Fe films. The dominant genera in water samples were concentrated in cluster III, which included *Curvibacter*, *Hydrogenophaga*, *Leptothrix*, *Pseudomonas*, and unclassified *Burkholderiaceae* and *Rhodocyclaceae*. The distribution of microbial abundance in sediments was relatively balanced and genera with higher abundances were *Geobacter*, *Dyadobacter*, *Methylophilus*, *Comamonas*, and *Kaistia* (Fig. 4). In floating Fe films and rhizosphere samples that formed Fe oxides in the present study, although the biological Fe(II) oxidation occurred at the oxic-anoxic interface, the dominant genera for Fe(II) oxidation showed a distinct difference because the sediments could provide more nutrients and energy for microorganism growth in the rhizosphere. The organic acids secreted by plant roots and a certain amount of nitrate in sediments can support the growth of heterotrophic FeOB that are not dominant in floating Fe films (Wang et al., 2012; Liu et al., 2019). Since the micro-environments differed greatly between the water surface and rhizosphere, such as pH, oxygen concentration, and Fe oxidation state, and the floating Fe films and water could react and exchange closely through direct contact, the following discussion mainly focused on the microbial community change among the floating Fe films, water column, and sediments.

To assess the taxonomic difference between the floating Fe films and associated water and sediments at the genus level, linear discriminant analysis (LDA) effect size multivariate analysis was used to describe the microbial depletions and enrichments in these samples. The results are shown in Figs.5 and S4. Compared with the microbial community in water column and sediments, *Methylococcales*, *Geobacter*, *Gallionella*, and *Ferriphasclus* were enriched in floating Fe films. These enriched genera were classical FeOB and FeRB (*Geobacter*, *Gallionella*, and *Ferriphasclus*) and methane-oxidizing bacteria (*Methylococcales*). These results suggest that special microorganisms could move to the water surface and adapt to the specific physico-chemical conditions of the floating Fe films with a high availability of nutrients (Sánchez-España et al., 2023). *Lepohtrix*, as a microaerophilic FeOB, was enriched in water but was depleted in sediments. In addition, while *Lepohtrix* was enriched in the floating films, it was at a lower level than that in the water sample. This might be due to the oxic-anoxic interface in the Fe(II)-oxygen opposing gradient from sediments to the water surface (Sobolev and Roden, 2001). The abundances of the enriched FeOB in sediments were low, suggesting that these FeOB were not dominant genera and that sediment environments limited the growth of FeOB. The sub-oxic conditions created by Fe films and Fe-enriched conditions may be the niches occupied by FeOB in wetland environments. *Methylococcales*, as a typical methanotroph, can oxidize CH_4 into organic matter under oxic or microaerobic conditions (Kato et al., 2013a; Quaiser et al., 2014). Although the methane-oxidizing bacteria were not directly involved in the formation of floating Fe films, they could act as primary products to provide necessary organic matter for the growth of heterotrophic FeOB and FeRB. These results indicate that Fe-cycling and methane-oxidizing bacteria may play an important role in floating Fe film formation.

Among the microorganisms detected from floating Fe films, typical FeRB (*Geobacter*, 6.8 %) and neutrophilic FeOB (*Gallionella*, 6.9 %, Ferriphasclus, 1.1 %, and *Leptothrix*, 0.8 %) were found to be the dominant genera, and were thought to be responsible for the coexistence of Fe(II) and Fe(III) and the dominance of different morphotypes. Such microbial community logically differed from that found in floating Fe films from acidic conditions where *Ferrovum*, *Acidithiobacillus*, and *Leptospirillum*, the well-known acidophilic FeOB genera were dominant (Sánchez-España et al., 2023). Difference in microbial communities led to different compositions of Fe oxides in Fe films. For example, ferrihydrite was the dominant minerals in Fe films formed in

Fig. 4. Community structure of bacteria at genus level in four samples that associated with the formation of floating Fe films. The iron film, water, sediment, and root represent the microbial communities from floating Fe films, water column, sediment, and rhizosphere, respectively.

the present study while the Fe films mainly consisted of schwertmannite or jarosite under acidic conditions (Fernandez-Rojo et al., 2017; Sánchez-España et al., 2023). *Gallionella*, *Ferriphasclus*, and *Leptothrix* are known as neutrophilic, microaerophilic FeOB that grow at the oxic-anoxic interface (Kato et al., 2013a; Chan et al., 2016). In the early stage of the formation of floating Fe films, the oxygen consumption by chemical Fe(II) oxidation resulted in low dissolved oxygen partial pressure in the initial floating Fe films and beneath these films (Roden et al., 2004; Weber et al., 2006). The low oxygen concentration promotes the growth of microaerophilic FeOB, which can successfully compete with chemical Fe(II) oxidation, and accounts for 50–60 % of the total Fe(II) oxidation (Neubauer et al., 2002; Rentz et al., 2007). Even in wetlands, biological activity could account for up to 90 % of Fe(II) oxidation with microaerophilic FeOB (Sobolev and Roden, 2001). The biologically formed Fe oxides by *Gallionella* and *Leptothrix* can be morphologically distinct (Fig. 2). These results confirmed the crucial role of microaerophilic FeOB in the formation of floating Fe films. However, these insoluble Fe(III) oxides would be readily available for dissimilatory Fe(III) reduction below the depth of oxygen penetration via chemical oxidation and FeOB activity. *Geobacter* species have been described as the first dissimilatory Fe(III) reducers, and members of the genus *Geobacter* are capable of coupling the oxidation of organic matter to Fe(III) reduction (Coates and Lovley, 2015). The clustering of *Geobacter* species within their exudates, which are mostly composed of extra-polymeric substances, can form a biofilm that accelerates electron transport between Fe(III) reducers and Fe(III) oxides and adsorbs some FeOB to avoid the encrustation of the cell surface with the Fe(III) minerals that they produce (Schädler et al., 2009; Esther et al., 2015). Therefore, a unique potential mechanism is that the activity of FeOB might regulate the Fe(III)-Fe(II)-oxygen reaction system, so as to generate preferable conditions for rapid microbial Fe(II) oxidation and reduction at the microscale (Sobolev and Roden, 2001; Yang et al., 2021). These processes may minimize the reaction zones between the sites of Fe(II) oxidation and reduction, leading to the enrichment of FeOB and FeRB in floating Fe films.

In addition to FeOB and FeRB, the methanotrophs (*Methylococcales*, 12.8 %) were also the major bacteria in the floating Fe films. *Methylococcales*, together with the *Geobacter*- and *Gallionella*-related bacteria, have been found in Fe-rich flocs, flooded wetlands, and freshwater (Wang et al., 2012; Kato et al., 2013a, 2013b; Myllykangas et al., 2020). Previous reports have shown that these methanotrophs can thrive at the redox interface and utilize CH_4 as a carbon and energy source (Semrau et al., 2010; Wang et al., 2011). Methanotrophs likely act as primary producers to provide energy for heterotrophic bacteria in floating Fe films, such as FeRB. Patzner et al. (2022) reported that the increasing abundance of classical FeRB was accompanied by an increase in the relative abundance of methanotrophs in the mineral-rich layer. Because the environmental distribution and nutrient demands of methanotrophs are similar to those of microaerophilic FeOB, methanotrophs exhibit a very similar distribution to FeOB in flooded wetlands (Wang et al., 2012; Quaiser et al., 2014). Laboratory culture has also revealed that autotrophic FeOB have similar growth rates compared to methanotrophs (Dedysh et al., 2007; Weiss et al., 2007; Belova et al., 2011). In the present study, although the CH_4 concentrations in water or floating Fe films were unknown, another dominant genus *Acetobacteroides* (2.5 %) could promote CH_4 production in floating Fe films and/or the surrounding water (Zhang et al., 2015; Ozbayram et al., 2017). These results indicate that microbial Fe(II) oxidation and CH_4 oxidation occur simultaneously in floating Fe films and that both groups of microorganisms are major players in the formation of floating Fe films under

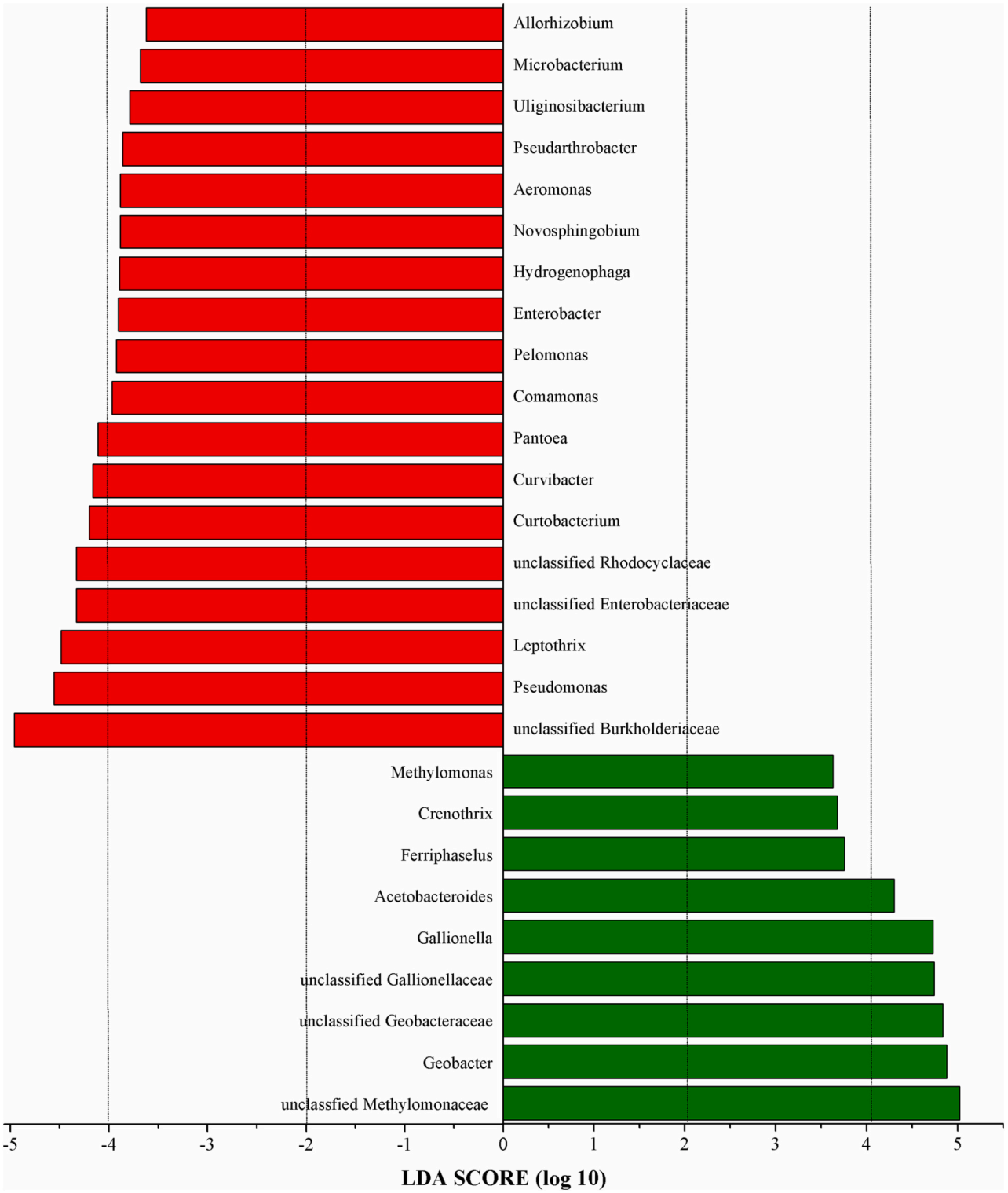

Fig. 5. LEfSe measurements identify differentially abundant bacteria between the floating Fe films and water columnsamples at genus-level taxa. The LDA score is presented as the average value of the three replicates.

circumneutral conditions.

As discussed above, the dominant redox processes associated with the formation of Fe films were microbial Fe(II) oxidation, Fe(III) reduction and carbon fixation. To evaluate the potential contributions of these processes, the copy numbers of *Gallionella*-related FeOB, *Geobacter*-related FeRB and carbon fixation genes (summary of *cbb*L and *pmo*A ratios) were determined via qPCR (Fig. 6). The results showed that the relative abundances of Fe-cycling-related bacteria in Fe films were significantly higher than that in water and sediment samples in all sampling locations. The rations of *Gallionella*-related FeOB in iron films ranged from 11.8 % to 15.8 % for different locations, which were significantly higher that the results of previous reports of paddy soil and wetlands (Xiao et al., 2021; Watanabe et al., 2021). In this study, the high ratios of *Gallionella*-related FeOB were consistent with the enriched

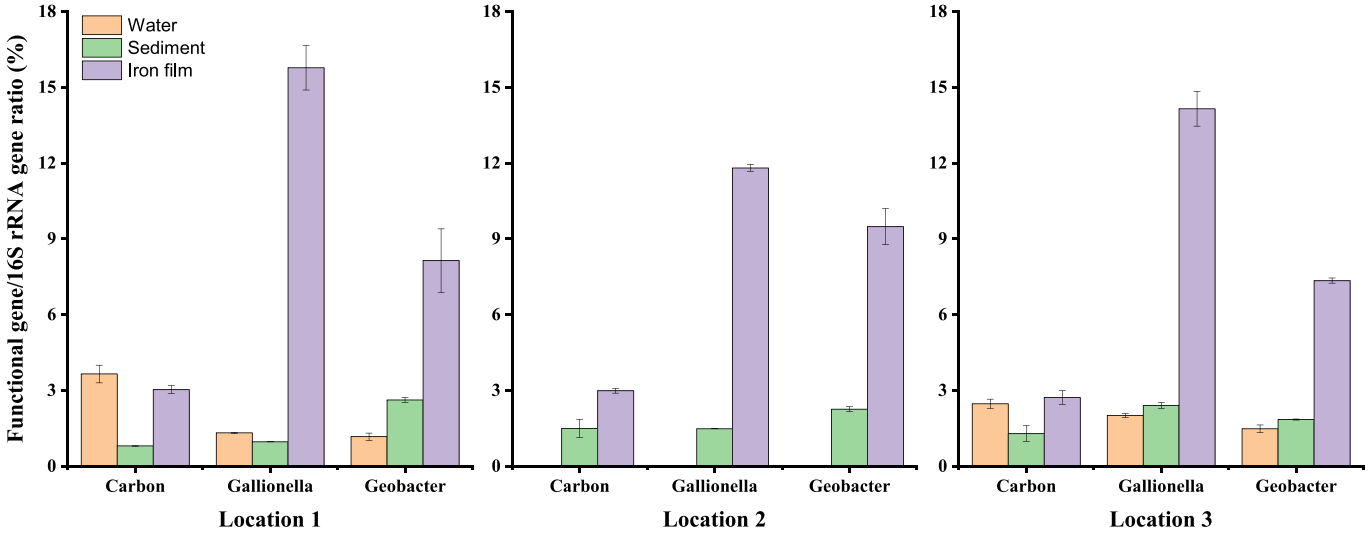

Fig. 6. The ratios of *Gallionella*-related FeOB, *Geobacter*-related FeRB and carbon fixation genes to total bacteria of 16S rRNA genes from water, sediment and Fe films in different sampling locations. The carbon represented the summary of ratios of *cbb*L and *pmo*A to total bacteria of 16S rRNA genes. The iron film, water, sediment, and root represent the microbial communities from floating Fe films, water column, sediment, and rhizosphere, respectively.

microaerophilic FeOB from 16S rRNA data. These high ratios suggest that *Gallionella*-related microorganisms are potential contributors to Fe (II) oxidation, leading to the formation of Fe films. Similar trends were observed in the changes of *Geobacter*-related FeRB, suggesting the important role of FeRB in the formation of Fe films. Additionally, the ratio of *Gallionella*-related FeOB was much higher than *Geobacter*-related FeRB (Fig. 6), indicating the microbial Fe(II) oxidation was competed with the Fe(III) reduction, thereby accumulating a certain amount of Fe (III) oxyhydroxides. The ratios of carbon fixation genes were similar in water column and Fe films, but higher than that in sediment. The *cbb*L might be associated with microorganisms that can fuel autotrophic CO_2 with light and/or Fe(II) as electron donors while *pmo*A might be involved with *methanotrophs* that can assimilate CH_4 (Berg, 2011; Ma et al., 2013). The higher ratios seem to be more active with microbial CH_4 and CO_2 fixation to provide energy for the growth of heterotrophic microorganisms in water column and Fe films.

3.4. Proposed biological pathway for the microbial formation of floating Fe filmsunder circumneutral conditions

In the freshwater systems, an aggregate-enriched biofilm environment with distinct microbial communities could be formed at the boundary interface between the atmosphere and water (Cunliffe et al., 2011; Sánchez-España et al., 2023). The microbial populations in the film can impact gas and nutrient exchange through specific biogeochemical processes mediated by particular microbial groups such as methanotrophs and Fe-metabolizing bacteria (Cunliffe et al., 2011). The presence of floating films observed in acidic water has been attributed to the presence of acidophilic FeOB such as *Ferrovum*, *Acidithiobacillus*, and *Leptospirillum*. However, the microbial communities in the studied floating Fe films have rarely been subject to specific study under circumneutral conditions. It has been reported that the mechanisms for the formation of floating Fe films involve chemical and microbial oxidation, radical reduction, photo-redox cycling, and mineral transformation (Grathoff et al., 2007; Sobolev and Roden, 2001; Perkins et al., 2016). The present study focused on the microbial diversity and composition of floating Fe films and their effects on the formation of floating Fe films under circumneutral conditions. As illustrated in Fig. 7, the microbial carbon and Fe cycling by special microorganisms could maintain the growth of floating Fe films either as a unique phase or potentially as an intermediate carrier for geochemical cycling of other nutrient elements in wetlands. Usually, these Fe films are found in wetlands where Fe(II)-

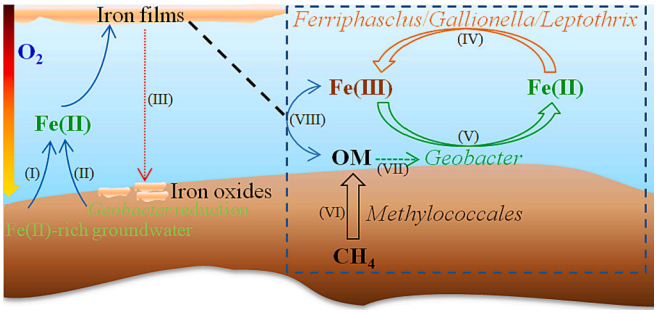

Fig. 7. Schematic illustration of possible biological pathway for formation and persistence of floating Fe films in water-sediment system. Brown represented the sediment at the bottle while the blue represent water phase. The dotted square represented the reactions that may occur in the floating Fe films. (I) and (II) the Fe(II) pumping from groundwater and Fe(III) reduction; (III) the precipitation and deposition of Fe oxides; (VI) microbial Fe(II) oxidation; (V) microbial Fe(III) reduction; (VI) microbial methane oxidation; (VII) the organic matter as carbon source for microbial Fe(III) reduction; (VIII) formation the floating Fe films contained organic matter.

rich groundwater is discharged and pumped to the water surface, as illustrated in process (I) in Fig. 7 (Kleja et al., 2012). In this study, the high concentrations of Fe(II) in pore water can diffuse to the water surface for biological Fe(II) oxidation (Table S1). Furthermore, these films are believed to be the precursor phases for the formation of a solid phase during Fe(II) oxidation. After deposition and precipitation, the solid phase could be reduced by dissimilatory FeRB and could act as an Fe(II)-source for the further formation of Fe films (processes II and III). In sediments, the abundance of *Geobacter* (1.2 %) was much higher than those of other genera (Fig. 4), indicating that the microbial Fe reduction process was active. Meanwhile, the extractable Fe(II) from poorly crystalline phases may also be released into water due to the reduction of Fe oxides (Tables S1, Wang et al., 2011). These potential Fe(II) sources could provide more Fe(II) to the formation of floating Fe films. These processes constitute the complete Fe cycle during the formation and deposition of floating Fe films in wetlands and reveal the strong links among Fe and other nutrient elements.

During the formation of floating Fe films, the oxygen consumption by chemical Fe(II) oxidation and diffusion-limited oxygen transport by Fe films could lead to the low partial pressure of oxygen which created

microaerobic conditions for the growth of microaerophilic FeOB. As discussed in Section 3.3, the microaerophilic FeOB, such as *Gallionella*, *Ferriphasclus*, and *Leptothrix*, were the dominant genera involved in microbial Fe(II) oxidation (process IV). These genera could produce distinct twisted stalks and sheath structures associated with Fe oxides (Chan et al., 2011, 2016), which was consistent with the observations in the present study. In the presence of FeOB activity, anaerobic microzones might be generated on the periphery of the Fe films, resulting in the activity of FeRB at some distance from the water surface (Sobolev and Roden, 2002). The results showed that FeRB associated with *Geobacter* had high abundances in the Fe films and a small amount of Fe(II) was detected (process V). Compared to the Fe (II) concentration in the sediments or pore water, the lower dissolved Fe(II) concentration in Fe films may be due to the acceleration of Fe(II) scavenging by microaerophilic FeOB activity. The high abundances of microaerophilic FeOB and FeRB in the floating Fe films suggest that microbial Fe(II) oxidation and reduction activities co-exist in the formation of floating Fe films. Because FeRB requires organic matter as carbon source to trigger the Fe (III) reduction, special microorganisms that can produce organics from inorganic carbon to support FeRB growth are believed to play an important role in the formation of Fe films. The present study showed that methanotrophs that could utilize CH_4 as a carbon and energy source to produce organics were enriched in Fe films (process VI). These organics can not only provide energy for Fe cycling, but also form Fe oxide-organic matter (ferrihydrite-OM, processes VII and VIII) that suppresses the formation of higher crystalline Fe(III) phases and the hydrolysis of Fe oxides (Perkins et al., 2016; Adusei-Gyamfi et al., 2019). Additionally, the presence of organic matters may promote the formation of small ferrihydrite nanoparticles by hampering the nucleation or polymerization reactions, which is beneficial for stabilizing the Fe films (Kleja et al., 2012; Gentile et al., 2018). The discussion of the dominant genera in the floating Fe films indicates the co-contribution of the heterotrophic and autotrophic microbial communities to Fe transformation and the formation of floating Fe films.

4. Conclusions

In wetlands investigated in this study, the floating Fe films formed at circumneutral pH (6.3–7.0) at the interface of oxygen and Fe(II) gradientsfrom Fe(II)-bearing sediment and groundwater. Ferrihydrite was the dominant Fe(III) phase in Fe films, accompanied bymoderate levels of carbon and silicon.The Fe film morphology with stalk and sheath structures supports the widespread distribution of neutrophilic, microaerophilic FeOB in wetlands. The microbial community structure of floating Fe films showed that the dominant genera were FeOB, FeRB, and methanotrophs, including *Leptothrix*, *Ferriphasclus*, *Gallionella*, *Geobacter*, and *Methylococcales*. *Leptothrix*, *Ferriphasclus*, and *Gallionella*, as classical microaerophilic FeOB, can oxidize Fe(II) with limited oxygen and form special structures that are closely associated with the formation of Fe films. The presence of *Geobacter* would provide a source of Fe (II) for FeOB growth and the *Methylococcales* would result in CH_4 oxidation to provide energy for Fe cycling. The high ratios of *Gallionella*- and *Geobacter*-related microorganisms and carbon fixation genes confirmed the contribution of potential of Fe cycling and autotrophic microbial community to the formation of Fe films. The suboxic and anoxic microenvironments within or beneath the Fe films may stimulate more localized cycling of Fe and carbon (Kato et al., 2013a). Although Fe cycling and CH_4 oxidation are not necessarily linked, the co-occurrence of these processes in wetlands indicates that Fe film formation might promote carbon fixation as well as other nutrient elements, which requires further investigation. Based on the enrichment of microaerophilic FeOB, FeOB are believed to be responsible for the deposition of Fe(III) phase and the formation of Fe films in wetlands. However, more work is needed to isolate and identify these functional microorganisms from floating Fe films, which would provide a better understanding of the microbial mechanism of Fe film formation and would help to establish the networks between Fe cycling and other nutrient elements in wetlands.

CRediT authorship contribution statement

Hui Tong: Conceptualization, Methodology, Supervision, Writing-review & editing, Funding acquisition. Leheng Dong and Manjia Chen: Methodology, Writing-original draft, Investigation, Writing-review & editing. Chengshuai Liu and Xugang Wang: Supervision, Writing-review & editing. Yahui Lv: Date curation, Investigation. Qingkai Lei: Investigation, Sample collection. Yujuan Fang: Investigation.

Declaration of competing interest

The authors declare no competing financial interests or personal relationships that would influence the work presented in this research.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

The sampling locations (Fig. S1); sample collection (Fig. S2); PCA compares microbial composition to four samples (Fig. S3); LEfSe measurements identify differentially abundant bacteria between the floating Fe films and water samples (Fig. S4); parameters of associated water and sediment (Table S1); Mössbauer spectral fitting parameters (Table S2). Supplementary data to this article can be found online at doi:https://doi.org/10.1016/j.scitotenv.2023.167711.

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