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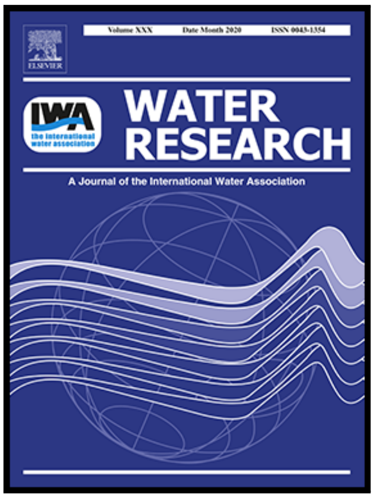

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Highlights

- State-of-the-art of Fe(0)/Fe(II) mediated autotrophic denitrification are reviewed.
- Various iron sources, including liquid-phase and solid-phase, are discussed.
- Mechanisms of Fe(II) oxidization and microbial interactions are comprehensively analyzed.
- Advancements in natural and engineering application are presented.
- Key factors influencing denitrification performance are discussed.

A review in Fe(0)/Fe(II) mediated autotrophic denitrification for low C/N wastewater treatment

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Abstract

Fe-mediated autotrophic denitrification has emerged as a promising technology for low carbon-to-nitrogen (C/N) wastewater treatment due to its cost-effectiveness, operational safety, and independence from organic carbon. While several reviews have addressed this process, there is still a lack of comprehensive analyses that connect iron materials, denitrification performance, and microbial communities. The practical applicability of solid-phase iron has received little attention, and the key controversial issue - Fe(II) oxidation pathway - has not been specifically examined. This review systematically examines both liquid- and solid-phase iron sources, with particular emphasis on the practical applicability of solid-phase iron, and further provides an integrated discussion of denitrification performance and associated functional microbes. In addition, this review summarizes a range of iron-oxidizing denitrifiers and highlights several key genera in detail. It also provides an in-depth analysis of Fe(II) oxidation pathways, with particular attention to the ongoing debate regarding the involvement of enzymatic mechanisms. Moreover, the latest advancements in both natural and engineered applications are reviewed. Operational parameters such as temperature (T), pH, dissolved oxygen (DO), Fe/N ratio, and other influencing factors are discussed. Finally, several critical challenges that fundamentally affect Fe-mediated autotrophic denitrification are highlighted. This review aims to support the practical implementation of Fe-mediated autotrophic denitrification in low C/N wastewater treatment and contribute to the sustainable development of environmentally friendly biotechnologies for advanced nitrogen control.

Keywords

Autotrophic denitrification; Fe; Fe(II); Microbes; nitrate removal; cell encrustation

Contents

1. Introduction

Nitrate pollution is one of the most serious water quality issues worldwide, resulting from extensive agricultural and human activities (Hu et al., 2020). Excessive agricultural fertilization, septic tanks, wastewater treatment plants, and other activities can contribute to elevated nitrate levels in water bodies (Modi and Kasher, 2024; Zirrahi et al., 2024). Excessive nitrate damages the natural ecosystem and pose a threat to public health (Bai et al., 2023a). The World Health Organization (WHO) recommends a maximum nitrate level of 50 mg/L NO_3^- (11.3mg/L NO_3^--N) for drinking water (Zirrahi et al., 2024). Therefore, water contaminated with nitrate must undergo appropriate treatment to meet the requirements before being discharged into surface water and groundwater due to its potential risks.

Compare with physicochemical processes such as ion exchange, adsorption, reverse osmosis and chemical reduction (An et al., 2024; Lu et al., 2024), biological denitrification requires limited energy and has demonstrated high selectivity for nitrogen gas (N_2). This process utilizes nitrate as the electron acceptor and requires electron donors to provide reducing power. It is further categorized into heterotrophic and autotrophic denitrification, depending on whether organic or inorganic electron donors are used (Pang and Wang, 2021b). Heterotrophic denitrification is a well-established technology; However, external carbon sources are required when the BOD_5/TN ratio is less than 2.47, inevitably increasing operational costs and the risk of secondary pollution (Peng et al., 2020a). Moreover, the high sludge yield is another drawback of heterotrophic denitrification, leading to more complex post-treatment processes (Deng et al., 2020b). As a result, autotrophic denitrification has emerged as an alternative approach to address these potential problems.

Autotrophic denitrification mainly involves H_2-mediated, S-mediated, and Fe-mediated autotrophic processes, which utilize H_2, reduced sulfur compounds (H_2S, HS^-, S^{2-}, S^0, $S_2O_3^{2-}$),

Fe^{2+}/Fe^0, as well as iron sulfides (FeS, $Fe_{1-x}S$ and FeS_2) as electron donors (Pang and Wang, 2021b). Although H_2 serves as a clean, low-biomass yield electron donor with a high energy content, its low solubility and explosiveness causes low bioavailability and safety risks, thereby restricting its application (Chen et al., 2023; Prosnansky et al., 2002). Meanwhile, the inevitable alkalinity consumption and sulfate production in sulfur autotrophic denitrification require proper control strategies for its practical application (Pan et al., 2022).

Fe-mediated autotrophic denitrification is a process that utilizes Fe^0 or Fe^{2+} as electron donors to reduce NO_3^- or NO_2^- into N_2 via autotrophic denitrifying bacteria. This process is widespread in natural environments, such as in mineral water springs (Hegler et al., 2012), marine sediments (Laufer et al., 2016b), lake sediments (Pang et al., 2021), and soil (Li et al., 2016). Meanwhile, many engineering systems also utilize various forms of iron sources, including solid-phase (iron scraps, zero-valent iron (ZVI), iron powder, iron sulphides), or liquid-phase (Fe-EDTA, $FeCl_2$, $FeSO_4$) sources, to treat nitrate-containing wastewater (Chen et al., 2023; Di Capua et al., 2019). The Fe-mediated autotrophic denitrification process shows promising potential for practical application due to its high safety, low-cost iron sources, absence of secondary pollution, and the ability of its reaction products to adsorb phosphorus and heavy metals.

Microbes are a key component of the Fe-mediated autotrophic denitrification process. Straub first reported the simultaneous oxidation of ferrous iron with nitrate reduction in 1996 (Straub et al., 1996). Since then, more Fe(II)-oxidizing denitrifiers have been isolated over 30 years, although most of them require additional organic matters as co-substrates (Bryce et al., 2018; Chen et al., 2023). Whether the reduction process of these "mixotrophic bacteria" is an enzyme-catalyzed biological process or a chemical reaction between ferrous and active nitrogen intermediate products remain a controversial issue (Carlson et al., 2013; Klueglein et al., 2014; Pang et al., 2021). Current research still lacks strong evidence to prove that Fe (II)-

oxidizing nitrate-reducing bacteria or cultures are completely autotrophic (Tian and Yu, 2020). In addition, there is a need for a comprehensive introduction and comparisons of different iron-related electron donors, as less attention has been paid to solid-phase iron sources. These solid-phase sources hold significant potential for engineering applications, but their low solubility and dissolution rate must be considered for microbial utilization. Meanwhile, although reviews have summarized Fe(II)-oxidizing denitrifiers under mixotrophic and strictly autotrophic conditions, most of the reported microbes rely on liquid-phase Fe(II) electron donors ($FeSO_4$ and $FeCl_2$) for nitrate reduction (Kiskira et al., 2017a). A comprehensive exploration of microbes capable of utilizing solid-phase iron, along with corresponding denitrification rates under various electron donors, has not been sufficiently explored.

Therefore, this review presents the current knowledge on Fe-mediated autotrophic denitrification and provides an overview of various iron sources and iron-oxidizing bacteria, along with their Fe oxidation mechanisms. Following this, the natural occurrence and engineering applications are summarized. Moreover, the key influencing factors from environmental and water quality perspectives, among others are discussed. Finally, several key challenges of the Fe-mediated autotrophic denitrification are highlighted to promote its sustainable application in the future.

2. Biochemistry of iron-mediated autotrophic denitrifications

2.1 Biochemistry

Iron (Fe), an abundant redox-active metal in the natural environment, is utilized in wide range of environmental processes, involving electron transfer between Fe^0 and Fe^{2+}/Fe^{3+}, which profoundly impacts contaminant removal and biogeochemical cycling (Ahn et al., 2008; Wu et al., 2024). Fe^0 is oxidized in anaerobic environment to form Fe^{2+}, resulting in the reduction of water to produce H_2 (Eq. 1) (Kielemoes et al., 2000). It has been shown that *Paracocus denitrificans* and mixed cultures can utilize hydrogen from iron corrosion as an electron donor and energy source for autotrophic denitrification (Eq. 2) (Till et al., 1998; Kielemoes et al., 2000).

In addition, Fe-mediated autotrophic denitrification involves both biotic and abiotic reactions, which influence nitrate removal and products selectivity. The NH_4^+ is the dominant product in the abiotic process when Fe^0 is used as an electron donor in an acidic solution (Eq. 3). Over 93% NH_4^+ selectivity (reduction of nitrate to ammonia) has been observed in many previous studies (Song et al., 2020; Suzuki et al., 2012; Zhao et al., 2022). However, autotrophic denitrification with microbial assistance (Eq. 4) is thermodynamically more feasible than abiotic reduction (Eq. 3) (Till et al., 1998; Kielemoes et al., 2000). The combination of anaerobic Fe^0 corrosion (Eq. 1) and hydrogenotrophic denitrification (Eq. 2) is more favourable for producing N_2, while NH_4^+ production cannot be avoided due to abiotic reduction (Eq. 3) (Till et al., 1998). Therefore, strategies are still needed to minimize the ammonia selectivity.

$$Fe^0 + H_2O \rightarrow H_2 + Fe^{2+} + 2OH^- \qquad (1)$$

$$2NO_3^- + 5H_2 \rightarrow N_2 + 4H_2O + 2OH^- \qquad (2)$$

$$4Fe^0 + NO_3^- + 7H_2O \rightarrow 4Fe^{2+} + NH_4^+ + 10OH^- \quad \Delta G^{0\prime} = -620kJ \qquad (3)$$

$$5Fe^0 + 2NO_3^- + 6H_2O \rightarrow 5Fe^{2+} + N_2 + 12OH^- \quad \Delta G^{0\prime} = -1147kJ \qquad (4)$$

$$5Fe^{2+} + NO_3^- + 7H_2O \rightarrow 5\mathrm{FeOOH} + 0.5N_2 + 9H^+ \quad \Delta G^{0\prime} = -277\ kJ/mol \qquad (5)$$

Fe(II) can also serve as an electron donor in autotrophic denitrification for nitrate removal, as described in Eq. 5, a process known as nitrate-dependent anaerobic ferrous oxidation (NAFO) (Kiskira et al., 2017a). This process converts Fe(II) to Fe(III), and electrons are transferred to nitrate to produce N_2, with nitrogen intermediates such as NO_2^-, NO, and N_2O potentially forming as by-products (Wang et al., 2023). In addition, phosphorus and other pollutants (such as arsenic, zinc) can be simultaneously removed in situ through co-precipitation or adsorption by Fe(III) (hydr)oxides (Hu et al., 2020; Wang et al., 2023).

2.2 Iron sources

Different iron sources strongly affect the kinetics of biological denitrification. The Fe(0)/Fe(II) used in autotrophic denitrification can be derived from various iron sources, including solid-phase and liquid-phase sources (Table 1). Liquid-phase iron sources such as $FeCl_2.4H_2O$, $FeSO_4$, and Fe(II)-EDTA have been used as electron donors for the NAFO process (Kiskira et al., 2017b; Su et al., 2019; Tian and Yu, 2020). Kiskira et al. (2017b) investigated Fe(II)-mediated autotrophic denitrification by different denitrifying bacteria using $FeCl_2.4H_2O$ as the iron source. The results showed that the nitrate removal rates of activated sludge, *Pseudogulbenkiania* strain 2002, and *Thiobacillus* denitrificans were 0.321, 0.13, and 0.362 mM/g VSS/day, respectively, when Fe(II) was used as the sole electron donor. Furthermore, in large-scale biofilters based on sponge iron, the addition of Fe(II)-EDTA can prevent cell encrustation while achieving a nitrate removal rate of 90%, whereas the addition of $FeSO_4$ alone can only obtain 12.22% (Zhou et al., 2016). Although Fe^{2+} is cheaper compared to acetic acid, methanol, or hydrogen, its high specific substrate utilization rate, at 19.9 kg electron donor/kg NO_3^--N, means that a significant amount of supplementation is required to sustain continuous nitrate removal, thereby increasing complexity and the operation cost (Di Capua et al., 2019).

Compared with liquid-phase iron sources, solid-phase iron sources facilitate microbial attachment and growth (Wang et al., 2020a). They also offer economic benefits by utilizing abundant reserves of iron minerals and recycling waste materials, which provide great potential for practical applications in Fe-mediated autotrophic denitrification. Sponge iron, iron filings, and iron shavings are various forms of Fe^0, each containing over 90% Fe^0 content (Liu et al., 2020; Wang et al., 2019; Wang et al., 2022b). They are more transportable and storable compared to ferrous ion, although they may develop an iron oxide layer due to improper storage or natural oxidation. These solid-phase Fe^0 sources can continuously and effectively produce Fe^{2+} and electrons, eliminating the need for intermittent supplementation of ferrous salts to maintain continuous denitrification (Wang et al., 2022b). It was shown that denitrifying bacteria can not only directly extract electrons from Fe^0 for autotrophic denitrification, but the Fe^{2+} produced can also continue to serve as an electron donor to drive the NAFO process (Zhang et al., 2017). Using sponge iron as the electron donor, Wang et al. (2022b) achieved a nitrate removal rate of over 90% within 25 hours (initial 50 mg/L NO_3^--N) in batch experiments. Furthermore, a stable nitrate removal rate of 38% was maintained after 90 days in long-term bioreactor operations. Liu et al. (2020) achieved an average nitrate removal rate of 90.2% over 156 days of operation (initial 20 mg/L NO_3^--N) by using iron scraps as a substrate to further reduce nitrate in secondary effluent.

Research on solid-phase Fe(II)-mediated autotrophic denitrification has been relatively limited (Wang et al., 2020a). Minerals such as illite, nontronite, siderite, and natural $FeCO_3$, and iron sulfides which contain reduced iron or both reduced iron/sulfur, have been explored for their roles in biological redox iron cycling and their potential for nitrate removal (Yang et al., 2018; Zhao et al., 2017; Zhao et al., 2013; Zhu et al., 2019). *Pseudogulbenkiania* sp. strain 2002 was capable of linking the oxidation of structural Fe(II) in montmorillonite (IMt-1) with the reduction of 6.5 mM nitrate to N_2, showing a higher denitrification rate than other iron minerals (Zhao et al., 2017). The autotrophic enrichment culture KS can utilize Fe^{2+} in the form of microbially reduced goethite, biogenic magnetite (Fe_3O_4), and chemically precipitated siderite ($FeCO_3$), though its ability to oxidize biogenic siderite is limited (Weber et al., 2001). More importantly, these iron minerals are abundant in nature and cheap, with siderite costing only $60 per ton, making them more accessible in practical applications (Yang et al., 2018). While the consumption of electron donor is as high as 10 and 19.9 kg electron donor/ NO_3^--N for Fe^0 and Fe^{2+}, the associated cost is minimal compared to other electron donors. However, it is still unclear how microbes utilize solid-phase iron minerals in Fe-mediated autotrophic denitrification. Previous studies have shown that electron transfer can occur through direct contact between *Pseudogulbenkiania* sp.strain 2002 and nontronite, and the degree of bio-oxidation varies significantly among different minerals (Zhao et al., 2017). Meanwhile, there are different hypotheses explaining how bacteria utilize pyrite (Pang and Wang, 2021b). Hu et al. (2020) proposed that FeS_2 is initially attacked by Fe^{3+}, leading to its dissolution into $S_2O_3^{2-}$ before being utilized by denitrifiers, while Yan et al. (2019a) suggested that denitrifiers might only utilize the element sulfur (S^0) on the surface of FeS_2. Pang et al. (2020) also proposed that FeS_2 is first oxidized to bioavailable sulfur through microbially produced electron shuttles, and then utilized by sulfur-oxidizing denitrifiers. Although Fe(II) is involved in the autotrophic denitrification process when pyrite is used as the electron donor, most studies primarily focus on microbial sulfur utilization, while the regulatory role and utilization pathways of Fe(II) are often overlooked.

2.3 Iron oxidizing denitrifiers

2.3.1 Diversity of iron-oxidizing denitrifiers

The Fe(II)-oxidizing denitrifier was first found by Straub in 1996 (Straub et al., 1996). Since then, more bacteria have been identified or isolated that can obtain energy from ZVI, divalent iron salts or iron minerals for nitrate reduction, which are summarized in Table 2. The common criteria for strictly autotrophic denitrifying microorganisms are those that: (i) require no organic carbon source, (ii) show cell growth with only Fe(II), nitrate, and CO_2 provided, (iii) maintain Fe(II) oxidation over several transfers without the addition of organic carbon, and (iv) demonstrate labelled CO_2 uptake into biomass during Fe(II) oxidation (Bryce et al., 2018; Wang et al., 2023). To date, only the enriched culture KS has been proven to be a strict autotrophic Fe(II)-oxidizing denitrifying consortium. The dominant genus of the KS culture is *Gallionellaceae*, accompanied by complementary heterotrophic communities, and it can grow with nitrate as the sole electron acceptor, CO_2 as the sole carbon source, and ferrous iron as the sole electron donor (Pang et al., 2021). Since the KS culture was isolated, it has been continuously cultivated for over 20 years in two laboratories: the University of Tuebingen, Germany (culture KS-Tueb) and the University of Madison, USA (culture KS-Mad), with transfers of 1% and 10% (vol/vol), respectively (He et al., 2016). Interestingly, due to differences in inoculation concentrations, the relative abundance of the *Gallionellaceae* strain is 96% in KS-Tueb and 42% in KS-Mad, with significant variation in the heterotrophic flanking communities (Tominski et al., 2018). Previous studies have shown that *Gallionellaceae* sp. lacks NO and N_2O reductase genes, while heterotrophic flanking species possess complete denitrification pathways (He et al., 2016).

Therefore, it is hypothesized that autotrophs fix CO_2 for the heterotrophic community members, while the heterotrophic organisms detoxify nitric oxide for the autotrophs. This cooperative relationship is supported by several metabolite-substrates interactions (Fig. 1). The first one is, organic carbon: *Gallionellaceae* oxidizes Fe(II) to gain energy and fixes CO_2 into organic matter. These autotrophically produced organics serve as carbon and energy sources for heterotrophs. Second, *Gallionellaceae* may accumulate N intermediate products due to the lack of NO and N_2O reductase genes. These N intermediates can be further reduced to N_2 by heterotrophs, serving a detoxification role. Third, heterotrophs can produce extracellular polymeric substances (EPS) to form closely associated aggregates with *Gallionellaceae*, providing physical protection and reducing encrustation and environmental stress. The inability to isolate a pure autotrophic Fe(II)-oxidizing culture highlights the interdependence of the autotrophic culture on the heterotrophic flanking species. In addition, the microbial communities in Fe(II)-mediated autotrophic bioreactors inoculated with anaerobic granular sludge and activated sludge are similar to those identified in the enrichment KS culture (Wang et al., 2017; Zhang et al., 2016).

Thiobacillus denitrificans is a well-known autotrophic denitrifier belonging to the *Proteobacteria*, capable of using a range of reduced sulfur compounds and iron sulfides for the reduction of various forms of oxidized nitrogen, such as NO_3^-, NO_2^-, N_2O, etc (Hu et al., 2020). It has also been demonstrated to drive the NAFO process using Fe^{2+} as the electron donor (Pang and Wang, 2021a). Although *T.denitrificans* can continuously oxidize ferrous without organic carbon sources, there is currently no evidence of cell growth or uptake of labelled CO_2 (Bryce et al., 2018). Similarly, other isolated strains, such as *Azozrus* Strain ToN1, *Pseudomonas stutzeri*, *Aquabacterium* Strain BrG2, *Marinobacter*-related species, and *Hyphomonas*-related species, have demonstrated the ability to autotrophically oxidizing Fe(II) without requiring acetate as an additional electron donor or carbon source (Table. 2). However, there is no information available regarding the other three criteria. In addition, the *Acidovorax* genus, belonging to the β- Proteobacteria, has been proven unable to maintain sustaining Fe(II) oxidation across multiple culture transfers (Chakraborty et al., 2011).

Autotrophic NAFO bacteria, which utilize ferrous ions as an electron donor to obtain energy and fix carbon dioxide without the need for organic carbon, are relatively rare. As shown in Table 2, most of the bacteria capable of utilizing Fe(II) for growth and autotrophic denitrification have not been proven to be completely independent of an extra organic carbon source. In natural environments, mixotrophic iron-oxidizing bacteria are more commonly found. Pang et al. (2022) highlighted the presence of mixotrophic NAFO bacteria in diverse settings such as rivers, lake sediments, water treatment reactors, and constructed wetlands, primarily belonging to α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, and δ-Proteobacteria. It has been observed that, in the presence of organic carbon, 90% of nitrate-reducing bacteria can oxidize ferrous ions, indicating that NAFO has considerable potential for practical applications (Pang et al., 2022).

2.3.2 Mechanisms of Fe(II) oxidation

As the oxidation mechanism of zero-valent iron has been covered in Section 2.1, this section primarily discusses the oxidation mechanism of Fe(II). There are three distinct ways for NAFO bacteria interact with Fe(II) (Fig. 2). First, autotrophic denitrifiers utilize Fe(II) as an electron donor for energy generation and CO_2 fixation, without the need for organic carbon. In contrast, mixotrophic denitrifiers, which have been shown to oxidize Fe(II), require an organic substrate such as acetate to sustain Fe oxidation for nitrate reduction through an enzymatic process. Alternatively, some microbes catalyze Fe(II) oxidation via abiotic reactions with reactive nitrogen-containing by-products of heterotrophic denitrification, without any enzymatic involvement (Bryce et al., 2018). These so-called “chemodenitrifiers” are microbes that are capable of simultaneously oxidizing Fe(II) and reducing nitrate but lack evidence of enzymatic involvement.

Although there is controversy regarding the oxidation pathways of ferrous, enzyme-catalyzed ferrous iron oxidation has been reported to be feasible, effective, and dominant (Chen et al., 2023; Straub et al., 1996). NO_3^- is entirely ionized to produce spatially symmetric disposition of N-O bonds, and the chemical oxidation of Fe(II) to Fe(III) is slow due to its weak oxidizability (Yang et al., 2021). However, the chemical oxidation effect cannot be neglected, which raises questions about the existence of enzymatic Fe(II) oxidation: are theses strains “mixotrophic” or simply “chemodenitrifiers” that require additional organic carbon?

(Klueglein and Kappler, 2013). Fig. 3 illustrates the proposed mechanisms for Fe(II) oxidation through chemo-oxidation (abiotic chemical process) and bio-oxidation (biotic NAFO process), respectively. In chemo-oxidation, organic carbon, such as alcohols, undergoes a series of metabolic conversions before entering the Tricarboxylic Acid Cycle (TCA cycle), where it is further oxidized to release electrons. NO_3^- accepts the electrons and is then reduced to NO_2^-, which may leak out at any steps and subsequently react with Fe(II) both outside and inside of the cell. Fe(II) can crosse the outer membrane into the periplasm via channel proteins, where it chemically reacts with NO_2^- to produce NO or N_2O, without any enzymatic component involved in this process. Meanwhile, denitrification enzymes simultaneously participate in the conventional stepwise reduction of NO_2^- to N_2, a process independent of the abiotic oxidation of Fe(II).

In the autotrophic NAFO process, Fe(II) is oxidized extracellularly through outer membrane Fe(II) oxidoreductase, and the generated electrons are transferred via electron shuttles or cytochrome c to nitrate and nitrogen intermediates, coupled with the conventional heterotrophic electron transfer chain. Bryce et al. (2018) proposed a potential Fe(II) oxidation mechanism in the KS culture, where the electron produced from Fe oxidation is passed along the electron transport chain, where it reduces nitrate to NO intracellularly. The produced NO can either be consumed by the flanking community or react abiotically with the Fe(II) outside the cell (Bryce et al., 2018). Although no specific enzyme has been found to accept electrons from extracellular Fe(II), recent studies have attempted to identify potential dedicated outer membrane Fe(II) oxidoreductases in autotrophic Fe(II)-oxidizing microorganisms (Bryce et al., 2018; He et al., 2017). He et al. (2016) reported that homologues of the cytochrome c putative Fe(II) oxidase, Cyc2, were identified in the draft genomes of the *Gallionellaceae sp.* and in the *Rhodanobacter sp.* within the KS culture. Homologues of the porin cytochrome c porin complex, MtoAB, were also found in the *Gallionellaceae sp.* in the KS culture, as well as in

D. aromatica RCB (Bryce et al., 2018). Liu et al. (2019) also reported that cytochrome c (c-Cyts) is likely involved in the electron transfer between Fe(II) and iron-oxidizing microorganisms.

Determining which oxidation mechanism is associated with specific strains is an ongoing debate. Some studies suggest that Fe(II) oxidation is driven by abiotic chemical side reactions. Pang et al. (2022) noted that 90% of nitrate-reducing bacteria, including even *Escherichia coli*, can oxidize Fe(II) in the presence of organic matter (Brons et al., 1991). The abiotic reduction of nitrate by dissolved Fe(II) is slow, but the reduction of nitrite to N_2O by Fe(II) is kinetically favourable, especially in the presence of reactive chemical substrates acting as catalysts (Zhu-Barker et al., 2015). The viable surface of pyrite and cell surface can catalyse the reduction of N species by Fe(II) through heterogeneous surface catalysis (Bryce et al., 2018; Jones et al., 2015). Meanwhile, Zhang et al. (2016) observed through transmission electron microscopy (TEM) that both extracellular biotic oxidation of ferrous ion and intracellular chemical oxidation processes coexisted in the cultivated sludge used for NAFO process. Wang et al. (2020c) also demonstrated that bio- and chem-oxidation of iron coexisted, with the former dominating. Kappler et al. (2006) 's research also clearly concluded that the oxidation of Fe(II) in the experiment was catalysed by enzymes rather than by a non-biological reaction between nitrite and Fe(II).

3. Natural occurrence and engineering applications

3.1 Natural occurrence

Fe-mediated autotrophic denitrification has been widely observed in natural environments, such as freshwater sediment, lake sediment, groundwater and paddy soil, as summarized in Table 3. Straub et al. (1996) first observed that ferrous iron can be biologically oxidized by chemotrophic microorganisms and coupled with nitrate reduction in freshwater sediments, revealing the existence of iron-based autotrophic denitrification. Subsequently, more Fe-mediated denitrification processes were discovered in natural environments. In tropical mangrove sediments, Fernandes et al. (2013) found that denitrification activity is closely related to Fe content and generally decreases with depth, suggesting the occurrence of iron-mediated nitrate respiration. Han et al. (2018) also discovered significant Fe(II)-dependent anaerobic denitrification in paddy soil from the De'an region in southern China, with a nitrate reduction rate of 9.03 mg N kg^{-1} day^{-1}. Bai et al. (2021) analysed the concentrations of Fe^{2+}, NO_3^-, and NH_4^+ and other ions in the pore water of reservoir sediments, as well as the vertical distribution of functional microorganisms. The results showed that the 10-28 cm depth primarily constituted the denitrification and iron reduction zone, where *Acinetobacter* and *Pseudomonas* were the dominant nitrate-reducing microorganisms, utilizing nitrate as an electron acceptor to oxidize ferrous iron (Bai et al., 2021). Meanwhile, Laufer et al. (2016a) first provided evidence for the existence of autotrophic NAFO bacteria in the natural environment by using coastal marine sediment in ^{14}C-labeled incubations.

Oxygen is not abundant in all habitats on Earth. Many regions of oceans and lakes experience either seasonal or permanent anoxia, while in soil and sediments, it is detectable only a few millimetres below the surface. Similarly, the deep sea and deep continental crust are also oxygen-deficient. Microbial enrichment and isolation experiments have provided evidence for Fe-mediated autotrophic denitrification in these anaerobic natural environments. Sorokina et al. (2012) isolated a novel iron-oxidizing bacterium (*Hoeflea siderophile*) from iron-rich sediments in low-temperature, brackish water springs, which is capable of Fe(II) oxidation and NO_3^-/N_2O reduction under mixotrophic conditions. In the transitional zone of lakebed sediments, located at a depth of 1-2 meters in the northwestern arm of Lake Constance (between the brown surface and grey deeper sediments), *Acidovorax* sp. BoFeN1 was isolated (Kappler et al., 2006). It exhibits mixotrophic growth, requiring acetate as an organic co-substrate to continuously oxidize Fe(II), with nitrate as the electron acceptor. Similarly, other strains of *Acidovorax* sp. have also been found in freshwater sediments (Byrne-Bailey et al., 2010), ditch sediments and groundwater (Emerson and Moyer, 1997). Additionally, previous study showed that iron-oxidizing bacteria (FeOB) isolated from weathering environments, including rusty, reddish-brown oxidized sulfide chimney rubble, were capable of growing on a variety of solid rock and mineral substrates, such as FeS_2, FeO, and $FeCO_3$, using them as sole energy source and coupling with nitrate reduction under anoxic conditions. This suggests that FeOB may participate in the weathering of deep-sea rocks in natural environments and Fe(III) mineral formation in anoxic marine environments (Edwards et al., 2003).

In addition, iron sulfide-assisted denitrification (ISAD) is a crucial bioprocess for groundwater remediation in natural environments, particularly in aquifers and sediments where pyrite and other iron sulfides are present. This process is marked by the reduction of nitrate concentration with depth in aquifers, accompanied by the formation of sulfate, Fe^{2+}, and trace metals, as well as a slight increase in pH. Microbial studies have confirmed the presence of ISAD by showing heightened denitrification activity and the presence of specific denitrifying bacteria closely related to the *Xanthomonadaceae* species when pyrite is introduced (Torrentó et al., 2011). Additionally, bacteria associated with sulfur oxidation (*T. denitrificans*, *Thiotrix nivea*, *Thiotrix unzii*, *Pseudomonas stutzeri*) and iron oxidation (*Gallionella ferruginea*, BrG1) were identified in a heavily nitrate-contaminated, pyrite-rich sandy aquifer (Zhang et al., 2012).

These findings collectively demonstrate that Fe-mediated autotrophic denitrification is a widespread functional pathway in natural anaerobic environments. By coupling nitrate reduction with Fe(II) oxidation, this process facilitates nitrogen removal in sediments, aquifers, and other subsurface systems where organic carbon and DO are limited. Moreover, Fe plays a crucial role in the geochemical nitrogen cycle by providing an alternative inorganic electron donor for autotrophic denitrification, potentially mitigating nitrate pollution and influencing nitrogen cycling in iron-rich environments.

3.2 Engineering applications

3.2.1 Groundwater remediation

Fe-mediated autotrophic denitrification has been applied in groundwater remediation, which is known to be a valuable and relatively high-quality source of drinking water (Suzuki et al., 2012; Adimalla and Qian, 2021; Wang et al., 2009). Due to the extensive agricultural intensification and other non-agricultural N discharge sources, nitrate concentration in groundwater has continued to rise, often exceeding the regulatory limit for drinking water. A previous study has showed that excessive nitrate levels in groundwater contribute to 50%-60% of groundwater-related risks (Adimalla and Qian, 2021). ZVI has unique and irreplaceable advantages, such as low cost, high availability, and ease of transportation and storage, making it a key material in practical groundwater remediation (Table. 4). Lu et al. (2018) investigated nitrate removal in simulated groundwater using ZVI with *Enterobacter* sp. CC76, which is characterized by its anaerobic denitrification and iron-reduction performance (Su et al., 2016). The study demonstrated that strain CC76 tolerated the long-term presence of ZVI and the nitrate removal efficiency reached 82.15% under optimal conditions, as determined through response surface analysis. Numerous studies in recent years have also explored the removal of nutrients from groundwater using ZVI/nZVI (nanoscale zero-valent iron) (Wang et al., 2025; Xu et al., 2024b; Yu et al., 2024).

In addition, due to the complexity of groundwater pollutants, research has increasingly focused on the removal of other persistent contaminants. Yuan et al. (2022) investigated the coupling of microscale zero-valent iron (mZVI) and autotrophic hydrogen-bacteria (AHB) for the removal of trichloroethylene (TCE) in groundwater caused by industrial activities. The results showed that the mZVI-AHB system was significantly superior to mZVI or AHB alone in terms of TCE removal efficiency and complete dichlorination, while also facilitating the enrichment of functional dechlorinating microbes. Plagentz et al. (2006) demonstrated that a sequential permeable reactive barrier (PRB) composed of ZVI and granular activated carbon (GAC) could be effectively used for treating groundwater contaminated with chlorinated and brominated hydrocarbons, benzene, and chromate. Furthermore, Dong et al. (2019) provided a comprehensive review on the application of nZVI in combination with functional anaerobic bacteria, including organohalide-respiring bacteria (OHRB), sulfate-reducing bacteria (SRB), and iron-reducing bacteria (IRB), for groundwater remediation.

3.2.2 WWTP post-treatment

More stringent discharge standards pose challenges for existing WWTPs, as even advanced (tertiary) wastewater treatments is implemented to meet higher discharge requirements, the discharge limits (TN 10-15 mg/L, TP 0.5-1 mg/L) are still much higher than the thresholds that cause eutrophication (TN > 1.2 mg/L; TP > 0.1 mg/L) (Hu et al., 2017). As a result, many countries have adopted stricter discharge limits, such as the Netherlands, which requires TN < 2.2 mg N/L and TP < 0.15 mg P/L for sensitive water bodies (Hu et al., 2017).

Fe-mediated autotrophic denitrification is gradually being applied as a post-treatment unit in WWTPs to meet stricter nutrient discharge standards. Wang et al. (2024) developed a biomass-iron mixture that achieved 81% - 89% TN removal from secondary effluent, while simultaneously enriching iron-driven autotrophic denitrifiers (e.g., *Acidovorax* and *Citrobacter*). Meanwhile, constructed wetlands (CWs), as engineering facilities with a significant landscape value, can enhance the treatment efficiency of secondary effluent from WWTPs when iron-related substrates are incorporated, providing both functional and aesthetic benefits. Dong et al. (2024) conducted a comparative analysis of CWs using iron scraps and pyrite for nitrate removal. The results indicated that the iron scraps CW achieved a higher nitrate removal efficiency than the pyrite CW, with rates of 87.1% and 59.5%, respectively. Metagenomic analysis revealed a greater relative abundance of *Thiobacillus* and *Ferritrophicum* in the iron scraps CW, which contributed to the increased microbial diversity and may explain the enhanced nitrate removal. A lab-scale CW filled with iron scraps and biochar was used to purify synthetic secondary effluent with a C/N ratio of 0.5-2, achieving an average nitrate removal rate of 83.2% to 98.8% (Ji et al., 2022). Furthermore, the formation of insoluble Fe-P precipitates and the adsorption of phosphorus onto the large surface area of iron oxides and FeOOH reduced the TP concentration to less than 0.2 mg/L. The abundance of autotrophic denitrifying bacteria and iron cycle-related bacteria, such as *Dechloromonas, Ferritrophicum*, and *Thiobacillus*, increased significantly with the addition of iron scraps. Besides polluted groundwater and secondary effluent from WWTPs, low C/N ratio water resources are also commonly found in rivers, lakes, and man-made reservoirs, which typically supply drinking water. Similarly, wastewater produced from industrial activities such as mineral processing, electroplating, semiconductor manufacturing, and power plants has negligible concentrations of organic compounds (Di Capua et al., 2019). Therefore, Fe-mediated autotrophic denitrification technology offers an alternative method for effectively treating this broad range of low C/N wastewater.

3.2.3 On-site treatment and performance enhancement

Fe-mediated autotrophic denitrification is also used for on-site treatment applications. Visser et al. (2024) studied a nitrate-contaminated karstified aquifer in Oberes Gäu, Germany, using microbial trapping devices (MTD) filled with four different iron minerals, magnetic iron oxide-coated sand, zero-valent iron, pyrite, and Fe-rich biotite flakes, to investigate the microbial community composition after exposure to the groundwater for three to four months. The results indicate that MTDs with ZVI triggered more reactions due to its higher reactivity and exhibited greater complexity in microbial diversity. Genera containing putative Fe(II)-

oxidizing and Fe(III)-reducing species, such as *Leptothrix* and *Albidiferax*, were found in relatively high abundances. Additionally, increased abundances of *Hydrogenophaga spp.* and *Dechloromonas spp*., which are known to couple H_2 oxidation with NO_3^--N reduction, were observed. Ge et al. (2019) also constructed a pilot scale subsurface CW for in-situ river water treatment with natural pyrite and limestone as substrates and achieved an average TP and TN removal rates of 87.7% and 69.4%, respectively, over three years of operation.

Fe-mediated autotrophic denitrification can also serve as a supplementary technology to enhance the performance of existing wastewater treatment systems. Adding Fe^0 to a sulfur-based anoxic fluidized-bed membrane bioreactor reduced sulfate production by 29.3%-50.9% and offset the alkalinity consumed (Zhang et al., 2019). Combining Fe-mediated autotrophic denitrification with CW-MFC not only achieved higher nitrogen and phosphorus removal efficiencies, at 93.94% and 93.89% respectively, but also enhanced electricity recovery (Gan et al., 2024). In addition, some studies have focused on the removal of hazardous pollutants in groundwater, such as toxic heavy metals (eg., arsenic, chromium) (Jia et al., 2020; Li et al., 2021b; Liu et al., 2024; Lu et al., 2020), chlorinated organic compounds (eg., trichloroethane) (Zhou et al., 2014), and radioactive nuclides (eg., uranium) (Lu et al., 2023). Fe-mediated autotrophic denitrification can facilitate the removal of nitrate and these pollutants, or even multiple contaminants simultaneously.

Overall, the application of Fe-mediated autotrophic denitrification for treating low C/N ratio wastewater treatment has been validated through extensive engineering studies.

4. Influencing factors

The efficiency of Fe-mediated autotrophic denitrification is influenced by multiple factors, including environmental factors such as temperature (T), pH, and dissolved oxygen (DO); and operation parameters, such as the Fe/N ratio and organic carbon. These factors will be discussed in detail in the following sections.

4.1 Temperature

Temperature is a key factor influencing microbial activity. Most studies on Fe-mediated autotrophic denitrification have been conducted under moderate temperature conditions, generally considered suitable for the growth of most NAFO microbes, ranging from 28°C to 40°C. The nitrate removal efficiency gradually increased from 92.03% to 98.23% as the temperature rose from 25°C to 40°C when Fe(II) was used as an electron donor in an activated sludge system (Wang et al., 2016). Deng et al. (2022) observed that within the temperature range of 10-40°C, the nitrate removal rate gradually increased from 0.24 kg N/(kg VSS·d) to 0.67 kg N/(kg VSS·d), accompanied by a decrease in nitrite accumulation in Fe^0-mediated autotrophic denitrification. The combination of Fe^0 and the strain CC76 for nitrate removal also demonstrated that the optimal temperature was 30.44°C through response surface analysis (Lu et al., 2018). Lower temperatures can decrease microbial activity, as well as the solubility and redox processes of solid-phase iron substrates, negatively affecting electron transfer. However, Trouve et al. (1998) isolated several psychrophilic *Thiobacillus* denitrificans strains capable of using FeS and FeS_2 as electron donors to remove nitrate at 5°C in fixed cultures. Although Fe(II) is involved in sulfide-based autotrophic denitrification and *Thiobacillus* is believed to have the ability to reduce nitrate with Fe^{2+}, there is still no direct evidence that effective nitrate removal can be achieved in Fe-mediated autotrophic denitrification under low-temperature conditions.

4.2 pH

pH is a crucial parameter affecting both Fe corrosion and microbial activity in Fe-mediated autotrophic denitrification. The reduction of nitrate by Fe^0 is a strongly acid-driven chemical process. Under low pH conditions, the corrosion of iron and the dissolution of surface iron oxide layers promote the generation of more reactive sites, enhancing electron transfer. In contrast, neutral to alkaline conditions promote the formation of iron oxides, which hinders the reaction rate. Studies indicate that rapid chemical reduction is only observed in acidic solutions, and when the pH increases from 2 to 3, the efficiency of nitrate reduction drops significantly (Luo et al., 2014). Controlling iron dosing is often considered to achieve a balance between electron release and the minimization of pH rise.

Although the activity of Fe^0 is the highest under acidic conditions, microbial activity is strongly inhibited in such environments, making it essential to balance the influence of pH on both Fe^0 reactivity and microbial activity. Under neutral pH conditions, although electron release by Fe^0 is reduced, Fe^0 can still achieve efficient nitrate removal in bio-Fe systems, which is attributed to the presence of microbes (Deng et al., 2016; Xu et al., 2017). Previous studies have shown that the introduction of unacclimated activated sludge into Fe systems can achieve 75%-95% nitrate removal efficiency across a pH range of 2 to 10, while maintaining over 50% N_2 selectivity (reduction of nitrate to N_2) (Chang et al., 2024).

pH control is also crucial to the NAFO process, as Fe(II) oxidation produces acidity. The redox potential of Fe^{2+}/Fe^{3+} is much higher than that of NO_3^-–N/NO_2^-–N in acidic environments, which is not favourable for Fe(II)-mediated autotrophic denitrification (Kappler and Newman, 2004; Wang et al., 2023). It is generally recommended to maintain the pH between 6 and 8 (Kiskira et al., 2017a; Zhang et al., 2014). Oshiki et al. (2013) revealed that anaerobic ferrous oxidation has a broader suitable pH range, from 5.9 to 9.8. Studies have shown that the highest nitrate removal efficiency was achieved at a pH of 6.4 to 6.7 when Fe(II) was used as the electron donor (Straub et al., 2004). However, a higher pH accelerates the deposition of iron minerals on the cell surface, reducing their bioavailability, while a lower extracellular pH not only prevents Fe(III) precipitation but also enhances microbial utilization of Fe(II) minerals (Kappler and Newman, 2004; Yan et al., 2019b). This precipitation-inhibiting effect may be related to the influence of pH on the competition between aqueous Fe(III) ions (Fe(III)aq) and protons for binding sites on the cell surface (Hegler et al., 2010). Additionally, it is reported that pH can influence the efficiency of NAFO by affecting electron transfer between Fe(II) and cytochrome c, which is related to the effects of pH on Ec-Cyt, the direct involvement of protons, and the impact of mineral solubility on redox potential (Bird et al., 2011; Liu et al., 2017; Wang et al., 2023; Wu et al., 2016).

4.3 DO

In natural environments, Fe-mediated autotrophic denitrification typically occurs under anoxic conditions, such as in aquifers, deep seas, and lake sediments (Section 3.1). However, iron-amended treatment facilities (e.g., CWs, biofilters, or anaerobic sludge beds) are often unable to maintain strictly anaerobic conditions, making DO control crucial for optimizing denitrification performance.

The impacts of DO on iron-mediated denitrification are complex and can be either inhibitory or stimulatory. High DO conditions are generally believed to inhibit denitrification, mainly by inhibiting the synthesis of NO_2^--N reductase and N_2O reductase, with the expression of N_2O reductase being significantly inhibited when the DO level exceeds 2.5 mg/L (Yang et al., 2024). However, within a certain range, an increase in DO may enhance denitrification performance. For example, Deng et al. (2020a)'s study showed that the denitrification load increased from 0.12 kg-N/m^3/d to 0.34 kg-N/m^3/d when the DO increased from 0.1 mg/L to

1.6 mg/L, while the generation of NO_2^--N and N_2O was greatly decreased. Opportune DO (below 1.6 mg/L) enriched iron compound-transforming bacteria, such as *Sphaerotilus* and *Acidovorax*, and enhanced the biotransformation of Fe^{2+} and Fe_3O_4 into FeO(OH). This process reduced Fe_3O_4 coverage on the Fe^0 surface, thereby sustaining a continuous supply of electron donors (H_2/[H] and Fe^{2+}) in micro-electrolysis-mediated Fe^0-oxidizing autotrophic denitrification. Peng et al. (2020b) also found that simultaneous nitrification and Fe-mediated denitrification could be enhanced at DO levels between 0.08 and 1.56 mg/L, although oxygen-induced Fe passivation occurred when the DO increased further to 2.17 mg/L. Interestingly, the addition of Fe^{2+} into Fe^0 system promoted the Fe_3O_4 generation, which is a good electron conductor and facilitates electron transfer in the chemical reduction of nitrate by Fe^0 (Xu et al., 2012).

Moreover, the abundance of Fe-transformation related bacteria (FeTB), including *Acidovorax*, *Pseudomonas*, *Thermomonas*, and *Thiobacillus*, which utilize Fe^{2+} as an electron donor, along with other Fe(II)-oxidizing genera such as *Ferritrophicum*, *Ferriphaselus*, *Chloronema*, and *Bacillus*, increased from 12.9% at 0.08 mg DO/L to 32.3% at 1.56 mg DO/L (Peng et al., 2020b). Therefore, DO levels not only influence microbial community composition but also drive iron oxidation and transformation, thereby increasing the complexity of Fe-N cycle in biological systems.

4.4 Fe/N ratio

The Fe/N ratio is a key factor in determining nitrate removal and by-product production in Fe-mediated autotrophic denitrification. The optimal Fe(II)/NO_3^--N ratio is 5:1 according to stoichiometric relationships. When the ratio falls below 4, incomplete denitrification can lead to the production of intermediate products such as NO_2^--N and N_2O, whereas ratios exceeding

7.5 stimulate the chemical reduction of NO_3^--N to NH_4^+-N (Kiskira et al., 2017a; Li et al., 2014).

Regarding Fe^0-mediated autotrophic denitrification, factors such as microbial bioavailability of solid-phase iron sources, iron corrosion, particle size, and iron content can cause the Fe/N ratio to deviate from stoichiometric relationships. Wang et al. (2019) utilized iron scraps (purity > 99%) combined with sludge and found that the TN removal rate was the highest at 67.86%, and the nitrite concentration was the lowest when the iron dosage was 15 g/L. When the iron dosage exceeded 15 g/L, ammonia formation increased. Liu et al. (2020) used a column bioreactor filled with 7457 g of iron scraps and achieved a 97.2% nitrate removal rate when the influent nitrate concentration was 20 mg/L and the HRT was 24 hours. However, the ammonia concentration in the effluent was relatively high, averaging 10.8 mg/L (Liu et al., 2020).

In addition to the Fe/N ratio, the concentration of Fe(II) and nitrate also influence the efficiency of autotrophic denitrification. Since 19.9 kg of Fe(II) is required to reduce 1 kg of nitrate, the influent iron salt concentration generally ranges from 400 to 1500 mg/L and can reach as high as 6000 mg/L (Zhang et al., 2015). The addition of large amounts of iron salts, coupled with their susceptibility to oxidation, complicates transportation and storage, thereby increasing operational costs and the risk of secondary pollution. Theoretically, a high Fe(II) concentration can reduce the Em(Fe(II)/Fe(III)), enhancing the kinetic advantage of the NAFO process (Liu et al., 2017; Wang et al., 2023). An appropriate Fe(II) concentration of 0.25-8.3 mg/L can promote microbial growth, while excess Fe(II) concentration accelerates its hydrolysis, lowers the surrounding pH, disrupts enzymatic systems, and causes rapid cell encapsulation (Wang et al., 2023; Yang et al., 2024). These issues can lead to high residual Fe(II) in the effluent and sludge mineralization. Consequently, some studies have explored the use of iron minerals, such as pyrite, siderite, sponge iron, and iron filings, as slow-releasing sources of Fe(II). However, in some other studies, higher Fe(II) concentrations (1–5 mM (Liu et al., 2014) , 15 mM (Oshiki et al., 2013), 25mM (Lack et al., 2002) and nitrate concentrations (5.8–8 mM) have also been tested, and these levels do not inhibit microbial growth, the rate of Fe(II) oxidation, or nitrate removal.

4.5 Organic carbon

The majority of NAFO bacteria grow under mixotrophic conditions, and the addition of small amounts of organic carbon has been proven to enhance denitrification performance. Deng et al. (2020b)'s study demonstrated that when the organic carbon addition was 1 mg COD/mg TN, the nitrogen removal efficiency increased from $41.6 \pm 6.0\%$ to $90.7 \pm 2.3\%$ with denitrification load increased from 0.191 kg N/ ($m^3 \cdot d$) to 0.445 kg N/ ($m^3 \cdot d$). Additionally, the accumulation of N_2O and NO_2^--N decreased from 1.52 mg N/L and 6.86 mg N/L to 0.03 mg N/L and 0.31 mg N/L, respectively (Deng et al., 2020b). This improvement occurs because organic carbon reduces the passivation of Fe^0, increasing the production of H_2 and Fe(II). In batch experiments, the *Acidovorax sp*. strain 2AN strain was added to a medium containing 5-6 mM nitrate, 8-9 mM Fe^{2+}, and 1.5 mM acetate. Almost 90% of the Fe was oxidized within 7 days, accompanied by nitrate reduction and complete consumption of acetate (Chakraborty et al., 2011). Acetate is considered the most effective organic compound, however, while the optimal dosing has not yet been determined, as it varies with the type of organic compound and microbial species.

In addition, adding organic carbon can alleviate the cell encrustation in Fe-mediated autotrophic denitrification. Wang et al. (2020b) observed a reticular structure around denitrifying bacteria in a bioreactor with acetate addition after 40 days of operation, which can effectively keep Fe^{2+} away from the cells. Furthermore, organic carbon can be used to indirectly relieve stress on cells, as Fe(II) oxidation is a detoxification process rather than a metabolic one within bacterial cells, due to microbes preferentially using organics as electron donors for energy production (Liu et al., 2018; Wang et al., 2023). However, a high concentration of organic carbon may negatively impact the efficiency of Fe-mediated autotrophic denitrification by not only keeping periplasmic enzymes in a highly reduced state but also increasing the activity of metal efflux pumps, thereby hindering Fe(II) entry into cells (Wang et al., 2023).

4.6 Other factors

In addition to the factors mentioned above, there are other factors that also influence the efficiency of Fe-mediated autotrophic denitrification. For many lab-scale bioreactors and pilot-scale settings, a longer HRT can improve the denitrification efficiency, while it inevitably increases capital investment and operational costs. Therefore, determining an appropriate HRT should balance both the two aspects simultaneously.

Additionally, the use of chelating agents also affects the NAFO process. Zhou et al. (2016) conducted an experiment using an upflow bioreactor with sponge iron as the substrate and *Microbacterium sp*. W5 as the inoculum, supplemented with $FeSO_4$ as the electron donor. The results demonstrated that when Fe-EDTA was used to replace $FeSO_4$, the problem of cell encrustation at high Fe^{2+} concentrations (1100 mg/L–1500 mg/L) was significantly mitigated, and the nitrate removal rate increased substantially from 12.2% to nearly 90% (Zhou et al., 2016). Furthermore, chelating agents such as EDTA, oxalic acid, and NTA enhance microbial utilization of solid-phase Fe(II) by chelating with minerals such as pyrite or reduced nontronite. This improves the bioavailability of the solid-phase Fe(II) by enabling the use of chelated Fe materials as an electron shuttle (Wang et al., 2023).

Metal ions such as Ca^{2+}, Co^{2+}, Ni^{2+}, along with anions like Cl^{-}, SO_4^{2-}, HCO_3^{-}, and PO_4^{3-}, often coexist with NO_3^{-} and Fe^{2+} in the treatment systems. Microbes' high sensitivity to metals can significantly alter the expression of key functional genes and activate the efflux pump mechanism, enabling microbes to expel heavy metals from their cells, thereby reducing toxicity (Boopathy, 2000). Additionally, SO_4^{2-} and Cl^- can compete with NO_3^- for active reaction sites, potentially affecting by-product distribution by inhibiting the expression of *Nir* genes, which leads to nitrite accumulation (Yang et al., 2024). High concentrations of anions also pose risks by damaging cell membranes and altering cellular structure (Yang et al., 2024). Therefore, the impact of these coexisting ions on the NAFO process and the simultaneous removal of nitrate and heavy metals needs further investigation.

5. Challenges in Fe-mediated autotrophic denitrification

Despite ongoing discussions on various Fe^0/Fe^{2+} sources, microbes, and engineering applications, challenges persist in Fe-mediated autotrophic denitrification. The main challenges include a low denitrification rate, cell encrustation, and high ammonia production.

5.1 Low denitrification rate

In regard to autotrophic denitrification, H_2-mediated and sulfur-mediated autotrophic denitrification exhibit high nitrate removal rates, even surpassing heterotrophic denitrification in some cases. In a hollow fiber membrane reactor, H_2-mediated autotrophic denitrification can achieve nitrate removal rates of up to 2.42 g N/L·d, exceeding those of heterotrophic denitrification processes using ethanol, acetate, or glucose as electron donors (Pang and Wang, 2021b). Meanwhile, a denitrification rate of 0.98 g N/L • d was achieved in a moving-bed reactor using solid element sulfur (S^0) as the electron donor (Xu et al., 2024a).

In comparison, the denitrification rates for Fe^0/Fe^{2+}-mediated autotrophic denitrification are low, with the maximum observed denitrification rate of 0.33 g N/L·d when $FeSO_4$ is used as the iron source (Tian et al., 2020). Fe^0 and Fe^{2+} have a high electron donor consumption, reaching 10 and 19.9 kg electron donor/kg NO_3^--N, respectively, while sulfur and hydrogen are only 2.5 and 0.4 kg electron donor/kg NO_3^--N, respectively (Pang and Wang, 2021b). This necessitates the addition of large amounts of iron salts to maintain effective nitrate removal, thereby increasing costs and potential pollution risks. For solid-phase iron sources, the denitrification rate is even lower, ranging from 0.0015 to 0.057 g N/L·d, depending on the type of iron substrates (Table. 1). When natural pyrite is used, the denitrification rate can reach 0.01 to 0.07 g N/L·d (Hu et al., 2020).

The bioavailability of iron sources and the electron transfer rate significantly influence the autotrophic denitrification rate. The extremely low solubility and dissolution rate of solid-phase iron sources, such as FeS_2, severely limit its utilization by denitrifying microbes and hinder denitrification kinetics compared to other electron donors (Di Capua et al., 2019). nZVI generally has a larger specific surface area and higher surface reactivity than iron scraps or granular iron, making it more effective at releasing electrons (Zhang, 2003). Wang et al. (2020a) used sponge iron as a solid-phase iron source for treating low C/N wastewater, achieving very low nitrate removal rate, which was attributed to the formation of a dense, non-porous magnetic hematite coating following sponge iron oxidation, which restricted electron transfer and hindered nitrate reduction. In addition, the low Fe-mediated denitrification rate is related to the smaller difference of redox potential (ΔORP) compared to other autotrophic denitrification, such as sulfur- and hydrogen-dependent denitrification, indicating that microbes derive less energy from Fe oxidation (Pang et al., 2022). The slower growth of autotrophic bacteria, cause by the incomplete Krebs cycle and its lower ATP production, further limits denitrification (Mattes et al., 2013). The low reaction rate of Fe-mediated autotrophic denitrification makes it most suitable for slow processes, such as groundwater remediation. However, for applications requiring a high treatment capacity, such as WWTPs needing nitrate removal rates of 0.3 g N/m^3·d or above, it may be necessary to combine Fe^0/Fe^{2+} with other technologies to overcome the limitation of low reaction rates (Hu et al., 2020). To date, studies have focused on optimizing the composition of electron donors, such as by utilizing mixed inorganic electron sources (e.g., Fe^0/FeS, Fe^{2+}/S^0) or adding organic electron donors to simulate autotrophic denitrification (Hu et al., 2018; Pang and Wang, 2021a; Wang et al., 2018; Zhu et al., 2019). Some studies have also explored improving the denitrification rate by enhancing the properties of iron materials themselves or coupling them with other materials. Iron-carbon and Fe-bimetals have been investigated in various bioreactors to improve nitrate removal and overcome existing problems, such as low N_2 selectivity (An et al., 2010; Jia et al., 2020; Shen et al., 2019).

5.2 Cell encrustation

Another major issue is the formation of insoluble iron minerals through Fe(II) oxidation, which creates crusts that hinder microbial activity and reduce nitrate removal efficiency, as shown in Fig. 4 (Huang et al., 2023). To address these challenges, researchers have begun to explore the mechanisms behind cell encrustation and have found that it is closely related to the type of Fe(II) oxidation. Nordhoff et al. (2017) investigated the associations between cells and minerals under different conditions. The experimental results indicated that no cell encrustation occured in the KS culture under both autotrophic or mixotrophic conditions. However, heterotrophic nitrate-reducing isolates from KS culture, *Nocardioides* and *Rhodanobacter*, became encrusted, with the extent of encrustation correlated with Fe(II) concentration. As mentioned in Section 2.3.1, the KS culture has been proven to be a strictly autotrophic Fe(II)-oxidizing denitrifying consortium, indicating that enzymatic oxidation of Fe(II) (biotic NAFO process) does not cause cell encrustation. Meanwhile, the isolation experiment supported the hypothesis that encrustation may result from the abiotic reaction between Fe(II) and nitrite generated during the heterotrophic denitrification process (Nordhoff et al., 2017). Schmid et al. (2014) also demonstrated that the precipitation of Fe(III) minerals was due to the abiotic oxidation of Fe(II) by NO_2^- in cultures of the *Acidovorax* sp. BoFeN.

One possible reason for the phenomenon is that the controlled enzymatic oxidation of Fe(II) and the release of Fe(III) can prevent Fe(III) precipitation adjacent to the cells. In contrast, for mixotrophic or heterotrophic nitrate-reducing bacteria, controlling the location of Fe(III) precipitation is not feasible, and it may even occur in the pericellular region where Fe(II) is present (Klueglein et al., 2014; M. Nordhoff et al., 2017). Klueglein et al. (2014)'s research also supported the conclusion that the abiotic oxidation of Fe(II) with nitrite is the reason for cell encrustation. They observed that Fe(II) toxicity stimulated microbial secretion of extracellular polymeric substances (EPS), which serves as a strategy to mitigate iron encrustation. The biotic and abiotic pathways of Fe(II) oxidation are discussed in Section 2.3.2, and their mechanisms, along with the potential sites of encrustation, are illustrated in Fig. 3. However, encrustation has also been observed in some bacteria that were reported as autotrophic nitrate-reducing Fe(II) oxidizers, such as *Citrobacter freundii* PXL1(Li et al., 2015) and *Azospira oryzae* TR1 (Mattes et al., 2013). It remains unclear whether these strains are true autotrophic denitrifies.

In natural environments, heterotrophic and autotrophic denitrification typically coexist. However, cell encrustation is usually negligible due to the generally low concentrations of Fe(II). In engineered bioreactors that use iron as a substrate, the production of iron oxides can hinder nitrate removal by limiting active sites and binding to microbial cells. Despite this, no studies have specifically focused on cell encrustation in Fe-mediated bioreactors, and there are still no effective solutions to prevent or mitigate this crust formation. Further research is needed to address this issue.

5.3 High ammonia production

Ammonia is an undesirable by-product in Fe-mediated autotrophic denitrification, as nitrate is not truly removed but is instead converted into another form of nitrogen and retained in the system. The N_2 selectivity in nitrate chemical reduction is low, with over 90% of nitrate being converted to ammonia. Even composite materials such as Fe/Biochar are used, ammonia selectivity still exceeds 60% (Song et al., 2020). Furthermore, in systems using low-reactivity iron filings with larger particle size, ammonia selectivity remained at 27% and 73% in iron-carbon and iron systems, respectively (Chang et al., 2024).

The incorporation of microbes into Fe-mediated systems has been shown to significantly enhance N_2 selectivity, with various microbial species exhibiting this capability. In a system where iron filings were present alongside unacclimated activated sludge, ammonia selectivity decreased from 73% in the abiotic system to 58%, and further decreased to 16% when biochar was added (Chang et al., 2024). The coupling of nZVI with *Alcaligenes eutrophus* converted 33% of reduced nitrate to ammonium, significantly lower than the 100% conversion in the abiotic system (An et al., 2009). Moreover, H_2 produced by iron corrosion can be utilized by *Paracoccus* denitrificans to convert most of the nitrate into harmless N_2 gas rather than ammonia (Till et al., 1998). Even so, the accumulation of ammonia resulting from Fe-mediated chemical reduction is still difficult to avoid in biosystems. For example, nitrate removal in CWs using iron filings as the substrate is higher than in those with pyrite substrates. However, due to ammonia production in the iron fillings CWs, there is no significant difference in TN removal between the two CWs (Dong et al., 2024). Ji et al. (2022)'s study also indicated that the ammonia removal rate fluctuated significantly in CWs with iron filings as substrates. Although the reactivity of iron filings is relatively lower compared to nZVI, the issue of ammonia accumulation still needs to be addressed.

5.4 Recommendations for future research

Although iron is widely used in practical applications, the aforementioned issues still persist. Ammonia accumulation and the formation of iron oxides, which can lead to problems like low TN removal efficiency, passivation of iron materials, impaired sustained electron release, and solid by-products adhering to cells, reducing their activity and even causing severe cell encrustation. Therefore, some innovations are gradually being developed and applied to overcome these problems.

First, some studies have focused on the development and innovation of iron materials to address the issues of low denitrification efficiency and material passivation. An et al. (2022)'s study embedded mZVI into a chitosan (CS) gel, forming mZVI@CS, making the denitrification efficiency 11.33 times higher than mZVI alone when used as a substrate in constructed wetlands. An et al. (2024)'s study further demonstrated that the mZVI@CS material can effectively decrease the mZVI particle agglomeration and inhibit surface passivation, allowing for a sustained electron supply while achieving TN removal rates of 85%-96%. In addition, some bimetallic and polymer-coated Fe nanoparticles have been investigated for autotrophic denitrification, specifically for by-product production control. An et al. (2010) utilized nFe^0-Ni, nFe^0-Cu, chitosan-nFe^0, and oleic acid-nFe^0 particles to support autotrophic denitrification, in which the oleic acid-nFe^0 particles showed complete nitrate removal in 2 days and reduced ammonia production by 17% compared to nZVI. Wang et al. (2022a) suggested that iron-carbon micro-electrolysis (IC-ME) enhanced iron corrosion, which provided more electron donors, but also promoted the undesired formation of ammonium through chemical reduction. By developing a novel carbon-coated iron (Fe^0@C), an efficient simultaneous removal of both nitrate and TN was achieved, with removal rates of 95% and 84%, respectively (Wang et al., 2022a). This is attributed to the hydrophobic carbon shell, which prevented oxygen, nitrate and iron from interacting while also promoting electron production, thereby mitigating competitive chemical reactions. In addition, although solid-phase iron sources are valued for their economic benefits and wide applicability, most studies have focused on nitrate removal under stable influent conditions, with limited attention given to the impact of fluctuating nitrate loads in practical applications. Zhang et al. (2025) found that adjusting the Bed-Immersion Ratio (BIR) enabled directional regulation of nitrogen removal efficiency (NRE) in the condition of fluctuating influent nitrate concentrations. When the BIR was increased to 1.00, the system achieved maximum NRE, and controlled BIR variation reduced substrate consumption and allowed stable effluent nitrate concentration. This study provides valuable insights for engineering applications and offers a promising operational strategy for optimizing Fe-mediated systems using solid-phase iron sources.

To date, extensive studies have explored various modification and coupling strategies for iron-based materials to address the aforementioned challenges. From the substrate perspective, further studies are needed to evaluate their long-term stability, environmental adaptability, and the potential toxicity of Fe(II). It is important to consider whether complex modifications or composites significantly increase costs, thereby affecting their feasibility for large-scale engineering applications. For solid-phase iron sources with economic value and broad application potential, the issue of low bioavailability also deserves more attention. Meanwhile, research on operational regulation in Fe-mediated autotrophic denitrification remains limited. In addition, various factors can influence the denitrification efficiency and cell encrustation as previously discussed. Therefore, it is important to explore whether these issues can be addressed in existing systems by simply adjusting operational parameters - such as influent mode, loading rate, or HRT - without structural modifications. Furthermore, future studies should address practical challenges, such as fluctuating nitrate loads, low temperatures that impair microbial activity, and potential risks of heavy metal contamination - which may significantly limit the implementation of Fe-mediated autotrophic denitrification in complex environmental conditions.

6. Conclusions

Fe-mediated autotrophic denitrification has demonstrated great potential as a sustainable and effective approach for nitrate removal from low C/N wastewater, with applications in both natural occurrence and engineering applications. Among various iron sources, liquid-phase iron is widely used due to its high bioavailability, although it requires frequent supplementation to sustain continuous nitrate removal. In contrast, solid-phase iron is more economical but is limited by low denitrification rates, cell encrustation and practical challenges. The microbial mechanisms underlying the utilization of solid-phase iron remain unclear and require further in-depth investigation.

Enzyme-catalyzed Fe(II) oxidation is currently considered feasible, effective, and potentially dominant, yet chemical oxidation processes often coexist. A clear consensus on the dominant pathway in specific strains has yet to be established. Moreover, multiple parameters (e.g., temperature, pH, and DO) co-influence the efficiency of Fe-mediated autotrophic denitrification, while the regulatory effects of operational strategies remain insufficiently explored. To address persistent challenges, including low denitrification rates, cell encrustation, and high ammonia production, future study should focus on developing modified iron materials that are cheap and highly bioavailable, as well as practical and adaptable operational strategies to address complex real environments.

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Figure Captions

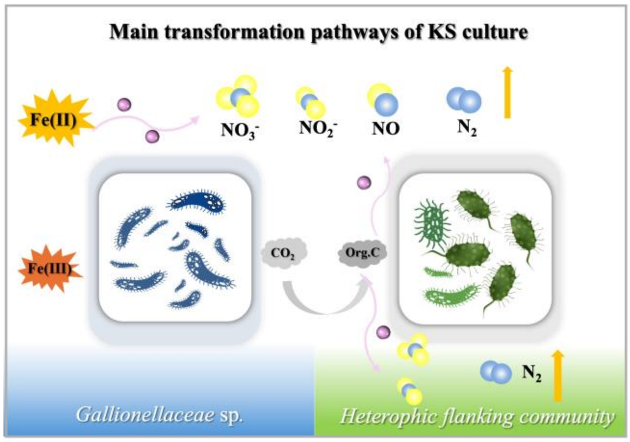


Figure 1. Main transformation pathways of Fe, N and C species in KS Fe(II)-oxidizing autotrophic denitrification (adapted from Tian et al. (2020)).

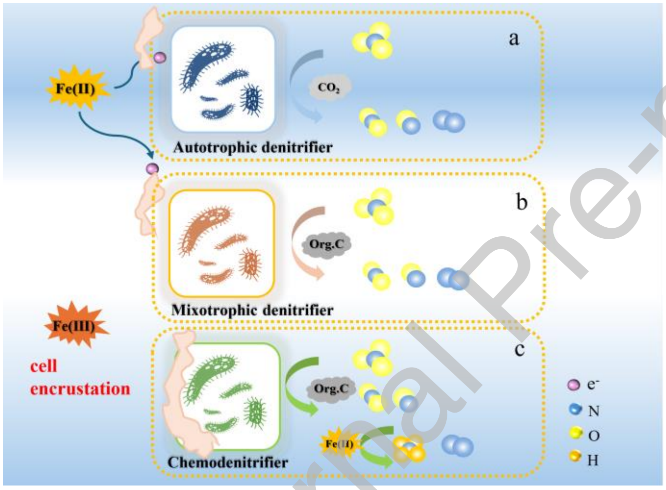


Figure 2. Three different types of interaction between nitrate-reducing bacteria and Fe(II): (a) autotrophic NAFO, where Fe(II) is oxidized enzymatically; (b) mixotrophic NAFO, involving both enzymatic and abiotic oxidation of Fe(II); (c) chemodenitrifiers, where no enzymatic oxidation of Fe(II) (adapted from Bryce et al. (2018)).

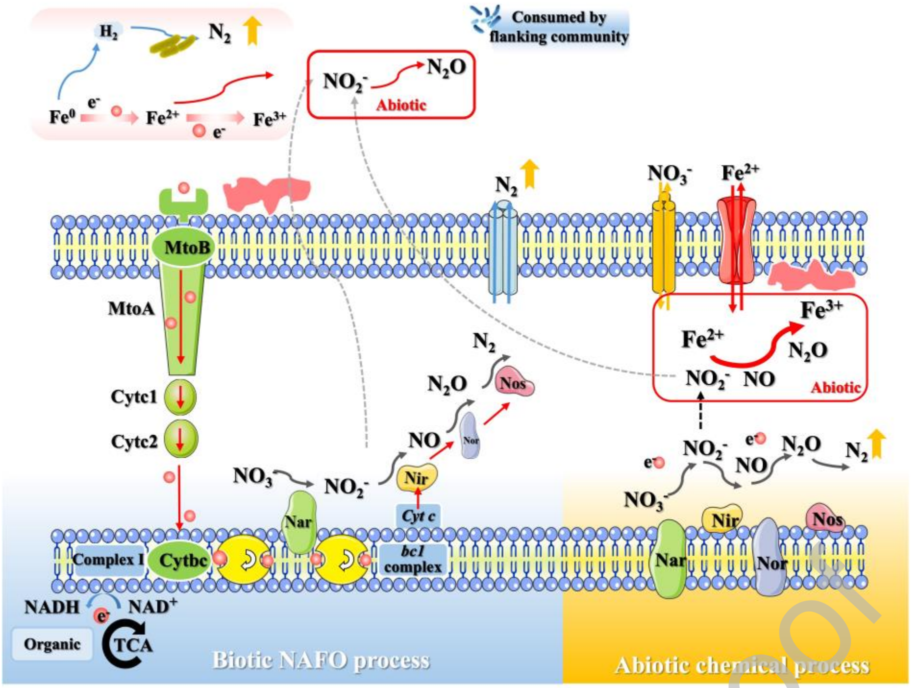


Figure 3. Proposed mechanisms for Fe(II) oxidation by biotic NAFO process and abiotic chemical process (adapted from Bryce et al. (2018); Wang et al. (2020); Pang et al. (2022)).

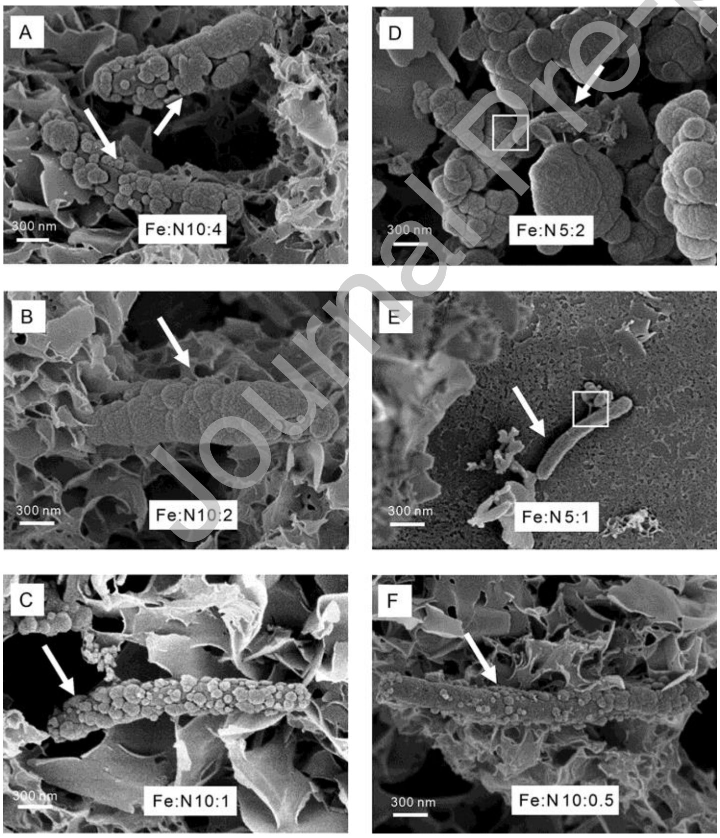


Figure 4. Scanning electron micrographs of cells in the autotrophic NRFeOx culture KS cultivated at different initial Fe/N ratios after 96 h of cultivation (Huang et al., 2023).

Table 1. Various solid-phase and liquid-phase iron electron donors for autotrophic denitrification: characteristics, application, and denitrification rate.

	Chemical composition	Reactor	Dosage/Fe^{2+} Conc.	Particle size	Bacteria	Initial Nitrate Conc.(mg/L)	Nitrate Removal (%)	Denitrification rate (g N/L·d)	Reference
Illite (IMt-1)	12.3% TFe, of which 10% was Fe(II)	Batch	13.3g/L	0.5–2μm	*Pseudogulbenkiania* sp. strain 2002	About 400	Over 99%	0.057	(Zhao et al., 2017)
Nontronite (Nau-2)	24% TFe, of which 0.6% was Fe(II)	Batch	6.3-6.8 g/L	0.02–0.5μm	*Pseudogulbenkiania* sp. strain 2002	558	Over 99%	0.028	(Zhao et al., 2013)
Siderite	Mainly $FeCO_3$ contain FeOOH, SiO_2 and illite	Up-flow biofilter	1158g/L	1-3mm	*Citrobacter freundii* strain PXL1	150	40.9% - 67.3%	0.0015-0.0025	(Yang et al., 2018)
Natural iron(II) carbonate	Fe>86%	Up-flow reactor			Mix culture (activated sludge and autotrophic bacteria)	20	72.29 %	0.029	(Zhu et al., 2019)
Sponge iron	Fe> 90% with TFe 96%-97%	Batch	83g/L	1-3mm	Cultured activated sludge (*Chloroflexi Acidobacteriota*)	50	90% TN: 25%-35%	0.045	(Wang et al., 2022a)
Iron scraps	Fe^0 purity>97 %	Lab-scale reactor	7457g	0.1-0.8cm	Soaked in wastewater for biomass enrichment	20.3	90.2%	0.018	(Liu et al., 2020)
Iron shavings	Fe^0 purity>99 %	Anaerobic reactors	15g/L		Sludge from anaerobic digester	TN 14	TN: 67.85 %	0.019	(Wang et al., 2019)
Pyrite		Constructed wetland	Pyrite:Rock 2:1		Activated sludge	33.9	61%	0.02	(Si et al., 2021)
Iron sulfide	FeS and S	Biofilter	Volume ratio 2:1	150μm	Sufure-based autotrophic denitrifiers	40		0.96	(Bai et al., 2023b)
Spong iron/$FeSO_4$		Up-flow biofilter	800mg/L Fe^{2+}	2-5mm	*Microbacterium* sp. W5	30	68.1%	0.02	(Zhou et al., 2016)
Spong iron/Fe(II)EDTA			1100mg/L Fe^{2+}				90%	0.027	
nZVI/Fe^{2+}		Lab-scale reactor	nZVI/Fe^{2+} 4g/L / 20mg/L		IRB strain CC76	10.57	93%	0.018	(Su et al., 2019)
$FeCl_2.4H_2O$		Batch	EDTA/Fe^{2+} 5.84g/L/0.56 g/L		*Pseudogulbenkiania* sp. strain 2002	124	55%	0.0068	(Kiskira et al., 2017b)
					T. denitrificans pure cultur	124	52%	0.0064	
$FeSO_4$		UASB			Activated sludge	50	81%	0.33	(Tian et al., 2020)
Pyrrhotite	Fe 56.69%, S 38.46%	Up-flow biofilter	Volume ratio 1:1	2.36-5.12mm	Sulfur-based autotrophic denitrifiers	TON 21.1 - 28	76.4% -91%	0.027	(Li et al., 2021b)
Pyrrhotite	Fe 56.02%, S 38.73%	Biofilter	50 g	250–425μm	Anaerobic sludge cultured in thiosulfate-based medium	28	99%	0.56	(Yang et al., 2017)

Table 2. Overview of isolates and enrichment cultures identified in previous studies as capable of autotrophic growth on Fe(II).

Species	Phylum	Isolation site	Growth pH	Temperature °C	Electron donor	Criteria for autotrophy 1-4				Reference
Azospira oryzae TR1	*β Proteobacteria*, *Rhodocyclales*, *Rhodocyclaceae*	Swine waste lagoons grass roots Bioremediation site	6.5	37	Fe^{2+}	-	-	-	n. a.	(Hunter, 2007; Mattes et al., 2013)
Acidovorax BoFeN1	*β Proteobacteria*	Lake sediments	6-9	4-37	Fe^{2+}	n. a.	+	-	n. a.	(Kappler et al., 2006)
Acidovorax strain BrG1	*β Proteobacteria*, *Comamonadaceae*	Town ditches; freshwater sediments	6-7	6-40	Fe^{2+}, FeS	n. a.	n. a.	-	n. a.	(Haaijer et al., 2012)
Aquabacterium strain BrG2	*β Proteobacteria*, *Burkholderiales*, *Comamonadaceae*	Town ditches; freshwater sediments	6-7	6-40	Fe^{2+}, FeS	n. a.	n. a.	+	n. a.	(Chen et al., 2023; Kiskira et al., 2017a)
Azoarcus strain ToN1	*β Proteobacteria*, *Rhodocyclales*, *Zoogloeaceae*	River sediment, Bremen; freshwater mud samples	7	15-28	FeS, $FeSO_4$	n. a.	n. a.	+	n. a.	(Straub et al., 1996)
Acidovorax sp. strain 2AN	*β Proteobacteria*	Iron-oxide-bearing sediment	6.8-6.9	30	$[Fe(II)EDTA]^{2-}$	-	n. a.	-	n. a.	(Chakraborty et al., 2011)
Citrobacter freundii PXL1	*γ Proteobacteria*, *Enterobacterales*, *Enterobacteriaceae*	Anoxic activated sludge of sewage plant, sewage sludge	7	30	$FeCl_2 \cdot 4H_2O$	n. a.	n. a.	-	n. a.	(Li et al., 2015)
Ferroglobus placidus (hyperthermophilic archaea)	*Archaeum*, *Archaeoglobi*, *Archaeoglobaceae*	Shallow submarine hydrothermal system	6-8.5	65-95	Fe^{2+}, FeS	+	-	-	n. a.	(Kiskira et al., 2017a)
Klebsiella strain FW33AN	*γ Proteobacteria*, *Enterobacteruaceae*	Nitrate and radionuclide contaminated aquifer	6.8	Room Temperature	Fe^{2+}, FeS	n. a.	n. a.	-	n. a.	(Kiskira et al., 2017a)

Pseudogulbenkiania sp. strain 2002	*β Proteobacteria, Neisseriales, Chromobacteriaceae*	Freshwater lake sediments	5-8	15-40	Fe^{2+}	+	-	+	+	(Weber et al., 2006)
Paracoccus ferrooxidans BDN-1	*α Proteobacteria, Rhodobacterales, Rhodobacteraceae*	Sewage sludge from bioreactor	5-8.5	10-45	$[Fe(II)EDTA]^{2-}$ $FeSO_4$	+	-	-	n. a.	(Kumaraswamy et al., 2006; Muehe et al., 2009)
Pseudomonas stutzeri	*γ Proteobacteria, Pseudomonadales, Pseudomonadaceae*	Soil and marine environments	7	15-28	$FeSO_4$	n. a.	n. a.	+	n. a.	(Muehe et al., 2009; Pena et al., 2012)
Pseudomonas sp. SZF15		Freshwater sediment	6.8-7	30	Fe2+	+	-	+	n. a.	(Su et al., 2015)
Thiobacillus denitrificans	*β Proteobacteria, Nitrosomonadales, Thiobacillaceae*	Pond, brackish mud, soil, marine sediment, sewage lagoon, digestion tank	6.8-7.4	28-32	Fe^{2+}, FeS_2 FeS	-	+	+	n. a.	(Beller et al., 2006; Shao et al., 2010)
Thiobacillus thioparus	*β Proteobacteria, Hydrogenophilales, Hydrogenophilaceae*	Pure culture, oil-contaminated sediment	7	30	FeS_2	n. a.	n. a.	+	n. a.	(Shao et al., 2010)
Enrichment culture KS	*β Proteobacteria, Gallionellales, Gallionellaceae*	Freshwater sediment	6.8 6.9-7.1	28-30	$FeCl_2$ Spong iron	+	+	+	+	(Claudia Tominski and Helene Heyer, 2018; He et al., 2016; Li et al., 2021a)

Criteria for autotrophy: (1) no need of organic carbon source, (2) cell growth with only Fe(II), nitrate, and CO_2 provided, (3) Fe(II) oxidation over several transfers, (4) CO_2 uptake demonstrated

Table 3. Fe-mediated autotrophic denitrification in Natural environments.

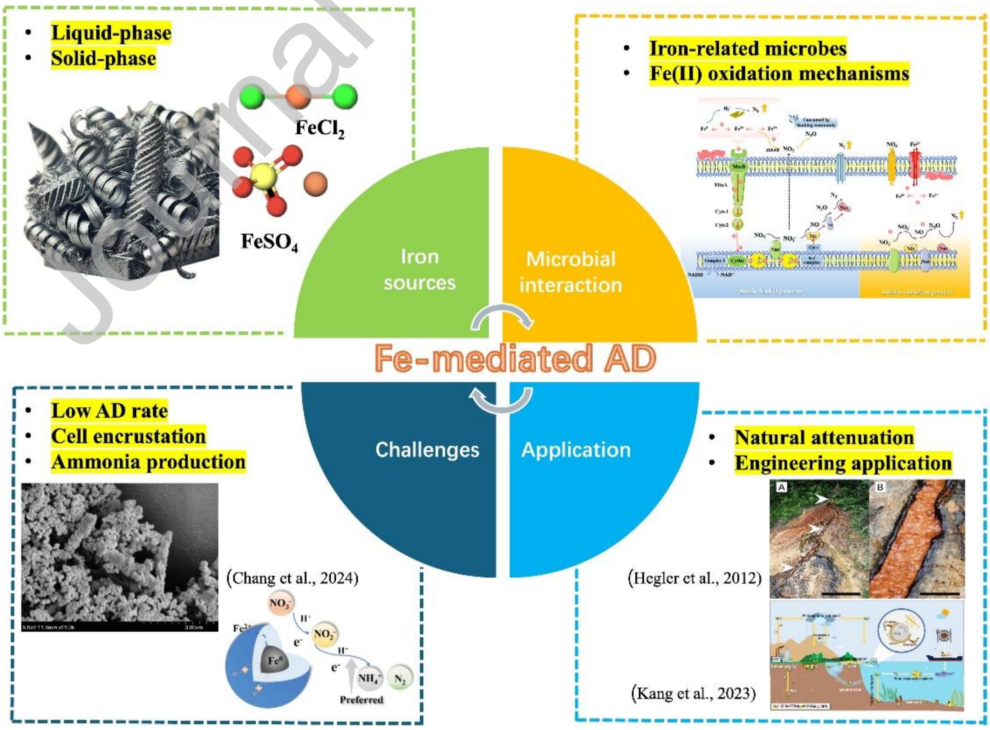
Location	Area	Electron donor	Fe Con. in original area	Evidence	Habitat/Culture Temperature (˚C)	Reference
Sediments of the brackish low-temperature spring	Novgorod region, Russia	Ochreous precipiates		Isolate *Hoeflea siderophile*	11-14/ 9-38	(Sorokina et al., 2012)
Groundwater basement tile drain	East Lansing, Michigan	FeS and $FeCO_3$	3-12μM	Isolate two axenic strains	10/21	(Emerson and Moyer, 1997)
Sediment				Isolation of BrG1		(Straub et al., 2004)
Groundwater	Oak Ridge			Stain TPSY grow mixotrophically with ferrous and 1mM acetate		(Byrne-Bailey et al., 2010)
Deep-water marine hydrothermal area	Juan de Fuca deep-sea	FeS_2, $FeCO_3$, FeO		Isolatiion of novel, psychrophilic, iron-oxidizing bacteria	4/ 3-10, up to 30	(Edwards et al., 2003)
Marine coastal sediment	Norsminde Fjord (Denmark)	Fe^{2+}	92±60 μM	Quantification of Fe(II) oxidation and nitrate reduction rates and the incorporation of 14C-labeled bicarbonate	NA /20	(Laufer et al., 2016b)
Plant rhizosphere		Iron deposits (Fe plaque)		4 strains of Fe plaque-associated FeOB were isolated		(Weiss et al., 2007)
Fuschna spring of Engadin region	Swiss Alps	Fe^{2+}, Fe(II) carbonates	High Fe^{2+}	Closely related to *Gallionella*	8.1-14.5/NA	(Hegler et al., 2012)
Lake sediment	Eastern China plain		Fe bound as Fe-Mn oxides 1243-1801 mg/kg	NO_3^- can still be reduced in Fe^{2+} treatments when it cannot be reduced by heterotrophic or chemodenitrifier	NA/25	(Pang et al., 2021)
Lake sediment	Constance	Fe^{2+}		Isolatiion of BoFeN1		(Kappler et al., 2006)
Paddy soil	Zhongsha city, China	Fe^{2+}	23 g/kg total Fe	dominant microorganisms (Zoogloea and Dechloromonas)		(Li et al., 2016)

Table 4. Application of iron in practical engineering wastewater treatment.

Application	Iron source	Reactor configuration	Wastewater	Experiment condition	Treatment performance	Main Bacteria	Reference
On-site River water treatment	Pyrite (FeS_2 89.2%, 5-20mm)	Horizontal subsurface CWs; Pyrite: Limstone (1:1 V/V)	COD 11.3-55.9mg/L NH_4^+ 0.3-7.4mg/L TN 8.9-19.6 mg/L TP 1.51-4.58mg/L	Pilot field test Three years HRT 72h	RE: COD 49.7-53.2%, NH_4^+ 38.4-83%, TN ± 21.4%, TP 88.2 ± 5.1%	*Anaeromyxobacter;Ramlibacter*	(Ge et al., 2019)
On-site groundwater treatment	Fe0 (3-8mm) Pyrite (0-3mm)	Microbial trapping devices	T: 10℃-19.3℃ pH: 7.3	Filled MTDs deployed into wells	0.67±0.13 mM 0.32±0.09 mM 0.01 mM in three different wells	*Hydrogenophaga* (14 %); Albidiferax (~20 %); Dechloromonas (22 %) *Thiobacillus* (27 %); *Pseudomonas* (~20 %)	(Visser et al., 2024)
Groundwater treatment	Iron scraps Pyrite	Vertical subsurface flow CWs	NO_3^-: 12.4mg/L	Hydraulic loading: 76.4 L/(m^2.d)	Effluent Con NO_3^-: 1.43mg/L TN RE: 59.1% Effluent Con NO_3^-: 4.28mg/L TN RE: 62.5%	High diversity *Thiobacillus* (2.3%); *Ferritrophicum; Hydrogenophaga; Actinobacteria* *Thiobacillus* (0.20%)	(Dong et al., 2024)
Municipal secondary effluent treatment	biomass-iron mixture (BIM)	Biofilter 500mm depth filter layer	NO_3^-: 10.68±0.11 COD: 33.07±2.96 NH_4^+: 0.54±0.02 TN: 11.67±0.33	HRT 3days	RE: TN 81%-89%	*Acidovorax; Citrobacter; Brevundimonas*	(Wang et al., 2024)
Nitrate and heavy metal-contained groundwater	Iron-carbon (0-1cm)	Vertical CWs Iron scarps:biochar 1:1 (mass ratio)	NO_3^-: 20mg/L Cr: 2mg/L Pb: 2mg/L	HRT 3days	RE: NO3- 87% TN 78% Pb and Cr : 75-97%	*Rhodobacteraceae; Rhodobacter*	(Jia et al., 2020)
Bioremediation of Cr(VI)-aquifiers	Mackinawite (FeS)	Bioreactor filed with 200g mackinawite	Con Cr(VI): 10mg/L 50mg/L	HRT 12-24h	RE: Cr(VI) 23.1-100%	*Geobacter; Syntrophobacteraceae; Acidovorax*	(Lu et al., 2020)

			NO_3^-: 10mg/L				
Arsenite and nitrate-contained groundwater	Pyrrhotite	Up-flow fixed-bed reactors; pyrrhotite, sulfur and limestone(6:3:1 v/v/v)	NO_3^- 30mg/L As(III) 200-10000μg/L	HRT 6h	Effluent Con NO3- 3.78±1.14 mg/L As(III) 7.84±7.29 μg/L	*Thiobacillus* (55.83%); norank-f-PHOS-HE36(12.16%); *Ferritrophicum*(4.46%)	(Li et al., 2021b)
		up-flow anaerobic sludge blanket (UASB)	As(III)800 μg/L NO_3^- 30mg/L	HRT 12h pH 7.2 250 days operation	Effluent Con. NO_3^- 3mg/L As(III) ≤10 μg/L	*norank_f_Gallionellaceae* *norank_f_Xanthobacteraceae* *Commonadaceae; Thermomonas*	(Liu et al., 2024)
Trichloroethylene-contaminated groundwater	mZVI (2-30 μm)	Batch experiment: mZVI-autotrophic hydrogen bacteria (AHB) 1-30g/L	TCE: 20mg/L		RE TCE: 75% at 20d, 97% at 60d	*Acetobacterium; Pseudomonas; Hydrogenophaga; Desulfomicrobium; Dechlorosoma*	(Yuan et al., 2022)

Graphical abstract



Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: