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RESEARCH ARTICLE

Visible light induces bacteria to produce superoxide for manganese oxidation

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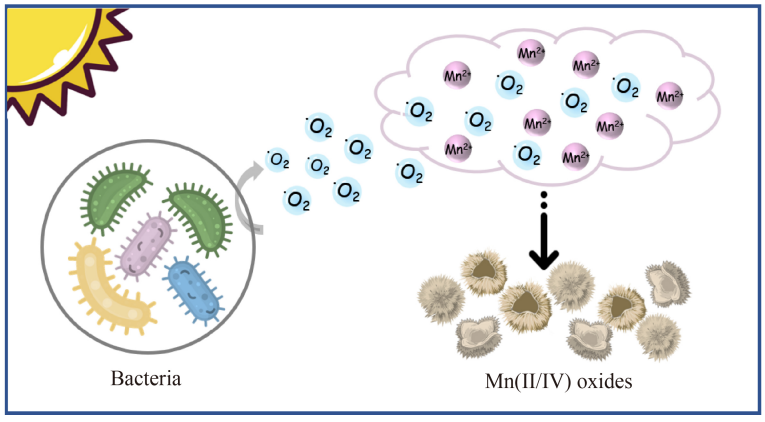
HIGHLIGHTS

- Term of manganese-oxidizing microorganisms should be reconsidered.
- Visible light induces heterotrophic bacteria to produce superoxide.
- Heterotrophic bacteria oxidize Mn(II) ions with a fast oxidation rate.
- Superoxide oxidizing Mn(II) ions is an unintended side reaction of bacteria.
- Superoxide is an important oxidation force of Mn(II) in the environment.

GRAPHIC ABSTRACT



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ABSTRACT

Manganese oxides are widely distributed in soils and sediments, affecting the migration and transformation of heavy metals and organic pollutants. The microbial conversion of soluble Mn(II) into insoluble Mn(III/IV) oxides is considered to be the initial source of manganese oxides in the environment; however, whether this process is related to a physiological role remains unclear. Here, we explored the microbial manganese oxidation process under visible light by using coastal surface seawater microorganisms. Visible light greatly promotes the oxidation rate of Mn(II), and the average rate reaches 64 μmol/(L·d). The generated manganese oxides were then conducive to Mn(II) oxidation, thus the rapid manganese oxidation was the result of the combined action of biotic and abiotic, and biological function accounts for 88 % ± 4 %. Extracellular superoxide produced by microorganisms induced by visible light is the decisive factor for the rapid manganese oxidation in our study. But the production of these superoxides does not require the presence of Mn(II) ions, the Mn(II) oxidation process was more like an unintentional side reaction, which did not affect the growth of microorganisms. More than 70 % of heterotrophic microorganisms in nature are capable of producing superoxide, based on the oxidizing properties of free radicals, all these bacteria can participate in the geochemical cycle of manganese. What's more, the superoxide oxidation pathway might be a significant natural source of manganese oxide.

1 Introduction

Manganese oxides are natural reactive minerals and widely spread in aquatic and terrestrial environments (Jung et al., 2020), affecting the fate of metals (such as As^{3+} and Cd^{2+}) and organic pollutants (such as phenols and diclofenac) through adsorption and oxidation in sewage treatment (Tournassat et al., 2002; Forrez et al., 2010; Song et al., 2019; Huang and Zhang, 2020; Yang et al., 2020). Usually, the manganese (III/IV) oxides in the environment are thought to be formed by the oxidation of dissolved Mn(II) through abiotic or biotic processes (Jung et al., 2020). Oxidation of aqueous Mn(II) by dissolved

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oxygen is thermodynamically favored, but the kinetic is slow due to the high energy barrier of the reaction from dissolved Mn(II) to Mn(III/IV) oxides (Nealson, 2006). The presence of microorganisms accelerates the oxidation rate, which is 4–5 orders of magnitude faster than the rate of abiotic chemical oxidation, therefore is considered as the initial source of manganese oxides in the environment (Carmichael and Bräuer, 2015).

Bacteria capable of catalyzing the oxidation of dissolved Mn(II) ions to undissolved Mn(III/IV) oxides are usually called manganese-oxidizing bacteria (Nealson, 2006). According to our summary of the previous reports (Fig. S1), there are many kinds of manganese-oxidizing bacteria, including the phyla of Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Tebo et al., 2005; Zhou and Fu, 2020). Tebo et al. (2004) divided the bacterial oxidation of Mn(II) ions into direct and indirect ways, and the process catalyzed by enzymes on the surface of microorganisms is called direct oxidation. Manganese oxidases belong to two families of proteins: multicopper oxidase, which has been described in *Bacillus*, *Leptothrix*, *Pseudomonas*, and *Pedomicrobium* (Zhou and Fu, 2020); and animal heme proteins, described in *P. putida* GB-1, *Erythrobacter* sp. Strain SD21 and *Aurantimonas manganoxydans* Strain SI85-9A1 (Anderson et al., 2009; Nakama et al., 2014; Geszvain et al., 2016). For indirect pathways, some bacteria can change their surrounding environmental conditions for Mn(II) oxidation (e.g., the pH and Eh) (Nealson, 2006; Zhou and Fu, 2020). *Roseobacter* clade has been demonstrated to oxidize Mn(II) by producing extracellular reactive oxygen species in recent studies (Learman et al., 2011; Andeer et al., 2015). However, reactive oxygen species are ubiquitous in the ocean due to biological and non-biological processes (Morris et al., 2022). Do other bacteria clades have similar Mn(II) oxidation processes with *Roseobacter*? Is the Mn(II) oxidation closely relative to the physiological process of bacteria?

In this study, we enriched heterotrophic bacteria from coastal seawater and investigated their manganese oxidation processes under visible light. The mechanism of manganese oxidation was investigated through community analysis, pH change, oxidation ability of cell-free supernatant, reactive oxygen species, and semiconductor minerals.

2 Materials and methods

2.1 Sample collection

Coastal seawater was collected from Jimei District of Xiamen City, China (24°34'59" N, 118°6'38" E). After 1 L of seawater was precipitated to remove large particles such as gravel and other impurities, a piece of 0.22 μm filter membrane was used to collect microorganisms in the seawater. Then the filter membrane was inoculated into a K-acetate medium with sodium acetate as the main nutrient for pre-enrichment. The K-acetate medium composition can be found in Table S1. After culturing for 2 d, the cells were transferred to a 2216E medium (Marine broth, BD, USA), cultured in a shaking incubator operated at 25 °C with 150 r/min for 24 h, and then preserved as seeds at −80 °C for subsequent experiments.

2.2 Manganese oxidation

The conserved bacteria were injected into the K-acetate medium and activated for 24 h before being inoculated with a 2 % inoculum into a fresh K-acetate medium. The manganese-added group was incubated in a light shaking incubator operated at 25 °C and 150 r/min (the light source used was LED light with a light intensity of 12 ± 2 mW/cm^2, wavelength range 450–460 nm), containing 1 mmol/L $MnSO_4$ (AR, Sinopharm Chemical Reagent Co. Ltd., China). Unless specified, the concentration of $MnSO_4$ used in this work was 1 mmol/L). The dark cultures were wrapped with aluminum foil to block the light. The sterile control group only added $MnSO_4$ without microbes inoculation. To measure the rate of manganese oxidation, changes in biomass, and pH during the cultivation, two milliliters of the culture medium were sampled at an appropriate interval.

To confirm the role of bacteria, the bacteria were precultured for 24 h without $MnSO_4$ presence in the dark. Then, $MnSO_4$ was added to the bacterial culture, mixed homogeneously, and transferred into the light culture. For the bacterial activity inhibition group, the inhibitor NaN_3 (AR, Sinopharm Chemical Reagent Co. Ltd., China) was added before light culture. Heat inactivation was also used to inhibit bacterial activity. After the bacteria were cultured in the dark without $MnSO_4$ presence for 24 h, the bacterial culture was heated in a 90 °C water bath for 20 min, after cooling, $MnSO_4$ was added and then placed in a light shaking incubator.

To explore the oxidation ability of the bio-generated Mn(III/IV) oxides to oxidize Mn(II) ions, the freeze-dried powder of generated Mn(III/IV) oxides was used in artificial seawater (Table S2) containing 0.5 mmol/L $MnSO_4$. Four milligrams of Mn(III/IV) oxides powder were added to 200 mL of $MnSO_4$-containing artificial seawater and ultrasonicated for 1 h to evenly disperse the oxides. Then the mixture was divided into 50 mL Erlenmeyer flasks, 20 mL in each, and cultured in a light shaking incubator. Samples were taken before and after 6 d of culturing to determine the concentration of manganese oxides in the culture medium. For the dark experiment, all the bottles were wrapped with aluminum foil.

2.3 Manganese oxides, biomass, and pH measurements

The concentration of manganese oxides was determined

by the Leucoberbelin Blue (LBB. AR, Sigma-aldrich, USA) colorimetric method (Yang et al., 2021). Briefly, 0.5 mL of specific color reagent LBB was added to 0.1 mL of culture medium (0.04 g LBB was dissolved in 45 mmol/L acetic acid solution, stored at 4 °C), and reacted in the dark for 15 min. After that, the mixture was centrifuged at 12000 r/min for 5 min, and the absorbance of the supernatant was determined at 620 nm. The standard curve was plotted using $KMnO_4$ (AR, Sinopharm Chemical Reagent Co. Ltd., China), and 1 μmol/L $KMnO_4$ was equivalent to 2.5 μmol/L MnO_2. The absorbance at 600 nm (Abs_{600}) was used to indicate the biomass during the whole culture process. To avoid the influence of the generated manganese oxides on the absorbance, 0.05 mL of 5 % hydroxylamine hydrochloride (AR, Sinopharm Chemical Reagent Co. LTD, China) was added into 0.75 mL of culture medium before absorbance measurements. One milliliter of culture medium was centrifuged at 12000 r/min for 5 min, and 0.9 mL of supernatant was taken for pH determination.

2.4 Community analysis

To investigate the community composition before and after dark/light incubation with and without Mn(II), the original inoculum and incubations under different conditions on the 8th day were collected. With the help of the Fast DNA SPIN Kit for Soil (MP Biomedicals, USA), the total DNA of these bacterial communities was extracted. The 16S rDNA gene was amplified with a set of primers (338F/806R) targeting the V3–V4 region of the 16S rRNA gene in a 20 μL PCR reaction system. After the PCR products were purified and quantification, the DNA libraries were sequenced using a HiSeq platform (Illumina Inc., USA) with a paired-end 250 strategy (Zheng et al., 2019) in Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). Detailed information is presented in the supporting information.

2.5 Cell-free supernatant Mn(II) oxidation

To investigate whether the bacteria produced extracellular metabolites to oxidize Mn(II) ions, we tested the manganese oxidation capacity of the cell-free supernatant. After the bacteria had been cultured in the dark for 24 h, the bacterial solution was centrifuged at 5000 r/min for 10 min. The supernatant was then transferred to a clean centrifuge tube and centrifuged again at 5000 r/min for 10 min. After filtering the supernatant through a 0.22 μm syringe filter, $MnSO_4$ was added and cultured under visible light. To avoid some of the metabolites being adsorbed by the filter membrane during filtration, we also performed an oxidation experiment on the supernatant after only two centrifugation procedures. The manganese oxides concentration was measured after 5 d.

2.6 Reactive oxygen species detection

2.6.1 Total reactive oxygen species detection

The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime, China) was used to detect the total reactive oxygen species (ROS) produced by microorganisms. The probe DCFH-DA can diffuse into the cell and be deacetylated by cellular esterases into a non-fluorescent compound that can be subsequently oxidized by ROS into 2',7'-dichlorofluorescein (Wu et al., 2020). The detailed procedure can be found in the supporting information, briefly, bacteria cultivated for 24 h were harvested, resuspended in a fresh medium, then incubated with 10 μmol/L DCFH-DA for 30 min to load the probe. Next, the cells were exposed to light for 2 h, and dark incubation without any treatments was used as the negative control. Finally, the fluorescence intensity was detected with a microplate analyzer (excitation and emission wavelengths were 488 nm and 525 nm, respectively) (Spark, TECAN, Austria). The change in the fluorescence intensity between the experimental groups and the negative control group was calculated.

2.6.2 Superoxide detection

The superoxide radical kit (Solarbrio, China) was used for detection. Before determination, the bacteria were cultured in the dark without $MnSO_4$ for 24 h and then transferred to the light for an additional 2 h. After light exposure, 0.3 mL of superoxide radical extract was quickly added to 5 mL of bacterial solution, and the determination was carried out according to the procedure of the kit. The biomass of each group was at the same level, and the absorbance at 600 nm was about 0.27.

2.6.3 Reactive oxygen species inhibition

To verify the role of ROS in the manganese oxidation process, ROS inhibitors were used in the manganese oxidation process. After the microorganisms were incubated in the dark for 24 h, $MnSO_4$ was added, mixed, and dispensed into a 50 mL Erlenmeyer flask, and each flask contained 20 mL. Then $CuCl_2$ (AR, Sinopharm Chemical Reagent Co. Ltd., China), dimethyl sulfoxide (DMSO. AR, Aladdin, China), and diphenylene iodonium chloride (DPI. AR, Macklin, China) (Learman et al., 2011) were added and incubated in the light incubator. The concentration of manganese oxides in each group was determined after 2 d of incubation. The inhibition rate between inhibitors with and without groups was calculated.

2.7 Manganese oxides characterization

The morphology, structure, valence, and optical

absorption properties of manganese oxides were investigated. Details are provided in the supporting information.

The photoelectrochemical properties were obtained by using the standard three-electrode system (Li et al., 2021) with a CHI 660D electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China), and details can be found in the supporting information.

3 Results and discussion

3.1 Visible light promotes bacteria manganese oxidation

Under light incubation, the concentration of manganese oxides continued to increase with time (Fig. 1(a)), and the color of the culture turned dark brown (Fig. S2). The average manganese oxidation rate in the first 7 d was 64 μmol/(L·d) (calculated by the generated manganese oxides). The scanning electron microscope (SEM) image (Fig. 1(b)) shows that the generated oxides were hollow spheres with a layered surface and were amorphous (Fig. S3). Fig. S4 displays the X-ray photoelectron spectroscopy (XPS) spectra of bio-generated manganese oxides and its fitting results. The fitting peak of Mn $2p_{3/2}$ at 641.5 eV and 642.6 eV corresponds to Mn(III) and Mn(IV) (Nesbitt and Banerjee, 1998), demonstrating that the generated Mn oxides are a mixture of coexisting multivalent states, with an average valence state of 2.8. However, no Mn(III/IV) oxides were detected in the dark-cultured bacterial system, indicating that visible light was indispensable in our experiments. In the abiotic control group, no Mn(III/IV) oxides were produced regardless of the presence of light, suggesting that the manganese oxidation process may be caused by the interaction of visible light and bacteria.

To confirm the above assumption, we verified the functions of bacteria and light in the manganese oxidation process, respectively. The cell activity was suppressed by heating or adding the inhibitor NaN_3 after they had been precultured in dark conditions until the stable period (approximately 24 h) (Cabrol et al., 2017; Cebrián et al., 2017). Fig. 1(c) demonstrates that the addition of 1 mmol/L NaN_3 could not completely inhibit microbial manganese oxidation, while the addition of 2 mmol/L NaN_3 could completely inhibit the generation of Mn(III/IV) oxides under light conditions. No Mn(III/IV) oxides were detected either after being heated. These results indicate that the presence of active bacteria is necessary for the oxidation of Mn(II) in the system.

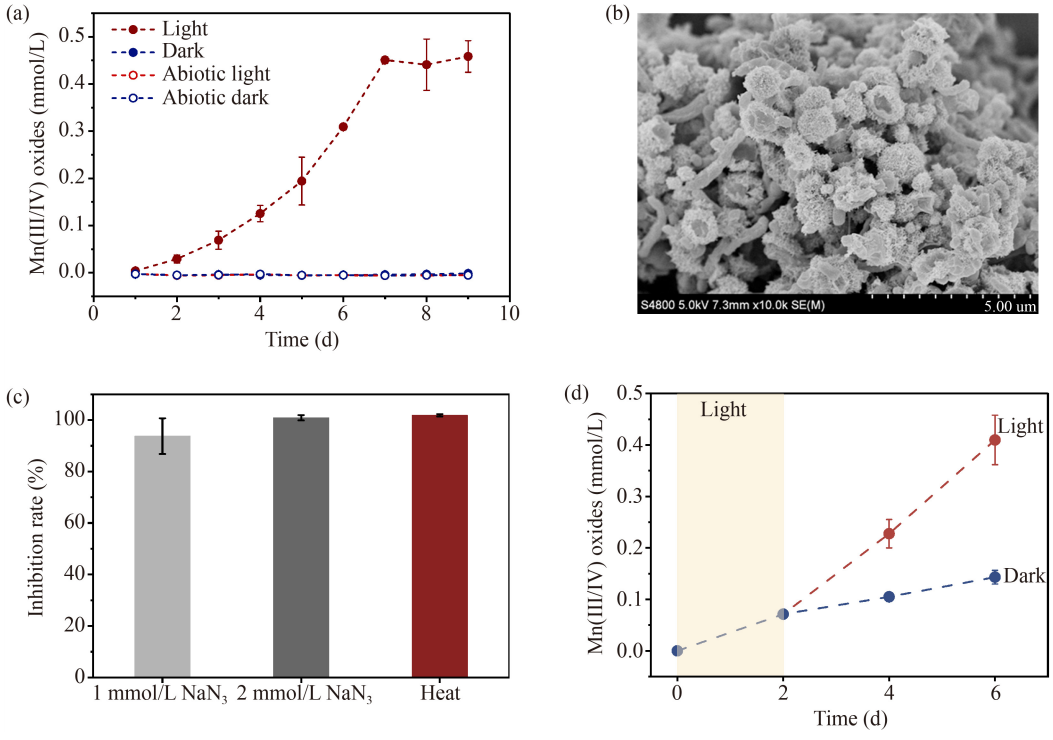

Fig. 1 Visible light promotes bacterial manganese oxidation. (a) The concentration change of Mn(III/IV) oxides with time: red and blue represent the light and dark culture conditions, respectively; solid and hollow symbols represent the bacteria and sterile control groups, respectively. Lines of dark, abiotic light, and abiotic dark groups are overlapped, due to Mn(II) oxidation has not occurred in the three groups; (b) SEM image of biogenic Mn(III/IV) oxides under light; (c) The manganese-oxidizing inhibition rate when the bacterial activity was inhibited by NaN_3 or heat treatment; (d) Change of Mn(III/IV) oxides content with time after light removal. Error bars represent the standard deviation of three replicates.

To confirm the role of visible light furtherly, we first incubated the bacteria with $MnSO_4$ under light for 2 d to obtain Mn(III/IV) oxides and then transferred half of the culture into the dark and the rest continued to be cultured under visible light. The increasing rate of Mn(III/IV) oxides content slowed down after light removal (Fig. 1(d)), indicating that the high rate of Mn(II) oxidation can not be separated from the effect of light. Overall, visible light promoted the oxidation rate of manganese.

3.2 Possibility of direct oxidation of Mn(II) by manganese-oxidizing bacteria

To explore whether the bacteria with direct manganese oxidation abilities were enriched under light conditions, high-throughput sequencing was used to analyze the original inoculum and the communities after 8 d of light/dark culture. *Vibrio* was the dominant genus in the initial inoculum, and the diversity of each group was calculated. After 8 d of cultivation, the Shannon index increased while the Simpson index decreased (Table S3), suggesting that the diversity of each community was improved after being cultured.

The community composition was similar after 8 d of light and dark culture (Fig. 2). But *Thalassospira* and norank_f_Flavobacteriaceae were more enriched, and the relative abundance of *Vibrio* was significantly reduced under light incubation. However, neither the genus of *Thalassospira* nor the family of *Flavobacteriaceae* has been reported to be able to oxidize Mn(II) ions directly. In addition, the relative abundances of *Thalassospira* and norank_f_*Flavobacteriaceae* in the dark culture accounted for 8.6 % ± 2.0 % and 3.8 % ± 1.7 %, respectively, however, no Mn(III/IV) oxides were detected, suggesting that the occurrence of manganese oxidation under light culture may not be specifically related to the bacteria of these two genera.

Moreover, manganese-oxidizing bacteria with direct oxidation capacity can oxidize Mn(II) ions by surface multicopper oxidase or animal heme enzymes, forming manganese oxides on the cell surface, even wrapping the cell (Nealson, 2006; Geszvain et al., 2016; Palermo and Dittrich, 2016). In this study, it was found that the generated Mn(III/IV) oxides existed independently and did not bind closely with the cells through SEM observation (Fig. 1(b)), suggesting that no bacteria with direct manganese oxidation ability were enriched under light culture conditions.

3.3 Possibility of forming a high pH microenvironment to oxidize Mn(II) ions

Some microorganisms can change the pH of their surrounding environment in the process of growth and metabolism. An alkaline environment (pH > 9) is favorable for the oxidation of Mn(II) ions by oxygen (Richardson et al., 1988). The changes in the pH of the culture medium during the whole culture process were tracked and measured (Fig. 3). The results showed that the pH of the culture medium gradually increased with culture time whether light and Mn(II) ions were supplied, and the final pH reached 8.4 ± 0.1. However, only when pH > 9 does the reaction of molecular oxygen oxidizing Mn(II) ions can quickly occur (Hansel et al., 2012). Notably, under the light culture conditions, Mn(III/IV) oxides can be detected after 1 d of incubation while the pH is only 7.4, suggesting that a small change in pH is not the main driving force for the oxidation of Mn(II) ions.

3.4 Possibility of Mn(II) oxidation by secreted in vitro metabolites

Some organic metabolites secreted from bacteria cells to the culture medium can chelate with Mn(II) ions and indirectly promote manganese oxidation (Duckworth and

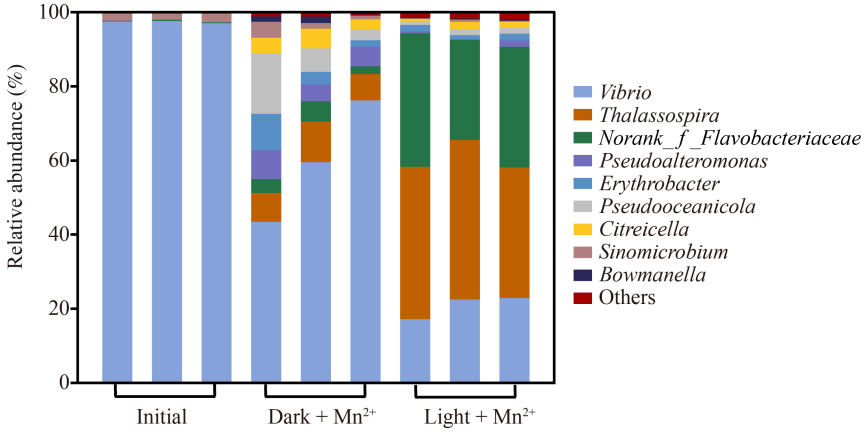

Fig. 2 Community composition before and after light/dark culture with manganese (genus level). Cultures with Mn(II) ions in the dark/light are marked as dark + Mn^{2+} and light + Mn^{2+}, respectively. The initial inoculum is marked as initial.

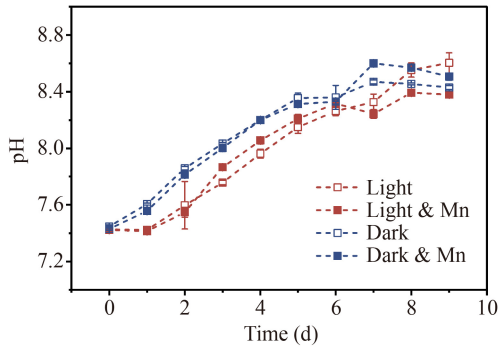

Fig. 3 Changes in pH during light/dark cultures with/without manganese. Red and blue represent the light and dark culture conditions, respectively; solid and hollow symbols represent the presence and absence of Mn(II) ions, respectively. Error bars represent the standard deviation of three replicates.

Sposito, 2005). However, when Mn(II) ions were added to the cell-free supernatant, no Mn(III/IV) oxides formed after 5 d of light incubation (Fig. S5), demonstrating that the bacterial community did not oxidize Mn(II) ions by producing in vitro metabolites or enzymes. The Mn(II) oxidation process was completely inhibited after the inhibition of microbial activity (Fig. 1(c)) also proving that there were no chelators or enzymes in the supernatant that could accelerate Mn(II) oxidation or directly oxidize Mn(II).

The above results demonstrate that manganese oxidation relies on the presence of bacteria, although not through a direct pathway. Comparing the community composition in the absence of manganese after 8 d of light/dark culture, the relative abundance of *Vibrio* decreased significantly under light conditions, from 97.4 % ± 0.3 % of the initial inoculum to 17.5 % ± 7.1 % of the light incubation (Fig. S6). While the relative abundances of *Thalassospira*, norank_f_*Flavobacteriaceae*, *Psudoalteromonas*, *Erythrobacter*, and other genera were significantly improved. There may be two possible reasons: first, light may be conducive to the growth of these bacteria. For example, some bacteria belonging to the genera of *Thalassospira* and *Erythrobacter* can fix CO_2 under the light driving to obtain additional carbon sources (Koblížek et al., 2003; Xia et al., 2021), which may improve their competitiveness and get more enrichment. Second, the light might inhibit *Vibrio* growth, thereby providing space for other bacteria to thrive. The photosensitive substances porphyrin and flavin in *Vibrio* cells can produce reactive oxygen species under light irradiation (Kumar et al., 2016), and excessive reactive oxygen species may not be conducive to the growth of *Vibrio* (Halstead et al., 2016). However, these reactive oxygen species may contribute to the oxidation of Mn(II) ions (Hansard et al., 2011). For instance, *Roseobacter* sp. AwzK-3bk secretes NADH into the culture medium to form superoxide, thereby oxidizing Mn(II) ions (Learman et al., 2011). Thus, we speculated that light may induce some bacteria to produce reactive oxygen species, resulting in the oxidation of coexisting Mn (II) ions.

3.5 Possibility of manganese oxidation by reactive oxygen species

Previous studies have shown that *Roseobacter* sp. AzwK-3b oxidized Mn(II) by producing superoxide, and Mn(II) oxidation by ascomycete fungus *Stilbella aciculosa* is associated with superoxide production during asexual reproduction (Learman et al., 2011; Hansel et al., 2012). Did the microbes produce reactive oxygen species under the light that led to Mn(II) oxidation? The fluorescent probe DCFH-DA was used to detect whether bacteria produced reactive oxygen species under light culture conditions (Tardu et al., 2017; Zhao et al., 2021). The fluorescence intensity increase ratio of the experimental group to the dark control group was calculated. When the increased intensity ratio was positive, reactive oxygen species were produced (Tardu et al., 2017). And the biomass of all experimental groups was the same to exclude the influence of biomass on fluorescence intensity (Fig. S7). As shown in Fig. 4(a), the fluorescence intensity of the initiators (Rosup) of the reactive oxygen species added group increased by 37.2 % ± 3.0 %, indicating that the experimental method was viable. After light stimulation, the fluorescence intensity increased by 10.1 % ± 0.3 %, demonstrating that light can cause bacteria to produce reactive oxygen species. Furthermore, with Mn(II) ions added under light, the fluorescence intensity increased by 15.5 % ± 3.9 %, suggesting that the addition of Mn(II) ions can make the bacteria produce more reactive oxygen species. Although manganese is an essential micronutrient for organisms, the amount added in the experiment (approximately 1 mmol/L) may be beyond the required level for organisms, and excessive Mn(II) ions may cause microbial oxidative stress reactions (Xu et al., 2020).

Generally, microbes can produce $\cdot O_2^-$ and H_2O_2 extracellularly or intracellularly (Cabiscol et al., 2000; Hansel and Diaz, 2021), and the role of these reactive oxygen species in manganese oxidation can be verified by adding scavengers of reactive oxygen species. When Cu(II) ions, an effective $\cdot O_2^-$ scavenger (Learman et al., 2011), were added (even only 25 μmol/L, which does not affect microbial activity (Yang et al., 2013)), the manganese oxidation process under light culture conditions can be completely inhibited (Fig. 4(b)), indicating that the $\cdot O_2^-$ played a decisive role in the Mn(II) oxidation process in our experiment. H_2O_2 always acts as a reducing agent for Mn(III/IV) oxides, detrimental to Mn(II) oxidation process (Learman et al., 2013). The addition of H_2O_2 in the culture medium did not enhance Mn^{2+} oxidation (Fig. 4(b)), and H_2O_2 was not detected in the culture medium under illumination

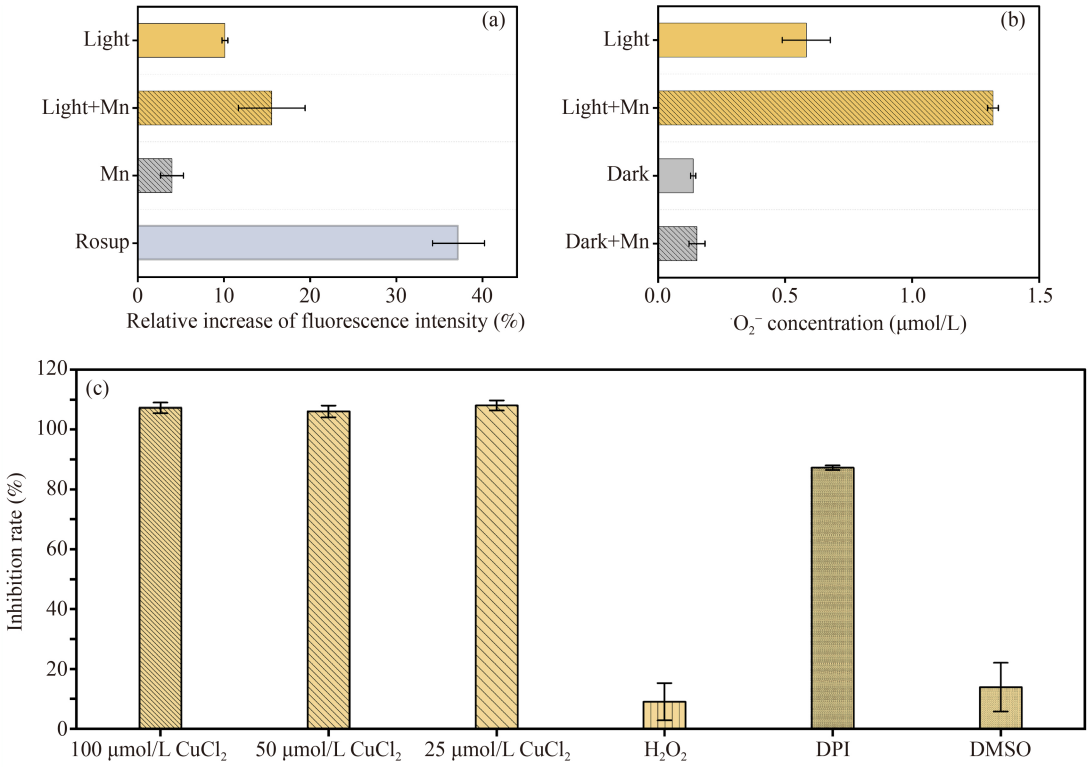

Fig. 4 Reactive oxygen species detection. (a) The increased fluorescence intensity ratio of light with/without Mn(II) ions and the addition of reactive oxygen species initiator (Rosup); (b) Superoxide concentrations were detected in light/dark conditions for 2 h in the presence/absence of manganese. (c) Inhibition rate of Mn(II) oxidation with different inhibitors. Error bars represent the standard deviation of three replicates.

(data not shown), thereby, H_2O_2 had no effect on the process of Mn(II) oxidation. Although, the addition of ·OH inhibitor dimethyl sulfoxide (DMSO) (Learman et al., 2011) slightly inhibited the Mn(II) oxidation rate, suggesting that ·OH contributed to the Mn(II) oxidation. However, bio-generated ·OH is usually transformed from $\cdot O_2^-$ (Hayyan et al., 2016), and the addition of $\cdot O_2^-$ inhibitor completely inhibited Mn(II) oxidation, demonstrating that the effective ·OH is generated from $\cdot O_2^-$.

We further tested the production of superoxide ($\cdot O_2^-$) under light stimulation. Microorganisms can produce superoxide during aerobic respiration (Hansel and Diaz, 2021); thus, 0.19 ± 0.09 μmol/L $\cdot O_2^-$ were also detected under dark conditions (Fig. 4(c)). The content of superoxide also increased slightly after the addition of Mn(II) ions in the absence of light, suggesting that the oxidative stress reaction caused by the addition of Mn(II) ions may lead to the generation of $\cdot O_2^-$. After light stimulation, 0.58 ± 0.09 μmol/L of $\cdot O_2^-$ was detected, which was 2 times higher than that in dark culture, indicating that light can induce more $\cdot O_2^-$ production by these bacteria. In particular, when Mn(II) ions were added, 1.32 ± 0.02 μmol/L of $\cdot O_2^-$ was detected after 2 hours of light culture, which was about 1.3 times more than light culture alone, demonstrating that Mn(II) ions can further stimulate the production of $\cdot O_2^-$, which is consistent with the determination of total reactive oxygen species. The above results also indicate that microorganisms can produce superoxide under light, which can oxidize Mn(II).

When diphenylene iodonium chloride (DPI), a commonly used microbial transmembrane oxidoreductase and NAD(P)H-binding enzyme inhibitor was added to a final concentration of ~50 μmol/L, the manganese oxidation process was inhibited (Fig. 4(b)), suggesting that superoxide formation may be related to NAD(P)H enzyme activity. NAD(P)H oxidases are inserted into the plasma membrane and can use cytoplasmic NAD(P)H to reduce O_2 outside the cell membrane to form superoxide (Hansel et al., 2012), therefore oxidizing the Mn(II) ions in the surrounding environment.

3.6 The role of biogenic manganese oxides

When manganese oxides were formed and transferred to dark cultures, their content continues to increase (Fig. 1(d)), however, these microorganisms were unable to oxidize Mn(II) in dark culture, indicating that abiotic Mn(II) oxidation was able to occur after Mn(III/IV) oxides generated. After the formation of manganese oxides, NaN_3 was added to inhibit the activity of microorganisms, and the content of manganese oxides

continued to increase (Fig. 5(a)), which might be because the newly formed Mn(III/IV) oxides have the ability to oxidize Mn(II) ions (Murray et al., 2007; Jung et al., 2020). To confirm whether the bio-generated Mn(III/IV) oxides could oxidize Mn(II) ions furtherly, the freeze-dried Mn(III/IV) oxides powder was used to investigate its oxidation capacity in artificial seawater. After 6 d of dark cultivation, the Mn(III/IV) oxides content hardly changed. However, the content of Mn(III/IV) oxides increased by approximately 35 % after 6 d of light cultivation, from the original 65 μmol/L to 83 μmol/L (Fig. 5(b)).

Manganese oxides are natural semiconductor minerals that can promote Mn(II) oxidation by photogenerated holes or reactive oxygen species under light irradiation (Xu et al., 2019). Ultraviolet-visible diffuse reflectance spectroscopy shows that the generated Mn(III/IV) oxides have good light absorption in the range of 310–600 nm (Fig. S8). The photocurrent test (Fig. S9) demonstrated that when the oxides were illuminated by visible light, electrons were quickly excited to the conduction band, resulting in the current increase. But after the light was cut off, the current immediately decreased, indicating that Mn(III/IV) oxides have well visible light responsiveness (Ding et al., 2018). The addition of hole scavenger oxalate (Ramadoss et al., 2021) and sulfite (Hou et al., 2018) during the reaction promoted the Mn(II) oxidation (Fig. 5(c)), suggesting that the Mn(III/IV) oxides may indirectly promote the oxidation of Mn(II) through photogenerated electrons. According to the Mott-Schottky analysis (Fig. S10) and the Tauc curve (Fig. S8), the conduction band potential of the generated Mn(III/IV) oxides is about −1.41 V, which is more negative than $O_2/\cdot O_2^-$ (−0.33 V). Therefore, photogenerated electrons can be transferred to O_2 to form $\cdot O_2^-$ and indirectly promote Mn(II) ions oxidation (Cui et al., 2018; Xu et al., 2019).

3.7 Mechanisms of manganese oxidation

A possible mechanism of Mn(II) oxidation in this study is shown in Fig. 6. Visible light induces bacteria to produce superoxide ($\cdot O_2^-$), which is crucial in Mn(II) oxidation. Bacteria produce excessive extracellular superoxide under visible light stimulation, which can be captured by Mn(II) ions dispersed evenly in the culture medium, meanwhile, forming high-valent manganese oxides. The generated Mn(III/IV) oxides have good photoelectric activity and can be excited by visible light to produce photogenerated electrons, which reduce the O_2 to $\cdot O_2^-$, oxidizing Mn(II) ions indirectly.

Superoxide is a powerful oxidant, its oxidation reaction of Mn(II) occurs under a wide range of pH conditions with a rate of 6×10^6–1×10^7 mol/(L·s) (Hansard et al., 2011; Hansel et al., 2012). Over 70 % of marine heterotrophic bacteria, including the phyla of Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes (Fig. S11), can produce extracellular superoxide, which is an important source of superoxide in the ocean (Diaz et al., 2013; Sutherland et al., 2020). Apart from heterotrophic bacteria, some phototrophs and fungi also produce superoxide. For instance, the Ascomycete fungus *Stilbella aciculosa* produces NADPH-dependent superoxide during its asexual reproduction, oxidizing Mn(II) incidentally (Hansel et al., 2012). In this study, the superoxide oxidation of the Mn(II) process does not promote the growth of bacteria (Fig. S12), and the process of bacteria producing superoxide does not require the presence of Mn(II). The oxidation of Mn(II) through superoxide is more likely an unintentional side reaction, like *Roseobacter* sp. AzwK-3bk and *Stilbella aciculosa* (Learman et al., 2011; Hansel et al., 2012). Hence, it is reasonable to question whether all these superoxide-producing bacteria should be classified as manganese-oxidizing bacteria.

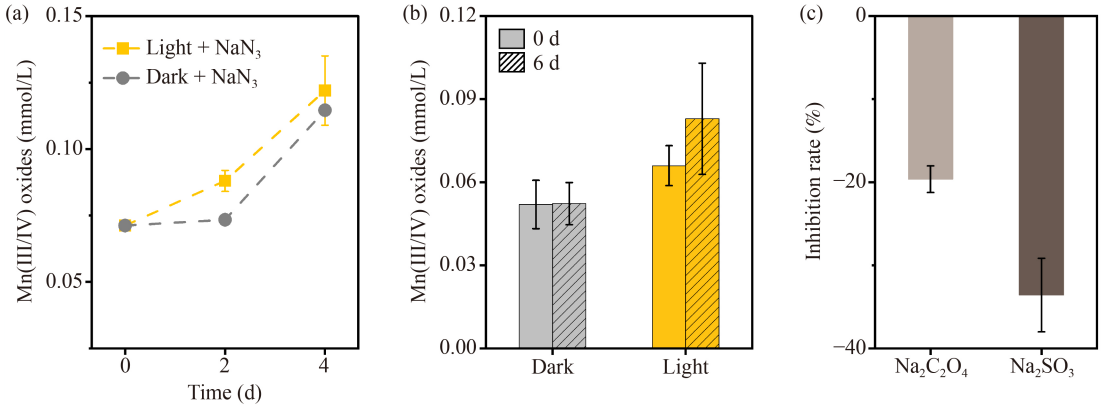

Fig. 5 Oxidation capacity of the generated Mn(III/IV) oxides. (a) Change of Mn(III/IV) oxides content with NaN_3 addition; (b) Content of Mn(III/IV) oxides before and after 6 d of light/dark culture; (c) Inhibition rate of manganese oxidation under light culture by adding the hole scavengers oxalate (1 mmol/L), sulfite (1 mmol/L). Error bars represent the standard deviation of three replicates.

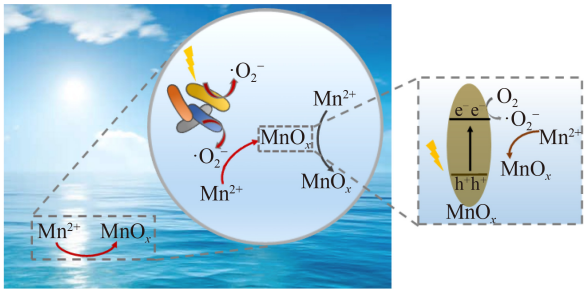

Fig. 6 Mechanism of manganese oxidation under light incubation.

In addition to the superoxide pathway, some bacteria oxidize Mn(II) through surface enzymes, or by changing the surrounding pH, which does not relate to the growth or metabolism either, their manganese-oxidizing behavior is also like a side reaction. For example, *Serratia marcescens* LG-1 could use CueO, a kind of multicopper oxidase, to oxidize Mn(II), but its expression is not modulated by the presence of Mn(II) (Queiroz et al., 2018). Cyanobacteria *Microcystis* sp. form a high pH (> 9) environment during photosynthesis regardless of the presence or absence of Mn(II) ions, and the manganese oxidation process does not enhance their photosynthesis (Richardson et al., 1988). However, these bacteria are all classified as manganese-oxidizing bacteria according to the previous definition. When microorganisms that oxidize manganese ions are independent of the metabolic process, it may not be conducive to a systematic understanding of their physiological role in the microbial manganese oxidation process. Perhaps a more precise definition of manganese-oxidizing bacteria should be proposed in the future.

Superoxide has a wide range of sources in the environment. In addition to biological sources, many photochemical reactions can also produce superoxide and participate in manganese oxidation. For example, NO_3^- in the ocean could produce superoxide under ultraviolet light irradiation, oxidizing the coexisting Mn(II) ions (Zhang et al., 2018). A large number of semiconductor particles in seawater can produce superoxide under sunlight illumination, indirectly promoting the oxidation of manganese (Xu et al., 2019). Natural organic matter can also form superoxide under sunlight irritation, oxidizing Mn(II) ions rapidly (Nico et al., 2002; Shi et al., 2020). The concentration of superoxide in seawater ranges from picomoles to hundreds of nanomoles, comparable to that of Mn(II) ions (1.8 nmol/L) (Bender et al., 1977; Sutherland et al., 2019). The rate of Mn(II) oxidation by $\cdot O_2^-$ is faster than the microbial oxidation rate (0.07–89 pmol/(L·h)) (Nico et al., 2002); therefore, the superoxide-based manganese oxidation process is an important source of manganese oxides in the environment.

4 Conclusions

Taken together, this study revealed an essential pathway for bacterial manganese oxidation. Heterotrophic bacteria produce superoxide under visible light irradiation and oxidize Mn(II) ions in the surrounding environment, which is the main source of manganese oxides. The bio-generated Mn(III/IV) oxides can also oxidize Mn(II) ions indirectly through abiotic reactions under light illumination. Moreover, the generation of superoxide caused by light does not require the presence of Mn(II), and the Mn(II) oxidation had no effect on microbial growth, demonstrating that the Mn(II) oxidation is an unintentional side reaction. Many bacteria in the environment that produce superoxide actively or passively may also oxidize Mn(II) in this way, suggesting that the manganese oxidation pathway through superoxide is a common behavior in the environment. In light of the oxidation properties and semiconductor properties of manganese oxides, this research will provide new ideas for the treatment of environmental pollution.

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References

Andeer P F, Learman D R, McIlvin M, Dunn J A, Hansel C M (2015). Extracellular haem peroxidases mediate Mn(II) oxidation in a marine *Roseobacter* bacterium via superoxide production. Environmental Microbiology, 17(10): 3925–3936

Anderson C R, Johnson H A, Caputo N, Davis R E, Torpey J W, Tebo B M (2009). Mn(II) oxidation is catalyzed by heme peroxidases in "*Aurantimonas manganoxydans*" strain SI85-9A1 and *Erythrobacter* sp. strain SD-21. Applied and Environmental Microbiology, 75(12): 4130–4138

Bender M L, Klinkhammer G P, Spencer D W (1977). Manganese in seawater and the marine manganese balance. Deep-Sea Research, 24(9): 799–812

Cabiscol E, Tamarit J, Ros J (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. International Microbiology, 3(1): 3–8

Cabrol L, Quéméneur M, Misson B (2017). Inhibitory effects of sodium azide on microbial growth in experimental resuspension of marine sediment. Journal of Microbiological Methods, 133: 62–65

Carmichael S K, Bräuer S L (2015). Microbial diversity and manganese cycling: a review of manganese oxidizing microbial cave communities. In: Summers Engel A, ed. Microbial Life of Cave Systems. Berlin: De Gruyter

Cebrián G, Condón S, Mañas P (2017). Physiology of the inactivation of vegetative bacteria by thermal treatments: mode of action, influence of environmental factors and inactivation kinetics. Foods,

6(12): 107

Cui H, Li B, Li Z, Li X, Xu S (2018). Z-scheme based CdS/$CdWO_4$ heterojunction visible light photocatalyst for dye degradation and hydrogen evolution. Applied Surface Science, 455: 831–840

Diaz J M, Hansel C M, Voelker B M, Mendes C M, Andeer P F, Zhang T (2013). Widespread production of extracellular superoxide by heterotrophic bacteria. Science, 340(6137): 1223–1226

Ding R, Yan W, Wu Y, Xiao Y, Gang H, Wang S, Chen L, Zhao F (2018). Light-excited photoelectrons coupled with bio-photocatalysis enhanced the degradation efficiency of oxytetracycline. Water Research, 143: 589–598

Duckworth O W, Sposito G (2005). Siderophore-manganese(III) interactions. I. Air-oxidation of manganese(ll) promoted by desferrioxamine B. Environmental Science & Technology, 39(16): 6037–6044

Forrez I, Carballa M, Verbeken K, Vanhaecke L, Schlüsener M, Ternes T, Boon N, Verstraete W (2010). Diclofenac oxidation by biogenic manganese oxides. Environmental Science & Technology, 44(9): 3449–3454

Geszvain K, Smesrud L, Tebo B M (2016). Identification of a third Mn (II) oxidase enzyme in *Pseudomonas putida* GB-1. Applied and Environmental Microbiology, 82(13): 3774–3782

Halstead F D, Thwaite J E, Burt R, Laws T R, Raguse M, Moeller R, Webber M A, Oppenheim B A (2016). Antibacterial activity of blue light against nosocomial wound pathogens growing planktonically and as mature biofilms. Applied and Environmental Microbiology, 82(13): 4006–4016

Hansard S P, Easter H D, Voelker B M (2011). Rapid reaction of nanomolar Mn(II) with superoxide radical in seawater and simulated freshwater. Environmental Science & Technology, 45(7): 2811–2817

Hansel C M, Diaz J M (2021). Production of extracellular reactive oxygen species by marine biota. Annual Review of Marine Science, 13(1): 177–200

Hansel C M, Zeiner C A, Santelli C M, Webb S M (2012). Mn(II) oxidation by an ascomycete fungus is linked to superoxide production during asexual reproduction. Proceedings of the National Academy of Sciences of the United States of America, 109(31): 12621–12625

Hayyan M, Hashim M A, AlNashef I M (2016). Superoxide ion: generation and chemical implications. Chemical Reviews, 116(5): 3029–3085

Hou H, Liu H, Gao F, Shang M, Wang L, Xu L, Wong W, Yang W (2018). Packaging $BiVO_4$ nanoparticles in ZnO microbelts for efficient photoelectrochemical hydrogen production. Electrochimica Acta, 283: 497–508

Huang J, Zhang H (2020). Redox reactions of iron and manganese oxides in complex systems. Frontiers of Environmental Science & Engineering, 14(5): 76

Jung H, Taillefert M, Sun J, Wang Q, Borkiewicz O J, Liu P, Yang L, Chen S, Chen H, Tang Y (2020). Redox cycling driven transformation of layered manganese oxides to tunnel structures. Journal of the American Chemical Society, 142(5): 2506–2513

Koblížek M, Béjà O, Bidigare R R, Christensen S, Benitez-Nelson B, Vetriani C, Kolber M K, Falkowski P G, Kolber Z S (2003). Isolation and characterization of *Erythrobacter* sp. strains from the upper ocean. Archives of Microbiology, 180(5): 327–338

Kumar A, Ghate V, Kim M J, Zhou W, Khoo G H, Yuk H G (2016). Antibacterial efficacy of 405, 460 and 520 nm light emitting diodes on *Lactobacillus plantarum, Staphylococcus aureus* and *Vibrio parahaemolyticus*. Journal of Applied Microbiology, 120(1): 49–56

Learman D R, Voelker B M, Madden A S, Hansel C M (2013). Constraints on superoxide mediated formation of manganese oxides. Frontiers in Microbiology, 4: 262

Learman D R, Voelker B M, Vazquez-Rodriguez A I, Hansel C M (2011). Formation of manganese oxides by bacterially generated superoxide. Nature Geoscience, 4(2): 95–98

Li Y, Chen L, Tian X, Lin L, Ding R, Yan W, Zhao F (2021). Functional role of mixed-culture microbe in photocatalysis coupled with biodegradation: Total organic carbon removal of ciprofloxacin. Science of the Total Environment, 784: 147049

Morris J J, Rose A L, Lu Z (2022). Reactive oxygen species in the world ocean and their impacts on marine ecosystems. Redox Biology, 52: 102285

Murray K J, Webb S M, Bargar J R, Tebo B M (2007). Indirect oxidation of Co(II) in the presence of the marine Mn(II)-oxidizing bacterium *Bacillus* sp. strain SG-1. Applied and Environmental Microbiology, 73(21): 6905–6909

Nakama K, Medina M, Lien A, Ruggieri J, Collins K, Johnson H A (2014). Heterologous expression and characterization of the manganese-oxidizing protein from *Erythrobacter* sp. strain SD21. Applied and Environmental Microbiology, 80(21): 6837–6842

Nealson K H (2006). Volume 5: Proteobacteria: Alpha and Beta Subclasses. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K H, Stackebrandt E, editors. The Prokaryotes. New York: Springer, 222–231

Nesbitt H W, Banerjee D (1998). Interpretation of XPS Mn(2p) spectra of Mn oxyhydroxides and constraints on the mechanism of MnO_2 precipitation. American Mineralogist, 83(3–4): 305–315

Nico P S, Anastasio C, Zasoski R J (2002). Rapid photo-oxidation of Mn(II) mediated by humic substances. Geochimica et Cosmochimica Acta, 66(23): 4047–4056

Palermo C, Dittrich M (2016). Evidence for the biogenic origin of manganese-enriched layers in Lake Superior sediments. Environmental Microbiology Reports, 8(2): 179–186

Queiroz P S, Ruas F A D, Barboza N R, de Castro Borges W, Guerra-Sá R (2018). Alterations in the proteomic composition of *Serratia marcescens* in response to manganese (II). BMC Biotechnology, 18(1): 83

Ramadoss G, Suriyaraj S P, Sivaramakrishnan R, Pugazhendhi A, Rajendran S (2021). Mesoporous ferromagnetic manganese ferrite nanoparticles for enhanced visible light mineralization of azoic dye into nontoxic by-products. Science of the Total Environment, 765: 142707

Richardson L L, Aguilar C, Nealson K H (1988). Manganese oxidation in pH and O_2 microenvironments produced by phytoplankton. Limnology and Oceanography, 33(3): 352–363

Song Y, Jiang J, Ma J, Zhou Y, von Gunten U (2019). Enhanced transformation of sulfonamide antibiotics by manganese(IV) oxide in the presence of model humic constituents. Water Research, 153: 200–207

Shi Y, Dai Y, Liu Z, Nie X, Zhao S, Zhang C, Jia H (2020). Light-

induced variation in environmentally persistent free radicals and the generation of reactive radical species in humic substances. Frontiers of Environmental Science & Engineering, 14(6): 106

Sutherland K M, Coe A, Gast R J, Plummer S, Suffridge C P, Diaz J M, Bowman J S, Wankel S D, Hansel C M (2019). Extracellular superoxide production by key microbes in the global ocean. Limnology and Oceanography, 64(6): 2679–2693

Sutherland K M, Wankel S D, Hansel C M (2020). Dark biological superoxide production as a significant flux and sink of marine dissolved oxygen. Proceedings of the National Academy of Sciences of the United States of America, 117(7): 3433–3439

Tardu M, Bulut S, Kavakli I H (2017). MerR and ChrR mediate blue light induced photo-oxidative stress response at the transcriptional level in *Vibrio cholerae*. Scientific Reports, 7(1): 40817

Tebo B M, Bargar J R, Clement B G, Dick G J, Murray K J, Parker D, Verity R, Webb S M (2004). Biogenic manganese oxides: properties and mechanisms of formation. Annual Review of Earth and Planetary Sciences, 32(1): 287–328

Tebo B M, Johnson H A, McCarthy J K, Templeton A S (2005). Geomicrobiology of manganese(II) oxidation. Trends in Microbiology, 13(9): 421–428

Tournassat C, Charlet L, Bosbach D, Manceau A (2002). Arsenic(III) oxidation by birnessite and precipitation of manganese(II) arsenate. Environmental Science & Technology, 36(3): 493–500

Wu L, Zhu G, Zhang X, Si Y (2020). Silver nanoparticles inhibit denitrification by altering the viability and metabolic activity of *Pseudomonas stutzeri*. Science of the Total Environment, 706: 135711

Xia W, Chen R, Li Y, Gao P, Li C, Jin T, Ma J, Ma T (2021). Photo-driven heterogeneous microbial consortium reducing CO_2 to hydrocarbons fuel. Journal of Cleaner Production, 326: 129397

Xu J, Zhao M, Pei L, Liu X, Wei L, Li A, Mei Y, Xu Q (2020). Effects of heavy metal mixture exposure on hematological and biomedical parameters mediated by oxidative stress. Science of the Total Environment, 705: 134865

Xu X, Li Y, Li Y, Lu A, Qiao R, Liu K, Ding H, Wang C (2019). Characteristics of desert varnish from nanometer to micrometer scale: a photo-oxidation model on its formation. Chemical Geology, 522: 55–70

Yang F, Zheng Y, Tian X, Liu Y, Li J, Shao Z, Zhao F (2021). Redox cycling of manganese by *Bacillus horikoshii* biET1 via oxygen switch. Electrochimica Acta, 375: 137963

Yang G, Ni W, Wu K, Wang H, Yang B E, Jia X, Jin R (2013). The effect of Cu(II) stress on the activity, performance and recovery on the Anaerobic Ammonium-Oxidizing (Anammox) process. Chemical Engineering Journal, 226: 39–45

Yang X, Peng Q, Liu L, Tan W, Qiu G, Liu C, Dang Z (2020). Synergistic adsorption of Cd(II) and As(V) on birnessite under electrochemical control. Chemosphere, 247: 125822

Zhang T, Liu L, Tan W, Suib S L, Qiu G, Liu F (2018). Photochemical formation and transformation of birnessite: effects of cations on micromorphology and crystal structure. Environmental Science & Technology, 52(12): 6864–6871

Zhao H, Wang Y, Liu Y, Yin K, Wang D, Li B, Yu H, Xing M (2021). ROS-induced hepatotoxicity under cypermethrin: involvement of the crosstalk between Nrf2/Keap1 and NF-kappaB/ikappaB-alpha pathways regulated by proteasome. Environmental Science & Technology, 55(9): 6171–6183

Zheng Y, Kappler A, Xiao Y, Yang F, Mahadeva G D, Zhao F (2019). Redox-active humics support interspecies syntrophy and shift microbial community. Science China Technological Sciences, 62(10): 1695–1702

Zhou H, Fu C (2020). Manganese-oxidizing microbes and biogenic manganese oxides: characterization, Mn(II) oxidation mechanism and environmental relevance. Reviews in Environmental Science and Biotechnology, 19(3): 489–507