Cooperative Mn(II) oxidation between two bacterial strains in an aquatic environment

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ABSTRACT

In natural or engineered environments, diverse interspecific interactions among two or more microbial taxa may profoundly affect the transformation of organic compounds in the media. Little is known, however, about how these organisms and interactions affect the transformation of heavy metals. Recently, we found an interaction between two non-Mn(II)-oxidizing (when in monoculture) strains, *Arthrobacter* sp. QXT-31 and *Sphingopyxis* sp. QXT-31, which, when cultured in combination, resulted in Mn(II)-oxidizing activity in synthetic media. In order to study the occurrence likelihood of cooperative Mn(II) oxidation in natural water and discharged effluent, we initially identified an optimal ratio of the two strains in a combined culture, as well as the impacts of external factors on the cooperative oxidation. Once preferred initial conditions were established, we assessed the degree and rate of Mn(II) oxidation mediated by the combined QXT-31 strains (henceforth referred to as simply 'QXT-31') in three different water types: groundwater, domestic sewage and coking wastewater. Results showed that Mn(II) oxidation only occurred when the two strains were within a specific ratios range. When introduced to the test waters at the preferred ratio, QXT-31 demonstrated high Mn(II)-oxidizing activities, even when relative abundance of QXT-31 was very low (roughly 1.6%, calculated by 454 pyrosequencing events on 16S rDNA). Interestingly, even under low relative abundance of QXT-31, removal of total organic carbon and total nitrogen in the test waters was significantly higher than the control treatments that were not inoculated with QXT-31. Data from our study indicate that cooperative Mn(II) oxidation is most likely to occur in natural aquatic ecosystems, and also suggests an alternative method to treat wastewater containing high concentrations of Mn(II).

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1. Introduction

In both natural and engineered environments, most microbes live in multispecies communities where interspecific interactions are not only common but continuous and highly varied. Recent research has focused on these interactions among two or more species since interesting novel biochemical properties may result. For example, a co-culture of *Gordonia* sp. strain MTCC 4818 and *Arthrobacter* sp. strain WY was able to bypass the limitations of each individual species with regard to degradation of butyl benzyl phthalate (Chatterjee and Dutta, 2008). Three gram-negative strains, including one strain of genus *Sphingopyxis*, are only

capable of degrading geosmin when they are combined as a whole group (Hoefel et al., 2006). The emergent biochemical properties generated from interspecific interactions contribute to our general knowledge of single strain functions, and provide us with an alternative method to accomplish various tasks, such as identification of new fungi-derived pharmaceuticals (Bertrand et al., 2014; Ochi and Hosaka, 2013) and degradation of organic contaminants (Liu et al., 2014; Wu et al., 2010; Zhang et al., 2013). However, research regarding transformations of certain metals (e.g. Mn) via these interactions is lacking.

Manganese oxides are widely distributed in the natural environment, including water, soil and sediment. Natural Mn oxides affect the transformation and migration of other metallic elements (Katsoyiannis et al., 2004; Miyata et al., 2007) and degradation of organic compounds (Forrez et al., 2010; Sunda and Kieber, 1994), due to their high oxidative and absorptive capacities. Most of Mn

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oxides in natural environment are believed to be biogenic, since microbes can accelerate the homogeneous oxygenation of Mn(II) by several orders of magnitude through enzymatic catalytic reaction (James, 2005). Although numerous microbes have been found to be capable of oxidizing Mn(II) by themselves, there are no data on the potential for Mn(II) oxidation via microbial interspecific interactions.

We recently found that Mn(II)-oxidizing activity was generated in the co-culture system of two non-Mn(II)-oxidizing bacterial strains, *Arthrobacter* sp. QXT-31 and *Sphingopyxis* sp. QXT-31. Through the analysis of the induced Mn(II)-oxidizing protein (identified as a multicopper oxidase) in the coculture and its encoding gene, we demonstrated they only belong to A. QXT-31. The Mn(II)-oxidizing gene was not expressed in A. QXT-31 monoculture, but transcribed when A. QXT-31 was co-cultured with S. QXT-31. It suggested that the Mn(II)-oxidizing gene was silent in A. QXT-31 monoculture, and activated by S. QXT-31 in the coculture. The RNA-seq data further indicated that type VI secretion system (T6SS) in S. QXT-31 may participate in the activation of the silent Mn(II)-oxidizing gene in A. QXT-31 (Liang et al., 2015). However, those data were generated using a pure laboratory culture, the occurrence likelihood of this cooperative Mn(II) oxidation in a natural aquatic environment remained uncertain.

The objectives of this study were to clarify (i) whether the cooperative Mn(II) oxidation by QXT-31 occurs in an aquatic environment, and (ii) the manner in which the two introduced strains interact with indigenous microbes. To achieve these goals, we first determined the optimal Mn(II) oxidation conditions of QXT-31 in pure culture, as well as the utilization of C sources and potential inhibitory factors for Mn(II) oxidation. Based on the determined optimal conditions, we further explored Mn(II) oxidation potential of QXT-31 in aquatic environments, and the interaction of two introduced strains with indigenous microbes, using chemical analysis and a molecular approach (pyrosequencing for 16S rDNA). Three different water types were selected for the experiments: (i) groundwater, (ii) domestic sewage and (iii) coking wastewater (representing low-nutrient conditions, high nutrient conditions and extreme water chemistry conditions, respectively). Since Mn(II) contamination is often present in groundwater (Jaudon et al., 1989), rivers (Akçay et al., 2003) and other surface water bodies, information from our study may also provide a potential method for treating Mn-polluted water, i.e., addition of QXT-31 to a biological treatment system.

2. Materials and methods

2.1. Bacterial strains and culture media

The bacterial strains A. QXT-31 (Gram positive, aerobic) and S. QXT-31 (Gram negative, aerobic) were isolated in 2011 from the soil around a Mn mine in Hu'nan province, China (Liang et al., 2015). A. QXT-31 and S. QXT-31 colonies on plate medium can easily be distinguished by color, which are white and yellow, respectively. The QXT-31 will grow in medium containing up to 1000 μ M Mn(II), and will oxidize up to 500 μ M Mn(II) (data not shown). The GenBank accession numbers of the 16S rDNA sequences of strains A. QXT-31 and S. QXT-31 are KC765085 and KC765086, respectively. The two strains are archived and maintained at the China General Microbiological Culture Collection Center (CGMCC) under CGMCC No. 6631 and CGMCC No. 6947, respectively.

The medium used in this study was modified PYG medium (Adams and Ghiorse, 1985), composed of 0.5 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 60 mg/L of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.25 g/L (each) of peptone, yeast extract and glucose. The pH was maintained between 7.0 and 7.2 using 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid) unless otherwise noted. For each experiment, a single isolated colony of the two strains on a solid medium plate was transferred to liquid PYG medium for 24 h cultivation, after which the suspension was used as inoculum. The initial ratio of the two strains varied depending on experimental requirement. After inoculation, the flasks were incubated on a rotary shaker at 170 rpm in dark at 30 °C unless otherwise noted.

2.2. The effect of initial bacterial strain ratio on Mn(II) oxidation

The two strains in QXT-31 were initially cultured separately for 24 h in medium without MnCl_2 . At the end of the culture period, the optical density of each culture was determined at 600 nm (OD_{600}). Each culture of 0.5 mL was then subjected to gradient dilution and spread onto solid medium for incubation for 5 d; the number of colony forming units (CFUs) of each strain was determined. The densities of each strain were calculated using the respective CFUs and the corresponding volume. The correlation coefficient between the OD_{600} value and CFU density was calculated.

Fresh cell suspensions of each strain were prepared according to the previously-described method; the OD_{600} in each culture was determined. Based on the correlation coefficient between the OD_{600} value and CFU density, the CFU density of each strain was calculated. In terms of the CFU density, co-cultures with different initial ratios of the two strains (1:4, 1:1, 4:1, 16:1 and 64:1 for A. QXT-31: S. QXT-31) were prepared. The co-cultures were cultivated for 24 h. Mn(II) oxidation by the co-culture was reflected by the decrease of soluble Mn(II) concentration. The suspensions were sampled at specific times, and soluble Mn(II) was determined after filtration with 0.22- μ m membrane. To monitor strains ratio variation during culturing, 0.5 mL suspensions at varying times were diluted and spread on solid medium for further cultivation. The number of each strain was counted based on the different colony color (A. QXT-31: white; S. QXT-31: yellow), and the living cell ratios of the two strains were calculated.

2.3. Effect of temperature, pH and metals on Mn(II) oxidation

The effects of temperature (10 °C, 20 °C and 30 °C) and pH (5.5, 6.0, 6.5, 7.0, 7.5 and 8.5) during QXT-31 culturing were investigated in order to assess the optimal conditions for Mn(II) oxidation. In addition, the effect of 18 toxic metals (Italic: AgNO_3 , AlCl_3 , BaCl_2 , NaAsO_2 , Na_2HASO_4 , $\text{Cd}(\text{NO}_3)_2$, CoCl_2 , CuCl_2 , $\text{Cr}_2(\text{SO}_4)_3$, K_2CrO_4 , FeCl_2 , KSeO_3 , Na_2SeO_3 , Na_2SeO_4 , $\text{Pb}(\text{NO}_3)_2$, ZnCl_2 , NiCl_2 , SnCl_2) on bacterial growth and Mn(II) oxidation of QXT-31 was evaluated by initiating cultures with three concentrations (5 μ M, 25 μ M, 125 μ M) of the metals. Aliquots of the metal stock solutions were added in appropriate amount to the co-culture following filtration sterilization. The OD_{600} was measured at 3 h, 6 h and 9 h, and the Mn(II) concentration was measured at 24 h, 48 h and 72 h.

2.4. Utilization of organic matter

Cells of QXT-31 were initially cultured without Mn(II), harvested at 16 h by centrifugation (4500 g, 5 min), washed three times with solution A (0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 60 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 46 mg/L NH_4NO_3 , 10 mM HEPES, pH = 7.2), and finally suspended in fresh solution A. When assessing water-soluble organic matter, they were first dissolved with solution A, and then added to cell suspension after filtration sterilization. When assessing non-water-soluble organic matter, they were initially dissolved in acetone and transferred to empty sterile Erlenmeyer flasks after filtration sterilization. After the acetone had volatilized completely, the same volume of cell suspension was added to the flasks. Varying quantities of organic matters were added to maintain a final C

concentration of 100 mg/L. The initial OD₆₀₀ value was approximately 0.15. Over the 4 d culture period, OD₆₀₀ was measured every day. The potential utilization of organic matter by QXT-31 was indirectly reflected by the differences in OD₆₀₀ values between the control and treatments to which organic matter was added. The Mn(II) concentration was also measured.

2.5. Mn(II) oxidation and removal of organic content in water

The cooperative Mn(II) oxidation of QXT-31 in water was studied by inoculating mixed bacterial strains (at a S. QXT-31: A. QXT-31 ratio of 8:1) into three water treatments: groundwater, domestic sewage and coking wastewater. The total organic carbon (TOC), total nitrogen (TN), and pH in the three types of water are as follows: groundwater (6.2 mg/L, 2.7 mg/L, 6.8), domestic sewage (248.9 mg/L, 142.3 mg/L, 6.7), coking wastewater (2438.5 mg/L, 1574.8 mg/L, 8.9). Coking wastewater was first diluted five times using groundwater before inoculation with QXT-31. This was necessary since the pH in raw water increased to >9.0 during culturing, when abiotic Mn(II) oxidation may become non-ignorable. The cells used for inoculation were prepared as follows: the bacterial inoculum (at 8:1 ratio) was prepared according to the method above and then transferred into fresh medium without MnCl₂ for further culturing. The cells were harvested by centrifugation, washed twice with solution B (0.5 g/L MgSO₄·7H₂O, 60 mg/L CaCl₂·2H₂O, 20 mM HEPES, pH 7.0), suspended in solution B and finally added to the individual water treatment being tested. Mn(II) and HEPES (pH 7.0) were added to achieve a final concentration of 100 μM and 20 mM, respectively. Mn(II), TOC and TN were measured during culturing. Solution pH was determined at 0 h and at the end of the experiment.

To investigate the effect of nutrient addition on Mn(II) oxidation, glucose and peptone (each at a concentration of 0.25 g/L) were added to the domestic sewage and coking wastewater. All other procedures were the same as described above. All experiments were conducted in triplicate.

For analysis of the indigenous bacterial community in water, subsamples of domestic sewage (300 mL) and coking wastewater (1 L) were collected and separately concentrated through a 0.45 μm pore-size polyether sulfone membrane (Jinteng, Tianjin, China) using vacuum filtration. During Mn(II) oxidation period of each treatment, 2 mL samples were collected at three time points: (3 h, 5 h and 7 h for raw domestic sewage; 7 h, 9 h and 13 h for domestic sewage + nutrient; 3 h, 5 h and 8 h for coking wastewater; 85 h, 90 h and 95 h for coking wastewater + nutrient). For each treatment, the nine samples obtained from three time period in the three parallel experiments were pooled into one tube for RNA extraction and subsequent pyrosequencing analysis.

2.6. Analytical and statistical methods

The presence of Mn oxides, produced in either liquid or solid culture media, was confirmed using leucoberbelin blue (LBB) (Krumbein and Altmann, 1973), which forms a blue color that absorbs at 620 nm after interaction with Mn(III&IV). We found that, in pure cultures, the decrease of Mn(II) concentration corresponded directly with the production of Mn oxides as determined by the LBB method. Although the potential LBB method interferences arising from the coking wastewater used in this study were unknown, the Mn(II) oxidation was indirectly reflected by the decrease of soluble Mn(II) concentration. After collection, samples were cooled in ice and centrifuged (10,000 g, 3 min, 4 °C). The supernatant was carefully removed, taking care not to resuspend the settled solids, and filtered at 0.45 μm. Mn(II) in the filtrate was measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-

OES 710, Agilent, USA). The OD₆₀₀ was determined spectrophotometrically (U-3010, Hitachi, Japan) and pH was determined with a pH meter (PHS-3C, Shanghai INESA & Scientific Instrument C., Ltd., China).

Samples for TOC determination were filtered with a 0.45-μm membrane and TOC in the filtrate was determined with a TOC analyzer (TOC-V_{CPH}, Shimadzu, Japan). Samples for TN determination were digested using alkaline potassium persulfate (Arnaldos and Pagilla, 2010) and the concentration of the generated nitrate nitrogen was determined using ion chromatography (ICS-2000, Dionex, USA). Samples were diluted with Milli-Q water when necessary to achieve a measureable concentration range.

Statistical analysis of data was performed with IBM SPSS Statistics 22.0 using one-way ANOVA followed by a least significant difference test in order to test for the significant differences in TOC and TN removal between the inoculated treatments and the controls.

2.7. RNA extraction and 16S rDNA 454 pyrosequencing

In order to analyze the active bacterial community in the collected samples mentioned above, the total RNA was extracted with TRNzol Reagent (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. Lysozyme was used to facilitate cell lysis. The first strand of cDNA from total RNA was synthesized using a QuantScript RT Kit (TIANGEN Biotech, Beijing, China), and stored at −80 °C until analysis.

The V3–V6 region of the 16S rDNA was amplified using bar-coded universal forward primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 1073R (5'-ACGAGCTGACGACARCCATG-3') containing the A and B sequencing adaptors. PCR amplification was performed in a ABI GeneAmp 9700 thermo-cycler (ABI, Foster City, USA) using the following program: 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and finally 72 °C for 5 min. Amplicons were subjected to emulsion-based clonal amplification PCR (emPCR). The 16S rDNA was analyzed through high-throughput sequencing by Roche GS FLX (Roche 454 Life Sciences, Branford, CT, USA).

2.8. Pyrosequencing processing and analysis

The raw sequences obtained from barcoded pyrosequencing were processed with the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso, 2010). Barcodes were first trimmed from the sequences, after which quality-filtering and putative chimeric sequences check were conducted according to the previous method (Bai et al., 2012). The remaining high-quality sequences were subjected to further analysis.

The sequences were initially clustered into operational taxonomic units (OTUs) using UPARSE (Edgar, 2013), with a similarity threshold of 97%. The representative sequences from individual OTUs were then aligned using the alignment algorithm PyNAST (Caporaso et al., 2010) and Greengenes (DeSantis et al., 2006) database. Ribosomal Database Project (RDP) (Wang et al., 2007) classifier was used to assign taxonomy, with a minimum confidence of 80%. Rarefaction, Shannon index, library coverage and Chao 1 were calculated for each sample.

The sequences were used to calculate the proportion of the two strains in the combined bacterial community. The V3–V6 region of the 16S rDNA sequences of the two strains were selected from previously-identified 16S rDNA sequences, and BLASTed against the high-quality sequences. All raw sequences generated from pyrosequencing were deposited in the NCBI Sequence Read Archive (SRA) database (accession no. SRP062579).

3. Results

3.1. Optimal conditions for cooperative Mn(II) oxidation

Results indicated there existed an optimal initial ratio range for the two strains that maximized Mn(II) oxidation. Mn(II) oxidation was (almost) completed at 6 h in the culture systems with initial ratios of 4:1 and 16:1 for *S. QXT-31*: *A. QXT-31* (Fig. 1). Compared to the two culture systems above, Mn(II) oxidation seemed delayed in 64:1 initial ratio system, since only a small portion (10%) of Mn(II) had been oxidized at 6 h. An obvious delay of Mn(II) oxidation was observed in 1:1 initial ratio system, and no Mn(II) oxidation was detected in the 1:4 initial ratio system during experimental time (24 h). The strain ratios varied with time due to asymmetrical growth of two strains and Mn(II) oxidation. At 3 h (in initial ratio systems of 4:1, 16:1, 64:1) and 12 h (initial ratio 1:1), just before Mn(II) oxidation in these four systems, the strain ratios fell in a range of 3.6–29:1, and then decreased after Mn(II) oxidation. In the no Mn(II) oxidation system (initial ratio 1:4), the strain ratios were always below 1.8:1 during the experimental time. The Mn(II)-oxidizing activities at initial ratios of 4:1 and 16:1 were found to be comparable and therefore a ratio approximately midway between these two (8:1) was selected for later experiments.

A temperature of 30 °C was found to be the most suitable for growth and Mn(II) oxidation of *QXT-31* (Fig. S1). In the study of media aliquots buffered (with HEPES) to pH values of 5.5–8.5, *QXT-31* was found to oxidize Mn(II) only when pH ranged from 6.5 to 8.5, with an optimum pH value of 7.5 (Fig. S2). To better simulate the natural environment where pH is often circum-neutral, a pH of 7.0 was used for later experiments. Interestingly, while no Mn(II)

oxidation occurred in the HEPES-buffered treatment with an initial pH of 5.5, Mn(II) oxidation did occur in non-HEPES medium when the initial pH was 5.5. A measured final pH of 6.8 in non-HEPES media suggested that, when cultured, the metabolic activity of *QXT-31* altered the pH to a range where Mn(II) oxidation is feasible.

3.2. Resistance to toxic pollutants and utilization of C sources

QXT-31 was capable of oxidizing Mn(II) in the presence of all the individual metal ions of 5 μ M, and 14 (of 18) individual metal ions of 25 μ M (Table 1). Below 25 μ M of added metal ions (except Ag), the OD₆₀₀ values increased more than six times the initial values (0.066), suggesting *QXT-31* grew well with these toxic ions. Although the specific molecular mechanism which is responsible for the tolerance of *QXT-31* to various metal ions was unknown, the extreme low nutrition concentration (100-times diluted modified PYG medium) and the high concentration of Mn(II) (100 μ M) used in the initial enrichment step for the isolation of the two species may partly explain their strong viability and tolerance to metal ions. Variability in OD₆₀₀ revealed that *QXT-31* could utilize various organic material as a carbon source (Table 2), including some toxic organic pollutants. The diverse metabolic capability of *QXT-31* facilitates the acquisition of C sources from the environment.

3.3. Mn(II) oxidation and removal of aquatic contaminants

Utilizing the fixed culture conditions described earlier, additional experiments were completed in three water types to explore the possibility of cooperative Mn(II) oxidation in aquatic environments. The final pH value in all of the treatments was maintained between 6.9 and 7.8, within which abiotic Mn(II) oxidation by oxygen is thermodynamically unfavorable (Nealson, 2006) and thus can essentially be ignored. Mn(II) oxidation occurred in all water treatments inoculated with *QXT-31* (Fig. 2). No measureable Mn(II) oxidation was observed in the control treatments without *QXT-31* (Fig. S3), thus suggesting that the observed Mn(II) oxidation activity was a result of *QXT-31* alone. Mn(II) oxidation occurred relatively quickly (less than 3 h) in the treatments without nutrient addition, while those treatments containing added nutrient demonstrated a delay in Mn(II) oxidation (Fig. 2d and f). The longest delay was found in coking wastewater with nutrient addition, where Mn(II) oxidation delayed for about 80 h. However, in the groundwater, which exhibited extremely low concentration of nutrient (low TOC and TN), only 40% Mn(II) was oxidized (Fig. 2b). The partial Mn(II) oxidation (85%) was also found in the coking wastewater without nutrient addition (Fig. 2e).

The contributions of added HEPES (20 mM) to TOC and TN were subtracted from each sample, and the percent removal of TOC and TN was calculated. The final TOC and TN contents in inoculated treatments were reduced by 5%–16% and 10%–17%, respectively, compared with the control treatments (Fig. 3). The contents of TOC and TN in inoculated treatments almost always lower than the corresponding controls during the cultivation (Figs. S4 and S5). Inoculation with *QXT-31* significantly ($p < 0.05$) enhanced the removal of TOC and TN in nutrient-added domestic sewage and coking wastewater in the end of the cultivation (Fig. 3).

3.4. Bacterial community diversity

The pyrosequencing of 16S rDNA amplification from eight samples yielded 20,616 valid reads; 11,250 high-quality reads remained following qualitative filtration (Table S1). Fifty-three OTUs were observed for the eight samples.

The dominant groups (more than 1% abundance) at the class level in the eight samples are displayed in Fig. 4a.

Fig. 1. Mn(II) oxidation and strains ratios in coculture systems with different initial strain ratios. Inoculation proportions in all treatments were 3% (v/v). Mn(II) oxidation was indirectly reflected by the decrease of soluble Mn²⁺ concentration in co-culture with initial bacterial strain ratios at 1:4 (squares), 1:1 (circles), 4:1 (up triangles), 16:1 (down triangles) and 64:1 (diamonds). The values were means $\pm$ standard deviations for triplicate assays.

Table 1
The effects of metal ions on the growth and Mn(II) oxidation of QXT-31.

	5 μ M		25 μ M		125 μ M	
	OD ₆₀₀	Mn(II) oxidation	OD ₆₀₀	Mn(II) oxidation	OD ₆₀₀	Mn(II) oxidation
Cd ²⁺	0.494	+	0.400	—	0.114	—
Co ²⁺	0.511	+	0.507	—	0.300	—
Cr ³⁺	0.529	++	0.484	—	0.450	—
Ag ⁺	0.076	+++	0.067	—	0.056	—
Cr ⁶⁺	0.515	+++	0.426	++	0.342	—
Se ⁴⁺	0.538	+++	0.542	+	0.447	+
Ni ²⁺	0.534	+++	0.524	+	0.496	+
Cu ²⁺	0.508	+++	0.466	+++	0.156	—
Sb ³⁺	0.525	+++	0.477	+++	0.166	—
Pb ²⁺	0.535	+++	0.542	+++	0.584	—
Al ³⁺	0.544	+++	0.545	+++	0.526	+
Se ⁶⁺	0.533	+++	0.531	+++	0.535	+
Zn ²⁺	0.539	+++	0.503	+++	0.159	+
Fe ²⁺	0.543	+++	0.532	+++	0.575	++
As ³⁺	0.531	+++	0.531	+++	0.486	+++
As ⁵⁺	0.527	+++	0.488	+++	0.323	+++
Ba ²⁺	0.541	+++	0.536	+++	0.540	+++
Sn ²⁺	0.527	+++	0.535	+++	0.549	+++

The initial OD₆₀₀ and Mn(II) concentration in all treatments was 0.066 and 100 μ M respectively.

—: no Mn(II) was oxidized within 70 h.

+: Mn(II) was partly oxidized within 70 h.

++: Mn(II) was completely oxidized within 30 h.

+++ : Mn(II) was completely oxidized within 20 h.

Table 2
Utilization potential of organic matters.

Treatments	OD ₆₀₀	Mn(II) oxidation
Control	0.132	—
Sodium formate	0.187	—
Hydroxyl sodium carboxymethyl cellulose	0.188	—
Urea	0.198	—
Sodium salicylate	0.21	—
Carbinol	0.22	—
Starch	0.22	—
Pyrene	0.225	—
Phthalic acid	0.239	—
Phenanthrene	0.245	—
Oxalic acid	0.246	—
Malonic acid	0.255	—
Acetic acid	0.258	—
Ethanol	0.271	—
Citric acid	0.325	+
Glycine	0.36	—
Succinic acid	0.361	—
Glucose	0.486	++
Sodium pyruvate	0.633	++
Glycerol	0.657	++

No organic matter was added in control treatment.

—: no Mn(II) oxidation within 12 d.

+: Mn(II) oxidation occurred between 6 d and 12 d.

++: Mn(II) oxidation occurred within 6 d.

Alphaproteobacteria and Actinobacteria were detected as dominant classes of bacteria in a pure QXT-31 culture, accounting for 100% of the observed bacteria. In the two raw waters, the dominant group was Betaproteobacteria, which accounted for 85% and 66% in raw domestic sewage and coking wastewater, respectively. The Shannon diversity index (Table S2) for inoculated domestic sewage varied slightly from the original value of 2.93 to 2.46 (no nutrient addition) and 2.99 (nutrient addition). In the inoculated coking wastewater, the Shannon diversity index decreased from the original value of 2.39 to 0.83 (no nutrient addition) and 1.23 (nutrient addition). The relatively high inoculum amount may have contributed to decreased Shannon diversity in coking wastewater.

Different similarity threshold values (97%, 98%, 99%) used for

selecting the putative *A. QXT-31* or *S. QXT-31* sequences were surveyed (Table S3). According to the percentage of QXT-31, a similarity threshold value of 0.98 was selected for distinguishing the two inoculated strains from indigenous microbes in the tested water. The relative abundances of the two inoculated strains are displayed in Fig. 4b. The relative abundance of QXT-31 (91.5%) in a pure culture was lower than the theoretical value (100%); this difference may partially be due to inherent error associated with pyrosequencing. In raw domestic sewage and coking wastewater, less than 0.7% of the sequences were similar (98% or greater) to the partial 16S rDNA sequences of the two inoculated strains. Indigenous microbial communities found in domestic sewage and coking wastewater, therefore, contained very few bacteria that were similar to the inoculated strains.

Nutrient addition decreased the relative abundance of QXT-31 by 43% in domestic sewage and by 98% in coking wastewater over the Mn(II) oxidation period, compared with the controls without nutrient addition (Table S3). Surprisingly, QXT-31 accounted for only 1.6% in coking wastewater into which nutrient was added (Fig. 4b), but the Mn(II)-oxidizing rate decreased by only 60% compared to a pure culture (Fig. 2a and f). These data suggest that even when the relative abundance of QXT-31 is low, the Mn(II)-oxidizing activity may be considerably high.

4. Discussion

In this study, we investigated the novel phenomenon of cooperative Mn(II) oxidation by two non-Mn(II)-oxidizing bacteria (QXT-31) in the aquatic environment. The optimal conditions for cooperative Mn(II) oxidation in a pure culture were initially investigated in order to establish the experimental parameters appropriate for conditions in environmental aquatic samples. Results from the aquatic experiments indicated that cooperative Mn(II) oxidation between the two strains may occur in natural or engineered settings. Data from our study also elucidated some underlying mechanisms of cooperative Mn(II) oxidation, thus supporting the possibility of its application in water treatment.

Fig. 2. Microbial growth (squares) and Mn(II) concentration variation (triangles) in (a) modified PYG medium, (b) groundwater, (c) domestic sewage, (d) domestic sewage + nutrient, (e) coking wastewater and (f) coking wastewater + nutrient. The cell for inoculation in actual water (b–f) was harvested at 16 h in modified PYG medium (a). Glucose and peptone (each at a concentration of 0.25 g/L) were added in nutrient-added treatments. The values were means $\pm$ standard deviations for triplicate assays.

4.1. Impacts of the ratio of the two strains on Mn(II) oxidation

In a pure culture, rapid Mn(II) oxidation occurred only when the ratio of the two bacterial strains was within a specific range (4:1 to

16:1). Once outside of the range, Mn(II) oxidation was delayed or even eliminated (Fig. 1). The mechanism involved in cooperative Mn(II) oxidation by QXT-31 may help to explain this phenomenon. In our previous study (Liang et al., 2015), it was found that *S. QXT-*

Fig. 3. The final TOC (a) and TN (b) concentrations in non-inoculated (white bars) and inoculated (gray bars) treatments in actual water with or without nutrient addition. “DS”: domestic sewage; “CW”: coking wastewater; “N”: nutrient addition (glucose and peptone, each at a concentration of 0.25 g/L). One-way ANOVA followed by a least significant difference test ($p < 0.05$) was used to test the significant differences in TOC and TN removal between the inoculated treatments and the controls. The values were means $\pm$ standard deviations for triplicate assays.

31 activated the silent (in monoculture) Mn(II)-oxidizing gene in *A. QXT-31*. We propose, therefore, that there is a threshold ratio for *S. QXT-31*: *A. QXT-31* that facilitates the activation of the Mn(II)-oxidizing gene. Below the threshold value, Mn(II)-oxidizing gene is not activated, while above the threshold (*S. QXT-31* present at a much higher concentration relative to *A. QXT-31*) the Mn(II)-oxidizing gene in *A. QXT-31* may still be activated, but the Mn(II) oxidation rate is restricted by *A. QXT-31* density. Based on the experimental results, we inferred that the threshold ratio should lie between 1.8:1 and 3.6:1 for *S. QXT-31*: *A. QXT-31*. An ideal ratio within a two-strain culture has been reported in previous research, which found that enhancement of the methane oxidation activity of *Methylocystis* sp. M6 by *Sphingopyxis* sp. NM1 is also density-dependent (Jeong et al., 2014). Methane oxidation was enhanced only at a 1:9 ratio (*Methylocystis* sp. M6: *Sphingopyxis* sp. NM1); there was no positive effect at ratios of either 9:1 or 1:1 (Jeong et al., 2014).

Waters of different origins and compositions appeared to affect the relative proportions of the two strains. We found that nutrient-enhanced domestic sewage and coking wastewater altered the ratio of the two strains in opposite directions (Fig. 4b). These changes may be associated with the distinct characteristics of the waters, including the presence and concentration of potential toxicants as

Fig. 4. Relative abundances of (a) dominate bacterial classes (>1%) and (b) the inoculated two strains (*A. QXT-31*, gray bars; *S. QXT-31*, black bars) in the period of Mn(II) oxidation. “PYG”: modified PYG medium; “GW”: groundwater; “DS”: domestic sewage; “CW”: coking wastewater; “I”: inoculation with *QXT-31*; “N”: nutrient addition (glucose and peptone, each at a concentration of 0.25 g/L).

well as interactions with indigenous bacteria, which could be synergistic, neutral, or antagonistic with the introduced strains. While domestic sewage generally tends to be much less toxic than coking wastewater, the competitive ability of indigenous bacteria in coking wastewater is higher, since they have naturally adapted to the extreme conditions and stress factors, such as low nutrient level and the presence of toxic organic and inorganic chemicals. In addition, the genus *Sphingopyxis* is well known for a small cell volume (Cavicchioli et al., 2003), which facilitates nutrient acquisition through a high surface-to-volume ratio, while the genus *Arthrobacter* is known to readily tolerate harsh environmental conditions, such as low nutrient availability and toxic chemicals (Mongodin et al., 2006).

4.2. Removal of organic pollutants by *QXT-31* and interaction of bacterial communities

The introduction of *QXT-31* accelerated TOC and TN removal in the tested water (Fig. 3). In view of the diverse metabolic capability of *QXT-31* (Table 2) and the oxidation of organic pollutants by the produced Mn oxides (Forrez et al., 2010; Xu et al., 2008), it was proposed that the inoculated *QXT-31* may reduce the quantities of both organics and inorganics in water via two pathways, i.e., the direct biodegradation by *QXT-31* and indirect oxidation by the produced Mn oxides. Organic compounds usually provide electrons for certain biochemical reactions and Mn oxides serve as an electron acceptor, resulting in the reduction to Mn(II). The generated Mn(II) is quickly re-oxidized to Mn oxides by catalysis of *QXT-31*. Through this action, the inoculated strains guaranteed the regeneration of Mn oxides, thus encouraging further degradation of organic compounds.

In the study of Mn(II) oxidation in different water samples, a relatively high amount of inoculum was used, leading to dominance

of the two strains in coking wastewater and domestic sewage (Fig. 4b). However, when nutrient was added, the relative abundance of QXT-31 decreased significantly due to the rapid growth of the indigenous microbes. The substantial decrease in relative abundance of QXT-31 when nutrient was added to the treatment solution implies a significant competition for nutrient between the inoculated and indigenous microbes. However, the Mn(II) oxidation rate in nutrient-added coking wastewater was still relatively high (equivalent to about 40% of the Mn(II) oxidation rate in the pure QXT-31 culture) at very low relative abundance (about 1.6%) of the inoculated bacteria (Fig. 4b). The seemingly inconsistent high Mn(II) oxidation rate versus the low relative abundance of QXT-31 suggests the possibility of cooperation with regard to Mn(II) oxidation between QXT-31 and the indigenous microbes. It has been speculated that physical contact (Schroeckh et al., 2009) or metabolic interactions (Zhou et al., 2011) were responsible for this apparent cooperation.

4.3. Potential for applying QXT-31 in water treatment

Considering the favorable adaptation of QXT-31 to waters with varying chemical characteristics, the diverse metabolic capacity of these combined strains relative to organic pollutants, and the strong oxidizing ability and adsorption capacity of biogenic Mn oxides (Forrez et al. 2010; Tani et al., 2004; Xu et al. 2008), it may be possible to add QXT-31 as a treatment step for wastewater that is high in Mn(II). Bioaugmentation, such as this, is a common method to accelerate and enhance the removal rate of specific pollutants through addition of bacterial cultures, and has been extensively studied by researchers (Bai et al., 2010). QXT-31 could be included as part of biological treatment chain in an activated sludge system (Henze et al., 1987) or a membrane bioreactor (Stephenson et al., 2000), where Mn(II) could be directly precipitated and bound to the solids.

Data from our study show that nutrient level has a substantive impact on Mn(II) oxidation by QXT-31. Mn(II) oxidation was limited by excessively low nutrient (C and N) levels in groundwater, and delayed when nutrient was artificially elevated in the tested waters (Fig. 2). Therefore, when nutrient level of Mn(II)-containing wastewater is particularly low (e.g., in some industrial effluents), biostimulation (adding specific nutrient or mixing nutrient-rich wastewater to stimulate microbial growth (Bento et al., 2005)) combined with bioaugmentation, to enhance Mn(II) oxidation and removal of organic compounds, may be efficacious. However, biostimulation should be approached with caution since the appropriate range of nutrient level may be quite narrow.

5. Conclusions

In summary, our study yielded three key conclusions. First, the cooperative Mn(II) oxidation between the two non-Mn(II)-oxidizing bacteria may occur in natural aquatic environments. Second, the ratio of the two strains in a combined culture is a crucial factor to optimize Mn(II) oxidation. Finally, QXT-31, representing the combination of two bacterial strains, substantially improves Mn(II) oxidation in different waters, even when its relative abundance is very low, thus minimizing impacts to the indigenous microbial community.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2015.11.062>.

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