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Engineered manganese redox cycling in anaerobic–aerobic MBBRs for utilisation of biogenic manganese oxides to efficiently remove micropollutants

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ABSTRACT

Freshly formed manganese oxides (MnOx) can oxidise various organic pollutants, but its reactivity is short-lived and therefore continuous pollutant removal by MnOx is not feasible. In this study, an engineered cycle of manganese reduction followed by oxidation was implemented in a two-stage moving bed biofilm reactor (staged MBBR), aiming to replenish MnOx for continuous micropollutant removal. In the anaerobic reactor, added MnO2 was reduced to Mn2+ by microbially-mediated reaction with organic matter in the sewage. The reduced Mn2+ was then re-oxidised to biogenic manganese oxides (BioMnOx) in the aerobic reactor, which precipitated into the suspended biofilm. Part of the BioMnOx was regularly recycled to the anaerobic reactor for the next round of manganese reduction. Mass balance assessment shows that manganese redox cycling functioned well during continuous operation, and BioMnOx was continuously regenerated within the aerobic reactor. A specific Mn-oxidising bacteria belonging to Stenotrophomonas sp. was isolated from suspended biofilm of the aerobic reactor and identified, and Illumina high-throughput sequencing analysis reveals a microbial community shift in the staged MBBR during manganese redox cycling. Furthermore, both batch and continuous-flow experiments show that specific micropollutants such as bezafibrate, diclofenac and sulfamethoxazole were efficiently removed (over 80%) by the staged MBBR.

1. Introduction

Mn(III&IV) oxides (MnOx, x between 1 and 2) can oxidise a large variety of organic compounds [1], owing to their relatively high redox potentials (500 and 600 mV). Biogenic manganese oxides (BioMnOx), i.e. MnOx generated by microorganisms [2,3], are thought to be among the strongest oxidants with low toxicity occurring in the natural environment [4]. Accordingly, BioMnOx-based technologies have attracted increasing attention as an effective solution to remove a wide range of pollutants in various matrices [5–8]. Up until now, these studies have mostly been batch experiments at bench scale, and focused on harvested or pre-oxidised BioMnOx, which is not feasible for continuous wastewater purification. In our recent study [9], an engineered MBBR coupled with BioMnOx (designated as BioMn-MBBR) was constructed as an efficient technology to polish micropollutants in wastewater effluent. However, the additional Mn(II) source for BioMnOx production was critical during the long-term operation, which limited its full-scale application in realistic wastewater treatment design.

The challenge in developing practical BioMnOx-based technologies remains, and engineering solutions through manganese redox cycling may offer new perspectives. To date, naturally-occurring manganese redox cycling has been widely investigated in diverse sediment-water systems, such as marine sediments and nodules [10–12], freshwater dams [13] and drinking water systems [14]. In general, manganese redox cycling is an internal cycle between Mn(II) and Mn(III&IV). That is, MnOx are reduced to soluble Mn(II) by Mn-reducing bacteria (MnRB), while soluble Mn(II) is in turn re-oxidised to BioMnOx by Mn-oxidising bacteria (MnOB) [15]. As the BioMnOx is in situ-regenerated during this redox reaction, and no additional Mn(II) source is required if this redox cycling is well-established, it therefore offers clear advantages over pre-oxidised BioMnOx for continuous pollutant removal. However, to our knowledge, the engineered application of manganese redox cycling for wastewater treatment is as yet still underexplored.

Moving bed biofilm reactor (MBBR) is a novel biological treatment process, in which biofilms attach and grow on suspended plastic carriers in a reactor. Ooi et al. [16] constructed a staged MBBR system treating
hospital wastewater, whereby the different stages facilitated the distinct operational conditions (i.e. anaerobic and aerobic). Hence, it is a reasonable expectation that manganese redox cycling could be induced in such staged MBBRs, allowing manganese reduction in the anaerobic reactor, and manganese oxidation in the aerobic reactor. Furthermore, the suspended biofilms with long solids retention time could avoid competitive weakness of slow-growing bacteria (such as nitrifiers and MnOB) with other fast-growing heterotrophs [17,18], and thus offering an additional advantage in enriching slow-growing MnOB.

Among the emerging organic pollutants, micropollutants have become a global issue over the last decades. Micropollutants are commonly found in wastewater at trace concentrations (ng/L to μg/L), including pharmaceuticals, personal care products, pesticides, and other compounds [19]. Since typical wastewater treatment plants was unable to remove micropollutants effectively, these contaminants may end up in aquatic and terrestial environments, becoming potential risks to human health [20]. To date, various treatment methods have been applied for enhancing micropollutant removal, including coagulation-flocculation, activated carbon adsorption, advanced oxidation process, and membrane bioreactors [19]. However, these processes required further operational costs in comparison to biological treatment processes. Thus, attached growth biofilm systems such as MBBRs have been developed as an alternative solution to remove micropollutants from contaminated wastewater [21,22], with comparison studies showing that the micropollutant removal performance was greater than that in typical activated sludge plants. Nevertheless, some refractory micropollutants such as diclofenac are not completely biodegraded by MBBR. Thus, the pursuit of an optimised biofilm system for enhanced micropollutant removal remains.

Herein, we proposed engineered manganese redox cycling in anaerobic-aerobic two-stage MBBRs as a continuous biofilm system for efficient nutrients (i.e. COD and ammonia) and micropollutant removal. The aims of this study are: (1) to examine the feasibility of engineered manganese redox cycling in the staged MBBR under laboratory conditions. In doing so, we also evaluated the mass balance of manganese in the staged MBBR and the reusability of the reformed BioMnOx; (2) to investigate the possibility of using this continuous biofilm system coupled with manganese redox cycling for micropollutant removal. Lastly, 16S rRNA gene-based sequencing was performed to explore the bacterial community shift in the staged MBBR during manganese redox cycling.

2. Materials and methods

2.1. Chemicals

Commercial MnO₂, leucobenelberin blue and formaldoxime were purchased from Merck (analytical grade, Germany). Synthetic sewage was prepared based on OECD Guideline 303 A (defined as OECD peptone sewage). The composition is as follows: 160 mg/L peptone, 110 mg/L meat extract, 30 mg/L urea, 28 mg/L KH₂PO₄, 7 mg/L NaCl, 4 mg/L CaCl₂ and 2 mg/L MgSO₄. The corresponding common parameters such as COD and ammonia are described in Table S1.

The 14 micropollutants investigated in this study are listed in Table S2. These compounds are divided into six groups: 1) analgesic (carbamazepine, diclofenac and mfenamic acid); 2) antibiotic (azithromycin, clarithromycin and sulfamethoxazole); 3) anti-depressant (citalopram, sertraline and venlafaxine); 4) anti-inflammatory (ketoprofen); 5) blood pressure regulator and lipid-lowering agent (atenolol, bezafibrate and metoprolol); and 6) immunosuppressant (mycophenolic acid). For the micropollutant analysis, methanol, acetonitrile, and formic acid were all HPLC grade (Merck, Germany).

2.2. Configuration of the staged MBBR

In this study, two custom glass MBBRs were set up under laboratory conditions (Fig. 1a). The first was an anaerobic reactor (5 L) to perform microbially-mediated manganese reduction (MnO₂ to Mn(II)). This reactor was completely sealed to guarantee anaerobic conditions, and a mechanical stirrer was used to keep the carriers in suspension. The second was an aerobic reactor (2.5 L) to perform microbially-mediated manganese oxidation (Mn(II) to BioMnOx). This reactor was based on the previous BioMn-MBBR, and the detailed description about biological manganese oxidation in the BioMn-MBBR can be seen in our previous study [9]. Anoxkaldnes™ K5 carriers (25 mm diameter × 4 mm thickness) used in the two reactors were obtained from a regional WWTP receiving municipal wastewater (Lyngby, Denmark), and the filling ratio was 25% for each reactor.

Synthetic sewage was used as the startup feed for the anaerobic reactor, which was refreshed every 24 h. The anaerobic effluent was subsequently used to start up the aerobic reactor, in which the reduced Mn(II) was utilised to perform manganese oxidation. The two reactors were operated separately in batch mode for 20 days, to facilitate manganese reduction and oxidation within the corresponding reactors (Fig. 1a). Dissolved oxygen (DO) was < 0.1 mg/L in the anaerobic reactor and 8.9 mg/L in the aerobic reactor.

Stable performance of both reactors (manganese reduction, manganese oxidation, and COD removal) during batch mode operation was confirmed after 20 days. The two reactors were then connected in series to construct the anaerobic-aerobic two-stage MBBR (staged MBBR, Fig. 1b). Herein, manganese redox cycling was induced in the staged MBBR. Synthetic sewage was used to feed the staged MBBR through a peristaltic pump (BT100-1F, Longer, China), and the whole system was then operated for 30 days in a continuous-flow mode, with an influent flow rate of 0.18 L/h. Owing to the different reactor volumes (5 L and 2.5 L), the HRTs of the anaerobic and aerobic reactors were 24 h and 12 h, respectively.

A 2 L settler was used for collecting the BioMnOx sludge from the aerobic reactor effluent (Fig. 1b), which was then recycled into the anaerobic reactor. Although this design is inspired by the classic recirculation approach for biomass distribution and nitrogen management, the main purpose of doing it in this study was simply to reuse the BioMnOx sludge as a manganese source for the next round of manganese reduction-oxidation.

2.3. Evaluation of manganese redox cycling

2.3.1. Manganese reduction and oxidation in separated MBBRs

The performance of manganese reduction and oxidation in the separated MBBRs was examined during the batch mode operation (20 days). For manganese reduction, commercial MnO₂ was added intermittently into the anaerobic reactor every five days to initiate manganese reduction. A pre-experiment found this to be a good balance between keeping sufficient MnO₂ available in the reactor, whilst avoiding shock effects from overloading (Text S1 in the supplementary information). The MnO₂ dosage was then varied (0.5 g, 0.25 g and 0.1 g) to evaluate its effect on manganese reduction performance. For manganese oxidation, the effluent of the anaerobic reactor was transferred to the aerobic reactor after each HRT. Duplicate 5 mL aqueous samples were taken daily from each reactor for determination of Mn(II) concentration.

2.3.2. Manganese redox cycling in the staged MBBR

The performance of manganese redox cycling in the staged MBBR was examined during the continuous-flow operation (30 days). To sustain manganese redox cycling, 0.1 g MnO₂ was added into the anaerobic reactor following the same time interval as was used for batch mode operation (i.e. every five days). Duplicate 5 mL aqueous samples were taken daily from each reactor for determination of Mn(II) concentrations. Furthermore, the mass balance of manganese through the staged MBBR was also investigated, and the corresponding calculation is described in Text S2.
2.3.3. Reuse of BioMnOx sludge

This experiment was designed to test the reusability of the in situ-regenerated BioMnOx to sustain manganese redox cycling. During the continuous-flow operation, BioMnOx sludge (100 mL) from the aerobic reactor effluent had been accumulated in the settler. This sludge was then utilised in the reuse experiment, where 10 mL of the BioMnOx sludge was recycled per day to the anaerobic reactor. In short, the reactor setup in this further experiment was operated the same way as in 2.3.2, but utilised the BioMnOx sludge in place of direct MnO$_2$ addition. The sludge return ratio was not considered in this experiment due to the negligible sludge volume (10 mL vs 5 L reactor volume).

2.4. Investigation into micropollutant removals

2.4.1. Experimental design

Micropollutant spiking experiments were conducted in the staged MBBR after the 30 days of continuous-flow operation, in order to investigate the corresponding micropollutant removal performance. Firstly, a batch experiment was set up to evaluate the anaerobic and aerobic reactors individual abilities to removal micropollutants. The two reactors were operated separately over 24 h in batch mode, where the COD loadings for the anaerobic and aerobic reactors were 165 mg L$^{-1}$ d$^{-1}$ and 15 mg L$^{-1}$ d$^{-1}$, respectively. A mixture of 14 micropollutants was spiked directly into each reactor, which yielded concentrations of micropollutants between 10 and 20 μg/L. After spiking, aqueous samples (5 mL) were taken in duplicate from each reactor at time intervals of 1 min, 3 h, 6 h, 9 h, 20 h and 24 h.

A continuous-flow experiment was subsequently set up to evaluate micropollutant removal through the staged MBBR as a combined system. The two reactors were reconnected into the staged MBBR setup and keep operating for 3 days. This experiment was then conducted over one HRT (36 h) of the staged MBBR (24 h of anaerobic reactor and 12 h of aerobic reactor). The micropollutant mixture was spiked into the synthetic sewage prior to experiment, and the initial concentrations of micropolliutants were again between 10 and 20 μg/L. Aqueous samples (5 mL) were taken in duplicate from the anaerobic reactor at 0 and 24 h, and again from the aerobic reactor at 36 h.

Additionally, the staged MBBR was continuously operated for a further two weeks, where the COD loading for the anaerobic and aerobic reactors was increased to 330 mg L$^{-1}$ d$^{-1}$ and 50 mg L$^{-1}$ d$^{-1}$, respectively. An extra batch experiment was then conducted to investigate the effect of COD loading on micropollutant removal.

2.4.2. Data treatment

The concentrations of all investigated micropolliutants in the batch experiments were plotted with first-order kinetics (Eq. (1)) using GraphPad Prism, where $k$ is the rate constant of first-order kinetics; $C$ and $C_0$ are the micropolliutant concentration at time $t$ and initial concentration, respectively.

$$C = (C_0) \cdot e^{-kt} \quad (1)$$

The removal of all investigated micropolliutants were calculated by Eq. (2), where $C_i$ and $C_e$ are the micropolliutant concentration in the influent and effluent of each reactor, respectively.
2.5. Analytic methods

2.5.1. Common parameters

The common wastewater parameters COD and ammonia were measured according to test kit protocols. The aqueous samples were filtered through 0.22 μm nylon syringe filters (Agilent Technologies, China) into 5 mL glass tubes. For COD and ammonia measurement, the filtered samples were mixed with Hach test kits (LCI 400, LCK 302, Hach, Germany) and subsequently measured with a Hach Lange DR 3900 (Düsseldorf, Germany). DO and pH were determined by calibrated electrodes (Orion Star A215, Thermo Scientific, American). The biomass of each reactor was estimated by a modified method from Tang et al. [23]. Briefly, five carriers were randomly selected from each of the anaerobic and aerobic reactors. The carriers were placed on tin-foil cups, dried at 105 °C overnight and weighed. The coated biofilms were then removed from the carriers by 60 min sonication, and dried at 105 °C, and reweighed. The difference in weight of carriers before and after biofilm detachment was calculated as the estimated biomass.

2.5.2. Manganese quantification

For soluble Mn(II), aqueous samples were filtered through 0.22 μm nylon syringe filters (Agilent Technologies, China) into 5 mL glass tubes. The measurement was based on a formaldimoxime method reported by Brewer and Spencer [24]. Briefly, Mn(II) reacts with formaldimoxime at pH 10, and the generated orange-red colour is quantified by absorbance at 450 nm (Cary 50 Bio Varian spectrophotometer, Denmark). A seven point calibration curve was constructed by a dilution series of Mn(II) concentrations between 0.1 and 30 mg/L. For particulate Mn, BioMnOx samples were collected from: (1) the suspended biofilms of five carriers in the aerobic reactor; and (2) the BioMnOx sludge in the settler - both of which had been dried at 105 °C overnight. The quantitative measurement was then carried out through a leukobacterin blue (LBB) method that has been verified within previous studies [17,25], where BioMnOx selectively reacts with LBB and is quantified by colorimetric analysis at 625 nm.

2.5.3. Micropollutant quantification

Samples taken from the batch and continuous-flow experiments were transferred into 10 mL glass centrifuge tubes (2120016, VWR, Denmark) by using a 5 mL pipette (Eppendorf, Germany), and acetoni-trile was added to quench further reaction. The samples were then stored at -20 °C prior to analysis. Micropollutant concentration was quantified on high-performance liquid chromatography (HPLC, Agilent 1290 Infinity, USA) coupled with a triple-quadrupole mass spectrometer (Agilent 6470 series, USA). The detection limit of internal standards and HPLC-MS/MS parameters are detailed within a protocol, and the DNA concentrations were measured by a NanoDrop ND-2000 spectrophotometer. The V4 regions of the 16S rRNA genes were amplified by using primers 515F (5'-GTGCCAGCMGCGGCGGTATA) and 806R (5'-GGACTACHVHHRHTCTTAA). Finally, the sequencing was executed on an Illumina MiSeq platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd, China), and then analysed on the free online platform of Majorbio Cloud Platform (https://www.majorbio.com).

2.7. Isolation and identification of functional bacteria

During the 30 days of continuous operation, the functional bacteria MnOB GKH-1 was isolated from the suspended biofilms of the aerobic reactor, and the detailed cultivation and identification procedures can be seen in the supplementary information (Text S3).

2.8. Toxicity assay

A Microtox test was conducted to assess the toxicity of the effluent from the staged MBBR, using Vibrio fischeri as the toxicity indicator [27]. This test was conducted during the continuous-flow micropollutant spiking experiment, and detailed experimental procedures can be seen in our previous study [9]. The dried bacteria Vibrio fischeri was obtained from ABOATOX (Finland). Furthermore, the one-way analysis of variance (one-way ANOVA) was conducted to determine the level of significance via SPSS 24.0 Statistics software (IBM Crop, USA). P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Manganese reduction and oxidation in separated MBBRs

Fig. 2 shows manganese reduction and oxidation performance in the MBBRs during batch mode operation over 20 days. The black cross (X) indicates the time points when varied amounts of MnO2 were added, i.e. at days 0, 5, 10 and 15. By day 1, Mn(II) was already observed in the anaerobic reactor effluent, indicating that manganese reduction from Mn(IV) had already started. Over the first four days, the Mn(II) concentration in the anaerobic reactor effluent was variable between 10 and 20 mg/L, however by day 5 it had decreased to 5 mg/L (Fig. 2). Subsequently, the MnO2 dosage was decreased to 0.25 g at day 5 and 0.1 g at days 10 and 15. As expected, the Mn(II) concentration in the anaerobic reactor effluent decreased in line with decreasing MnO2 dosage. Addition of 0.25 g MnO2 resulted in approximately 9 mg/L of Mn(II) production (days 6–10), whereas addition of 0.1 g of MnO2 (days 11–20)
resulted in approximately 3 mg/L Mn(II) (Fig. 2). In addition to this, an extra experiment (Text S4 in supplementary information) was conducted within the anaerobic reactor to investigate the contribution of manganese reduction to COD and ammonia removal. This shows major differences in COD removal with varied MnO₂ dosage (Fig. S1a). For example, by adding 0.5 g MnO₂ to perform manganese reduction, 100% of COD was removed by the anaerobic reactor within 12 h. However, when the MnO₂ dosage decreased to 0.1 g, only approximately 40% of COD was removed over the same time period. This was similar to the COD removal in the control (without MnO₂). Furthermore, the ammonia removal was limited in the anaerobic reactor, regardless of the MnO₂ dosage (Fig. S1b).

For manganese oxidation within the aerobic reactor, the feed was the effluent from the anaerobic reactor, meaning that the Mn(II) concentration in the aerobic reactor influent was equal to that in the anaerobic reactor effluent. Initially, no significant Mn(II) removal was apparent over the first four days, where the concentration stayed around 20 mg/L (Fig. 2). By day 5, the Mn(II) concentration had decreased to around 5 mg/L, and further decreased to below the detection limit by day 10. The LBB approach identified the existence of BioMnOx both in the suspended biofilm and the excess sludge of the aerobic reactor (Fig. S2). The characteristics of BioMnOx in the aerobic MBBR, including morphologies, elemental composition, crystalline structure and valence state, has been detailed in our previous paper [21].

3.2. Performance of staged MBBR

3.2.1. Removal of COD and nitrogen

After the batch mode operation, the two reactors were reconnected as the anaerobic-aerobic two-stage MBBR system coupled with manganese redox cycling (staged MBBR, Fig. 1b). Common parameters including COD and ammonia were measured regularly to evaluate the stability and performance of this system. As shown in Fig. 3a, over 90% of COD was removed by the anaerobic reactor, leading to a decrease from 165 to 15 mg/L. This residual COD was then further removed by the aerobic reactor to below 5 mg/L. In contrast, the removal of ammonia was low in the anaerobic reactor (from 50 to 42 mg/L), and most ammonia was removed by the aerobic reactor (from 42 to 12 mg/L, Fig. 3a). Other parameters including DO, pH and temperature were stable over the continuous-flow operation.

3.2.2. Performance of manganese redox cycling

Manganese redox cycling was induced in the staged MBBR by performing manganese reduction in the anaerobic reactor and manganese oxidation in the aerobic reactor. An overview of manganese redox cycling in this system is shown in Fig. 4. A MnO₂ dosage of 0.1 g (23.1 mg/L reactor volume) was added to the aerobic reactor every five days to sustain manganese redox cycling. This led to a stable cycle where Mn(II) concentration oscillated between 2 and 3 mg/L in the aerobic reactor effluent, and the lowest concentration was on the last day of each five day cycle (days 5, 10, 15, 20, 25 and 30). For manganese oxidation, over 99% of Mn(II) was removed by the aerobic reactor to below the detection limit, despite that the HRT of the aerobic reactor was half that of the anaerobic reactor (12 h vs 24 h) which thereby had double the corresponding Mn(II) load (Fig. 4).

The mass balance of manganese was further calculated through the staged MBBR (Fig. 3b, Text S2). In the anaerobic reactor, the daily mass outlet of dissolved Mn(II) was calculated to be 12.34 ± 0.26 mgMn/d (mean ± SD), which was close to the daily mass inlet of particulate Mn (MnO₂, 12.8 mgMn/d). In the aerobic reactor, the daily mass outlet of dissolved Mn(II) was < 0.1 mg/d during the aerobic reactor (Fig. 3b). For particulate Mn (i.e. BioMnOx), the corresponding daily mass of BioMnOx into the suspended biofilms and the BioMnOx sludge were measured to be, 7.05 ± 1.35 and 4.22 ± 0.53 mgMn/d (mean ± SD), respectively. Lastly, this BioMnOx (4.22 ± 0.53 mgMn/d) accumulated on the bottom of the settler, and there was no outlet of dissolved Mn(II) from the settler. Hence, the mass of manganese was balanced between dissolved Mn(II) and particulate Mn (MnO₂ and BioMnOx) in such staged MBBR, and the mass loss of manganese was negligible during manganese redox cycling (Fig. 3b).
The reusability of the BioMnOx was also investigated in a further experiment. This was carried out by recycling the BioMnOx sludge (10 mL/d) from the settler to the anaerobic reactor for 10 days (Fig. S3). This resulted in a Mn(II) concentration between 1 and 3 mg/L in the anaerobic reactor effluent, and no Mn(II) was measured in the aerobic reactor effluent over the experimental period.

3.3. Microbial community

The bacterial communities in the staged MBBR coupled with manganese redox cycling over continuous operation were analysed through Illumina Hiseq sequencing of 16S rRNA gene. The diversity and richness indices including observed species, Shannon, Simpson, ACE and Chao1, and Good coverage are presented in Table S3. The bacterial community shift during manganese redox cycling was evaluated according to the relative abundance of bacteria (>1%) in the two reactors at phylum and genus taxa (Fig. 5a and b). At the phylum level, Proteobacteria was the most dominant phyla in the anaerobic reactor, and the corresponding proportions decreased from 76% to 41% during manganese redox cycling. For the aerobic reactor, Bacteroidota and Proteobacteria were the most dominant phyla during manganese redox cycling (Fig. 5a). At the genera level, significant abundance changes of several genera can be observed in Fig. 5b. For the anaerobic reactor, the most dominant genera was Rhodanobacter (43%), which gradually decreased to 2% after inducing manganese redox cycling. For the aerobic reactor, the dominant genera were norank_f_Microscillaceae and unclassified_f_Chitonophagaceae, which subsequently became norank_f_Microscillaceae, Rhodanobacter, unclassified_f_Comamonadaceae, Labrys and others.

Fig. 4. The manganese profiles through the anaerobic-aerobic two-stage MBBRs coupling with manganese redox cycling during continuous-flow operation. The cross (X) indicates when 0.1 g MnO$_2$ was added (days 0, 5, 10, 15, 20 and 25) to sustain manganese redox cycling. (COD = 165 mg/L, ammonia = 50 mg/L, pH = 7.5).

Fig. 5. (a) Relative abundance of major bacterial phyla observed in staged MBBR; (b) Relative abundance of major bacterial genera observed in staged MBBR. Ana1 and Ana2 represent the samples taken from the anaerobic reactor before and after inducing manganese redox cycling. Ae1 and Ae2 represent the samples taken from the aerobic reactor before and after inducing manganese redox cycling.
3.4. Functional bacteria in the staged MBBR

To identify the potential roles of microorganisms for the in situ regeneration of BioMnOx, a MnOB (designated as GKH-1) was screened from the suspended biofilms of the aerobic reactor. This bacterium can grow on the peptone medium and tolerate 2 mM Mn(II). The colony of the strain GKH-1 was round and had turned a brown colour by 144 h (Fig. S5). The LBB assay further identified that these brownish products on the culture plates were BioMnOx. The nucleotide sequence of the corresponding gene is presented in the supplementary information (Text S5, 1358 bp). According to the EzBioCloud database (https://www.ezbiocloud.net), the strain GKH-1 is highly homologous with Stenotrophomonas maltophilia (similarity 99.41%), which belongs to the phylum Proteobacteria.

3.5. Micropollutant removal

3.5.1. Batch experiments

Batch experiments were conducted to examine the micropollutant removal capacity of individual reactors by spiking a mixture of 14 micropollutants into the anaerobic and aerobic reactors. The removals of 4 representative micropollutants (bezafibrate, carbamazepine, diclofenac and sulfamethoxazole) within the first batch experiment are shown in Fig. 6a, and the results of all investigated micropollutants are illustrated in Fig. S6. Some compounds were observed to be removed within both reactors (anaerobic, aerobic), including azithromycin (73%, 70%), clarithromycin (69%, 79%), ketoprofen (55%, 30%) from carbamazepine and diclofenac, metoprolol and venlafaxine did not exhibit good removal (<30%) from either reactor after 24 h of treatment within the batch experiment (Fig. S6).

Furthermore, the micropollutant concentration curves were plotted with first-order kinetics (Eq. (1)), and the rate constants (k) of the two reactors for all investigated micropollutants are presented in Table S4. In general, the micropollutant removal in the anaerobic reactor did not follow first-order kinetics (Fig. S6). Instead, the removals of most micropollutants fitted well to first-order kinetics \( (R^2 > 0.9) \) in the aerobic reactor. Moreover, the constant k was normalised to the biomass of each reactor to obtain \( k_{bio} \), in order to estimate the micropollutant removal capacity per unit biomass. The measured biomasses of the anaerobic and aerobic reactors were 1.75 g/L and 0.42 g/L (Table S5), respectively. As presented in Table 1, the \( k_{bio} \) of the aerobic reactor for bezafibrate \((3.6 \times 10^{-1} \text{ vs } 6.3 \times 10^{-3} \text{ L.h}^{-1} \text{ g}^{-1})\), mefenamic acid \((2.2 \times 10^{-1} \text{ vs } 5.7 \times 10^{-3} \text{ L.h}^{-1} \text{ g}^{-1})\) and mycophenolic acid \((1.2 \times 10^{-1} \text{ vs } 3.8 \times 10^{-3} \text{ L.h}^{-1} \text{ g}^{-1})\) were two orders of magnitude higher than that of the anaerobic reactor. Whereas the \( k_{bio} \) for carbamazepine, diclofenac, metoprolol and venlafaxine were relatively low in both two reactors.

A followed up batch experiment was done to see the impact of COD loading on micropollutant removal, as the unexpected low removal capacity of diclofenac in the first experiment. The COD loadings to the anaerobic and aerobic reactor were simply increased to 330 mg L\(^{-1}\) d\(^{-1}\) and 50 mg L\(^{-1}\) d\(^{-1}\), respectively (as opposed to 165 mg L\(^{-1}\) d\(^{-1}\) and 15 mg L\(^{-1}\) d\(^{-1}\)). In this instance, 100% removal of diclofenac was achieved within the aerobic reactor (Fig. 6b). The removals of other micropollutants, such as citalopram and ketoprofen, were also enhanced to varying degree in this batch experiment (Fig. S7).

3.5.2. Continuous-flow experiment

A concentration profile of four representative micropollutants through the staged MBBR in the continuous-flow experiment is shown in Fig. 7, and the detailed results of all investigated micropollutants are presented in Fig. S8. Among the four micropollutants, sulfamethoxazole was removed by both reactors, whilst it was clear that bezafibrate was more efficiently removed by the aerobic reactor (Fig. 7). In contrast, the removals of carbamazepine and diclofenac were limited within the both reactors. Overall, 10 of 14 compounds were efficiently removed by the staged MBBR (>80% removal) with a total HRT of 36 h (Fig. S8).

3.6. Toxicity assessment

To evaluate the potential environmental impacts from the staged MBBR effluent, a Microtox test was implemented using a Vibrio fischeri assay. Water samples were collected from the influent at 0 h and from the staged MBBR effluent at various times (3–36 h). Fig. 8 shows the bioluminescence inhibition percentage of Vibrio Fischeri in each sample with assay times of 10 min and 20 min. The initial inhibition percentage
Table 1
Summary of first-order constants $k$ normalised to the biomass per gram per litre ($k_{bio}$, L h$^{-1}$ g$^{-1}$) in this study and a comparison with previous reported staged MBBR systems. Methods for biomass measurement of anaerobic and aerobic reactors in the two batch experiments are shown in section 2.5.1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{bio}$ with 165 mg/L COD (L h$^{-1}$ g$^{-1}$)</th>
<th>$k_{bio}$ with 330 mg/L COD (L h$^{-1}$ g$^{-1}$)</th>
<th>$k_{bio}$ in control MBBR$^*$ (no BioMnOx) (L h$^{-1}$ g$^{-1}$)</th>
<th>$k_{bio}$ in Literature (L h$^{-1}$ g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Atenol</td>
<td>1.9 × 10$^{-2}$</td>
<td>7.9 × 10$^{-2}$</td>
<td>1.5 × 10$^{-2}$</td>
<td>1.8 × 10$^{-2}$</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>1.1 × 10$^{-1}$</td>
<td>2.1 × 10$^{-1}$</td>
<td>1.2 × 10$^{-2}$</td>
<td>1.8 × 10$^{-2}$</td>
</tr>
<tr>
<td>Bezafibrate</td>
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<td>3.6 × 10$^{-3}$</td>
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n.a. represents not available.

a. A control MBBR operated without BioMnOx in our previous study [9].
b. A laboratory scale three-staged MBBR system [22]. Stage 1 is an aerobic MBBR and stage 3 is also an aerobic MBBR in the last stage.
c. A laboratory scale hybrid biofilm and activated sludge system (Hybas) [28]. Stage 2 is an aerobic MBBR coupled with activated sludge and stage 4 is an aerobic polishing MBBR.  
d. A pilot scale five-staged MBBR system [16]. Stage 1 is an anaerobic MBBR and stage 5 is an aerobic MBBR.

Fig. 7. Concentration of four representative micropollutants through the staged MBBR in the continuous-flow experiment. The HRTs of the anaerobic and aerobic reactors were 24 h and 12 h, respectively. The error bars represent the standard deviation of two sample measurements from the same reactor (COD = 165 mg/L, initial micropollutant concentration = 10–20 µg/L, pH = 7.5).
was 43.56% (influent, assay time = 10 min), whereas the inhibition percentage of effluent samples were all around 25% during the 36 h of operation. The one-way ANOVA further shows a statistically difference ($p < 0.001$) in inhibition percentage between influent treated before and after the staged MBBR over time. This suggests that, as expected, the staged MBBR had the capacity to reduce wastewater toxicity. As reported by Zou et al. [29], an inhibition percentage below 20% was considered to have no significant harmful effects on the natural environment. Therefore, it can be concluded that the micropollutants were detoxified by the treatment process, and the toxic effect of the treated staged MBBR effluent was minimal.

### 4. Discussion

Initial investigations were carried out on the individual performance of manganese reduction, and manganese oxidation, in separated MBBRs during batch mode operation. For manganese reduction, MnO$_2$ was found to be continuously reduced to Mn(II) in the anaerobic reactor with varied MnO$_2$ dosage (Fig. 2). It has been reported that diverse MnRB can perform manganese reduction by using manganese oxides as an electron acceptor under anaerobic conditions, and thus obtain energy to support their growth [30]. Electron donors for this microbial respiratory process can be carbon sources, H$_2$ sulphide as well as ammonium [31,32]. For instance, *G. metallireducens* was found to be capable of coupling manganese reduction to the oxidation of toluene under anaerobic conditions [33]. A further batch experiment also confirmed that MnO$_2$ itself was unable to oxidise organic substrates from synthetic sewage abiotically (Text S1 in the supplementary information). Hence, we presume that manganese reduction in the anaerobic reactor was microbiologically-mediated by MnRB, in which MnO$_2$ and organic substrates served as the electron acceptor and donor, respectively.

For manganese oxidation, the removal of Mn(II) was low in the aerobic reactor during the first five days (Fig. 2). This may be due to the excessive manganese stress, which resulted in a decrease in activity of MnOB as reported by Zhang et al. [34]. When the initial Mn(II) was lowered from 20 mg/L to 3 mg/L, the aerobic reactor exhibited robust manganese oxidation performance from day 11 to day 20 (Fig. 2).

Moreover, microbiologically-mediated manganese oxidation was the only likely pathway in the aerobic reactor because the neutral pH (7.5) in the bulk phase completely prohibited chemical oxidation of Mn(II) [35]. In short, Mn(II) was first microbiologically-oxidised to BioMnOx in the aerobic reactor (mediated by MnOB), and thereafter the BioMnOx could physically absorb more Mn(II) as reported by Wang et al. [36].

Following on from the separated reactor studies, the anaerobic-aerobic two-stage MBBR coupled with manganese redox cycling was investigated as a combined system. The proposed mechanism of manganese redox cycling behind this system is illustrated in Fig. 9. In this mechanism, MnO$_2$ serves as a precursor to stimulate manganese reduction with the oxidation of organic substrates in the anaerobic reactor, and thereby produces Mn(II) in the anaerobic reactor effluent. Then, this Mn(II) enters the aerobic reactor and is re-oxidised to BioMnOx in the presence of O$_2$. Lastly, the regenerated BioMnOx is recycled to the anaerobic reactor for the next round of manganese reduction-oxidation. Although redox cycling of manganese in the natural environment is well-known to be coupled to carbon cycling within various natural habits [37–39], the utilisation of manganese redox cycling in environmental remediation has as yet received little attention. De Schamphelaere et al. [15] discussed the possibility of enhancing manganese redox cycling for sediment oxidation, and more recently Zhou et al. [40] constructed a Mn-cycling biosystem for ciprofloxacin degradation, in which the whole system was driven by a MnOB *P. putida* MnB1. This was conducted at bench scale and manganese redox cycling occurred both intra- and extra-cellularly. However, results of our combined system show that manganese redox cycling can function well in a staged MBBR setup under laboratory conditions (Fig. 4), which strongly supports the viability of manganese redox cycling within a real wastewater treatment system.

There are two specific advantages of utilising manganese redox cycling in a wastewater treatment system. The first advantage is the high oxidation capacity that the manganese reduction process creates in the anaerobic reactor. Manganese reduction is known to be a driving force for organic matter oxidation under anaerobic conditions, which has been shown for sediment core samples [41,42] and for litter decomposition in forest ecosystems [43]. Our study shows that when 0.5 g MnO$_2$ was added into the anaerobic reactor to perform manganese reduction, over 99% of COD was rapidly removed within 12 h, which was around twice the rate of the control (without MnO$_2$) (Fig. S1a). Accordingly, the anaerobic reactor combined with manganese reduction might be an efficient approach for COD removal in municipal or industry wastewater containing various organic pollutants. However, when the MnO$_2$ dosage was decreased to 0.1 g (Fig. S1a), the contribution by manganese reduction to COD removal was negligible, suggesting the MnO$_2$ dosage...
significantly affects COD removal in the anaerobic reactor. Furthermore, the ammonia removal was limited in the anaerobic reactor (Fig. S1b), which is in contrast to previous studies showing that manganese reduction could enhance ammonia removal under anaerobic conditions [32,44,45]. This is likely because at such a low dose, all of the available MnO\textsubscript{2} is being utilised for COD removal.

The second advantage is the more efficient source of Mn(II) for BioMnO\textsubscript{x} production within the aerobic reactor. Although some wastewater has been shown to contain a considerable concentration of Mn(II) [46,47], Mn(II) from an outside source were typically used to initiate MnO\textsubscript{x} production within the literature [3,48,49]. Likewise, the Mn(II) [46,47] , Mn(II) from an outside source were typically used to initiate MnO\textsubscript{x} production within the literature [3,48,49].

Regarding the aerobic reactor, the abundances of specific genera changed markedly (Fig. S4). For instance, nor\textsubscript{ank}\textsubscript{f}\_Holophagaceae, Aeromonas, Dechloromonas, Holophaga, nor\textsubscript{ank}\textsubscript{f}\_Aerolineaceae and Zoogloea were highly enriched in the anaerobic reactor after performing manganese reduction. In the literature, nor\textsubscript{ank}\textsubscript{f}\_Holophagaceae, Dechloromonas and Holophaga have been reported to be associated with microbiologically mediated manganese reduction processes [53,54], whilst Zoogloea and nor\textsubscript{ank}\textsubscript{f}\_Aerolineaceae were widely observed under anaerobic conditions with high MnO\textsubscript{x} tolerance [55,56]. Regarding the aerobic reactor, the abundances of Rhodanobacter, Rudaca, unclassified\textsubscript{f}\_Xanthomonadaceae, nor\textsubscript{ank}\textsubscript{f}\_Comamonadaceae and nor\textsubscript{ank}\textsubscript{f}\_Fimbriimonadaceae increased after performing manganese oxidation. Among these genera, Rhodanobacter, nor\textsubscript{ank}\textsubscript{f}\_Xanthomonadaceae and nor\textsubscript{ank}\textsubscript{f}\_Comamonadaceae have been reported to be involved in microbiologically mediated manganese oxidation processes [53,57]. Interestingly, the abundances of nor\textsubscript{ank}\textsubscript{f}\_Gemmataceae and nor\textsubscript{ank}\textsubscript{f}\_Chitinophagaceae decreased, which were previously reported as non-MnOB bacteria with intolerance of MnO\textsubscript{x} [53]. Since the elimination of MnO\textsubscript{x}-intolerant bacteria was greatly inhibited in the aerobic reactor, the anti-bacterial effect of MnO\textsubscript{x} was considered to be a competitive weakness of MnO\textsubscript{x}-intolerant bacteria with high-tolerance MnOB, and thus enabling MnOB to consume more organic substrates than MnO\textsubscript{x}-intolerant bacteria and be enriched in the aerobic reactor. In addition to community analyses, a specific MnOB GKH-1 was successfully isolated from the suspended biofilms of the aerobic reactor and further identified as Stenotrophomonas sp. This species is one of the dominant genera in iron and manganese-rich mine drainage, which plays a vital role in heavy metal remediation [58]. As reported by Barboza et al. [59], Stenotrophomonas sp. is able to oxidise Mn(II) to BioMnO\textsubscript{x} from mine water through an indirect non-enzymatic pathway. Further, Calderón-Tovar et al. [60] has recently applied Stenotrophomonas sp. as one of the inoculums in a biofiltration process for Mn(II) removal, and proposed that one isolate S-3 (Stenotrophomonas sp.) exhibited a direct Mn(II) oxidation pathway with high oxidation capacity. In our study, the MnOB GKH-1 was able to be enriched in the suspended biofilms of the aerobic reactor and thus could replenish the BioMnO\textsubscript{x} during continuous operation. However, the results of bacterial community analysis suggest that other uncultivated bacteria may also significantly contribute to the manganese oxidation process in the aerobic reactor (Fig. S4).

Lastly, we assessed whether manganese redox cycling is in fact able to efficiently remove micropollutants in the staged MBBR. As shown in Fig. 6 and Fig. S6, 10 of the 14 investigated micropollutants were removed to varying degrees by the staged MBBR in the batch and continuous-flow experiments. In general, the anaerobic reactor exhibited poor removal capacity towards most micropollutants over the batch experiments. This could be attributed to manganese oxides in the anaerobic reactor mainly being involved in manganese reduction coupling with organic substrate removal as mentioned before, while the biodegradation of most micropollutants are limited as experienced in previous anaerobic MBBRs [16,23]. Hence, the micropollutant removal in the anaerobic reactor did not follow first-order kinetics. For the aerobic reactor, despite having four times lower biomass than the anaerobic reactor (0.42 g/L Vs 1.75 g/L), the corresponding k\textsubscript{bio} for most micropollutants was much higher than that of the anaerobic reactor (Fig. S6). This result was further compared to a control MBBR operated without manganese oxides in our previous study [9], which shows that the removals of specific micropollutants such as bezafibrate and sulfamethoxazole distinctly improved in the aerobic reactor (Table 1). For instance, the k\textsubscript{bio} for sulfamethoxazole of the aerobic reactor (7.9 × 10^{-2} L h^{-1} g^{-1}) was one order of magnitude higher than that of the control MBBR (5.8 × 10^{-3} L h^{-1} g^{-1}). Since previous studies have reported that BioMnO\textsubscript{x} can readily oxidise bezafibrate, sulfamethoxazole and other compounds [1,61,62], we presumed that the enhanced micropollutant removal in the aerobic reactor was attributed to coupling effects of BioMnO\textsubscript{x} and microorganisms. The exception to this was diclofenac, where the removal efficiency was < 30% in the aerobic reactor (Fig. 6a). This is not consistent with our previous study where 95% of diclofenac was removed in the BioMn-MBBR [9]. Since both the biological activity of MnOB and abiotic oxidation by BioMnO\textsubscript{x} have previously been attributed to micropollutant removal in the literature [49,63], our contrasting result may be due to microbial activity inhibition in the aerobic reactor caused by low COD loading (15 mg L^{-1} d^{-1}), in comparison to 165 mg L^{-1} d^{-1} to the previous BioMn-MBBR). This was further verified by the extra batch experiment, which showed that diclofenac removal recovered to 99% in the aerobic reactor with a higher COD loading (50 mg L^{-1} d^{-1}) (Fig. 6b). Meanwhile, one specific micropollutant was sertraline, for which the corresponding concentration was rapidly decreased within the first 20 min in both anaerobic and aerobic reactors. This result is in line with our previous study which showed that sorption was the dominant removal pathway for sertraline [9]. However, the sorption ability of suspended biofilms to other micropollutants was negligible as reported in the previous literature [9,26]. Regardless, the other 3 of the 14 investigated compounds (i.e. carbamazepine, metoprolol and venlafaxine) exhibited low removal in the aerobic reactor over the experiments, also fitting poorly to first-order kinetics (Fig. S6).

In the literature, these compounds are well-known refractory compounds, e.g. carbamazepine with an electron-withdrawing functional group [64]. Although manganese oxides are efficient oxidants towards many organic pollutants, He et al. [65] reported that the oxidative transformation of carbamazepine by manganese oxides only occurred at low pH (i.e. pH = 2.7) and was completely prohibited under neutral conditions such as in our reactors. This suggests that other technologies...
are needed as a polishing step for these refractory micropollutants. Furthermore, three previously described staged MBBR systems for micropollutant removal were compared to our staged MBBR [16,22,28]. In general, the removals of serval micropollutants in the two reactors were much faster than those within the selected studies, in particular the aerobic reactor coupled with the in situ regenerated BioMnOx. For instance, the $k_{bio}$ for azithromycin within our aerobic and aerobic reactors were $1.1 \times 10^2$ and $2.1 \times 10^1$ L h$^{-1}$ g$^{-1}$ respectively (Table 1), whilst the $k_{bio}$ of anaerobic and aerobic stages in a five-staged MBBR were only $4.4 \times 10^2$ and $7.5 \times 10^1$ L h$^{-1}$ g$^{-1}$, respectively [16]. For sulfamethoxazole, the $k_{bio}$ of our aerobic reactor was approximately one magnitude higher than reported for both a three-staged MBBR, and a hybas MBBR stage [22,28]. Only the $k_{bio}$ of stage 5 in the five-staged MBBR was reported to be slightly higher than our system [16]. Therefore, it can be concluded that the removal of specific micropollutants, such as diclofenac and sulfamethoxazole, were significantly enhanced in our staged MBBR coupled with manganese redox cycling. Overall, in comparison to the previous study (BioMn-MBBR as a polishing technology to micropollutant in wastewater effluent), our two-staged MBBR coupled with manganese redox cycling exhibited high removal performance of both nutrients and micropollutants as a continuous biofilm system. Future investigations into this system should focus on microbial community shift over long-term operation, as well as the removal performance of total nitrogen and total phosphorus, in order to develop this continuous system into practical wastewater treatment designs.

5. Conclusion

In this study, engineered manganese redox cycling was established in anaerobic-aerobic two-stage MBBRs. This combined system functioned well during continuous operation, with great removal performance of COD and ammonia. BioMnOx was continuously regenerated in the suspended biofilm of the aerobic reactor, and was reused in the anaerobic reactor as an alternative to MnO$_2$. This indicates the possibility of manganese redox cycling as a practical application in wastewater treatment. The bacterial community shift was shown to be significant during manganese redox cycling, and a functional MnOB GHK-1 was isolated from the aerobic reactor and identified. Micropollutant spiking experiments showed the enhanced removal of specific micropollutants in our staged MBBR in comparison to the previously reported staged MBBR systems. Notably, diclofenac was also found to be effectively removed by the staged MBBR when a high COD loading was applied (330 mg L$^{-1}$ d$^{-1}$). Overall, our study has established a continuous biofilm system coupled with manganese redox cycling, which shows promise as an alternative to typical wastewater treatment technologies for efficient nutrients and micropollutant removal. Scale-up studies of this staged MBBR should be further investigated to gain broader perspective for engineered manganese redox cycling in wastewater treatment.

Declaration of Competing Interest

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.

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