ENHANCED SULFAMETHOXAZOLE REMOVAL USING ANAEROBIC AND AEROBIC SEQUENCING BATCH REACTOR WITH MAGNETITE

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Abstract. Sulfamethoxazole (SMX) is one of the most frequently used antibiotics. The capacity of conventional wastewater treatment plants to remove such antibiotics is limited, posing a risk of antibiotic resistance genes spreading into the environment. In this study, to carry out biological Fenton reaction under neutral conditions, an anaerobic and aerobic sequencing batch reactor (SBR) supplemented with magnetite was proposed. This process aims to enhance the treatment of antibiotics in addition to organic pollutants such as chemical oxygen demand (COD) without external addition of hydrogen peroxide (H_2O_2). Mixed anaerobic and aerobic sludge was exposed to alternative anaerobic and aerobic conditions in two identical SBRs with and without magnetite to treat the synthetic wastewater containing 1 mg·L^{-1} sulfamethoxazole. The experimental results showed that the H_2O_2 level increased to 34.9 μM under aerobic conditions in the system with magnetite, and similar COD removal was observed in both SBRs. Moreover, enhanced SMX treatment was observed in the SBR with magnetite, while removal efficiencies of SMX gradually decreased in the SBR without magnetite. The experimental results demonstrate that H_2O_2 generation under aerobic conditions and biological Fenton reaction that can produce hydroxyl radicals led to the enhanced treatment of SMX in the SBR with magnetite. Magnetite as the catalyst was not dissolved during the redox process, indicating the possibility of its reusability. Further studies are needed to analyse the reaction mechanisms and the kinetics in the proposed SBR.

Keywords: activated sludge process, magnetite, sulfamethoxazole, hydrogen peroxide, biological Fenton reaction.

1. Introduction

In recent years, the emergence of antibiotics as emerging contaminants (ECs) in natural water environment has attracted extensive attention. Antibiotics are widely used in the treatment of infectious diseases, however, after antibiotics enter the human body or animal body, 5% to 90% of them are excreted through urine and feces in the form of maternal structure or metabolites [1]. Sewage is considered as a main route for antibiotics entering the environment, while conventional wastewater treatment plants (WWTPs) have a limited performance to remove such ECs and the development of feasible and efficient technology to treat antibiotic wastewater is needed [2].

To date, the Fenton process is considered as a promising approach to oxidize ECs based on hydroxyl radicals (˙OHs) production, as shown in reaction (1). However, the issues such as comparatively high energy consumption and cost of operation and maintenance have limited its application [3]. Research efforts are put to overcome these shortcomings by applying heterogeneous catalysts and several studies have shown that magnetite (Fe_3O_4) was considered as a promising catalyst for the Fenton reaction in a neutral pH condition [4-6].

$$H_2O_2 + Fe(\text{II}) \rightarrow Fe(\text{III}) + OH^- + HO^{\cdot} \quad (1)$$

Ma *et al.* [7] observed effective removal of tribromophenol by adding submicron magnetite particles into a sequencing batch bioreactor and demonstrated that the supplemented magnetite could stimulate microorganisms to generate more H_2O_2 during the respiratory metabolism. Peng *et al.* [8] showed that the addition of magnetite and granular activated carbon promoted sludge hydrolysis and resulted in enrichment of iron-reducing bacteria (IRB) existing in anaerobic sludge. Peng *et al.* [9] have also reported IRB were able to produce H_2O_2 with Fe (III) oxides and therefore enhanced the Fenton reaction to produce ˙OHs. H_2O_2 may be produced via reactions (2), (3), (4), and (5) [10-12].

$$NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2 \quad (2)$$

$$Lactate + O_2 \rightarrow Pyruvate + H_2O_2 \quad (3)$$

$$Pyruvate + Pi\,(phosphate) + O_2 \rightarrow acetyl - phosphate + CO_2 + H_2O_2 \quad (4)$$

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (5)$$

It is expected from these studies that H_2O_2 may accumulate in a short period of time when the activated sludge is aerated, which may lead to continuous production of hydroxyl radicals with the addition of magnetite. To elucidate the effects of magnetite as a catalyst on antibiotics removal, as well as investigate the microbial H_2O_2 production by facultative anaerobes when oxygen is introduced, we conducted an advanced activated sludge process where magnetite particles were dosed to mixed anaerobic and aerobic sludge in a sequencing batch reactor under alternation between anaerobic and aerobic conditions. Sulfamethoxazole was used as a model antibiotic because of its poor removal in activated sludge process [13].

2. Materials and methods

2.1. Samples and chemical reagents

Anaerobic and aerobic sludges were collected from an anaerobic digester and return activated sludge, respectively, from two different WWTPs in the Kanto Region, Japan. Anaerobic sludge was stored in a refrigerator at 4 ºC before being used. Sulfamethoxazole (SMX) and magnetite powder (98%, particle size < 5 μm) were purchased from Sigma-Aldrich (Darmstadt, Germany). All other chemicals used in this study were of analytical grade. The composition of synthetic wastewater was ($mg \cdot L^{-1}$): casein peptone 150, glucose 150, sodium acetate 52, urea 52, $CaCl_2 \cdot 2H_2O$ 4, $MgSO_4 \cdot 7H_2O$ 2, NaCl 6, SMX 1. The COD concentration of the synthetic wastewater was 405 $mg \cdot L^{-1}$.

2.2. Batch experiment for microbial H_2O_2 production

Three experimental series (A, B and C) were carried out to investigate the microbial H_2O_2 production by using different types of inoculant activated sludge and iron catalysts. Each series contained 3 sets of experiments. Inoculant sludge and synthetic wastewater were added into centrifuge tubes with a working volume of 10 mL and the effective volume for all series was 7 mL. Table 1 shows the initial additions in terms of inoculant activated sludge, iron catalysts and COD concentrations for each series. The initial MLSS for all series was set as 3000 $mg \cdot L^{-1}$ (excluding iron catalyst). The experiment was operated for 7 cycles at room temperature (20-25ºC) and each cycle consisted of 3-day anaerobic period and 3-day aerobic period. Before starting the anaerobic stage, all centrifuge tubes were purged with nitrogen gas for 10 min at a flow rate of 0.03 $L \cdot min^{-1}$ to remove O_2 and then capped with matching covers. After 3 days of the anaerobic period, the tubes were opened and aerated to create an aerobic environment. At the end of each aerobic period, all tubes were centrifuged at 12000 rpm at room temperature for 10 min. One milliliter of supernatant was withdrawn to quantify H_2O_2 production. To maintain the total effective volume of 7 mL, one milliliter of synthetic wastewater was then replenished to the tubes.

Table 1

Initial conditions of synthetic wastewater, inoculant sludge, iron catalyst and COD concentrations in the batch experiments

	Synthetic wastewater, mL*	Inoculant sludge	Iron catalyst		Initial COD concentration, $mg \cdot L^{-1}$
			Fe_3O_4	$FeCl_3$	
A-1	3	4 mL aerobic sludge	-	-	171
A-2	3	4 mL aerobic sludge	1 $g \cdot L^{-1}$	-	171
A-3	3	4 mL aerobic sludge	-	0.1 M	171
B-1	6	1 mL anaerobic sludge	-	-	342
B-2	6	1 mL anaerobic sludge	1 $g \cdot L^{-1}$	-	342
B-3	6	1 mL anaerobic sludge	-	0.1 M	342
C-1	3.65	3 mL aerobic sludge + 350 μL anaerobic sludge	-	-	209
C-2	3.65	3 mL aerobic sludge + 350 μL anaerobic sludge	1 $g \cdot L^{-1}$	-	209
C-3	3.65	3 mL aerobic sludge + 350 μL anaerobic sludge	-	0.1 M	209

*In cycles 2 to 7, 1 mL of supernatant was replaced with 1mL of synthetic wastewater.

2.3. Determination of H_2O_2

H_2O_2 concentration was quantified using a modified chromogenic assay which is based on the oxidative coupling of DMAB and MBTH [14, 15]. Samples were centrifuged at 12,000 rpm at room temperature for 10 min and 1 mL of the supernatant was transferred into a cuvette. An aliquot of 400 μL of 12.5 mM DMAB reagent (51.6 mg of DMAB into 25 mL of 0.375 M sodium phosphate buffer solution), 80 μL of 1.3 mM MBTH followed by 40 μL of 5 units horseradish peroxidase were then added to the cuvette. The reaction mixture was incubated at room temperature for 5 min and then cooled at 0 ºC for 15 min to terminate enzyme activities. Absorbance readings were taken at 590 nm using a UV-Vis spectrophotometer (Shimadzu UV-160).

2.4. SBR experimental design for SMX removal

Two cylindrical column SBRs with a working volume of 1 L were established for the sequencing batch experiment, namely AS (no magnetite, control) and AS/Fe_3O_4 (containing 1g·L^{-1} of magnetite). Each reactor was fed with synthetic wastewater and inoculant sludge. The inoculant sludge consisted of anaerobic and aerobic sludge at a 1:1 ratio of MLSS and each SBR had an initial MLSS of 2 g·L^{-1}. The whole experiment was conducted at room temperature (20-25°C) and the pH of both systems was in the range of 7.0-8.0 without adjustment. The two SBRs were operated for four cycles and each cycle consisted of 1-day anaerobic period and 1-day aerobic period. The first cycle (i.e., day 0-2) was an acclimation period, followed by the three cycles (i.e., day 2-8). Two systems were purged with nitrogen to maintain dissolved oxygen (DO) at 0-1 mg·L^{-1} for anaerobic conditions and the air pump was used to keep aerobic environment with DO of 6-7 mg·L^{-1}. The operation started with the anaerobic period and at the end of each cycle, the stirrer and gas supply were stopped for 30 minutes to allow the sludge to settle, and the supernatant was replaced by new synthetic wastewater with a volume exchange ratio of 75%. Samples were withdrawn using a syringe prior to changing the conditions.

2.5. Analytical methods

COD, MLSS, mixed liquor volatile suspended solids (MLVSS) and the sludge volume index (SVI) were determined according to Standard Methods (2005) [16]. DO was measured using an Orion Star™ A223 DO meter (Thermo Fisher Scientific, Waltham, MA), pH was measured using an EutechTM PC 450 digital meter (Thermo Fisher Scientific, Waltham, MA). SMX concentration was determined using an Agilent 1260 Infinity II high-performance liquid chromatography (HPLC) equipped with a diode array detector at 268 nm. Before analysis, samples were filtered through a 0.45 μm syringe filter (25CS045AN, Advantec, Tokyo, Japan). 20 μL of supernatant was injected into the column from an auto-sampler. Separations were performed in a 4.6 x 150 mm L-column ODS (GL, Sciences, Tokyo, Japan) at 40°C. 0.3% acetic acid in acetonitrile and 0.3% acetic acid in ultrapure water were used as the mobile phase A and B, respectively, at a flow rate of 1 mL·min^{-1}. SMX was eluted by acetic acid in acetonitrile from 34% to 100% in 13 minutes. Total dissolved Fe concentration in supernatant was determined using an inductively coupled plasma optical emission spectroscopy system (5100 ICP-OES, Agilent Technologies, Santa Clara, CA). Prior to analysis, samples were diluted three times with diluted hydrochloric acid and then filtered through a 0.45 μm syringe filter. The COD and SMX removal efficiencies were calculated by the following equation:

$$Removal\ efficiecy = \frac{C_0 - C_{end}}{C_0} \times 100\%, \quad (6)$$

where C_0 and C_{end} – indicate the initial and final concentrations in each cycle, respectively.

3. Results and discussion

3.1. Microbial H_2O_2 production in batch experiments

During the whole experiment, control groups (A-1, B-1 and C-1) and experimental groups with magnetite (A-2, B-2 and C-2) were maintained at circumneutral pH (6.50-7.40) without adjustment. On the other hand, the pH of the experimental groups with $FeCl_3$ (A-3, B-3 and C-3) was in the range of 2.48-4.89. As shown in Figure 1, H_2O_2 produced in series A (aerobic sludge) was remarkably less than that in series B (anaerobic sludge) and C (mixture of aerobic and anaerobic sludge). In series B and C, the concentrations of H_2O_2 in control groups (B-1 and C-1 with no iron catalysts) were lower than those

in the experimental groups (B-2, B-3, C-2 and C-3). Anaerobic sludge and mixed sludge containing $FeCl_3$ (B-3 and C-3) produced more H_2O_2 compared with other groups during the first half of the experiment, while H_2O_2 concentrations decreased from the 3rd cycle to the end of the experiment. On the other hand, it is interesting to note that the concentration of H_2O_2 after aerobic periods in the groups with the addition of magnetite (B-2 and C-2) increased gradually in both anaerobic and mixed sludge and reached the peak concentrations of 15.0 and 16.4 μM, respectively. In B-2 and C-2, as magnetite consists of undissolved Fe (III) and Fe (II), structural Fe (III) in magnetite might be reduced by IRB to insoluble Fe (II), which was expected to enhance the production of H_2O_2 on the surface of magnetite particles [17, 18]. The redox of insoluble magnetite prevented the loss of iron even under neutral conditions. Furthermore, it was considered that IRB were enriched in the existence of magnetite particles during anaerobic periods, resulting in the increasing amount of H_2O_2 production via reaction (2), (3), (4) or (5). Due to the stable and promising H_2O_2 production under neutral pH in the groups with magnetite, it was applied as the catalyst in the subsequent sequencing batch experiment.

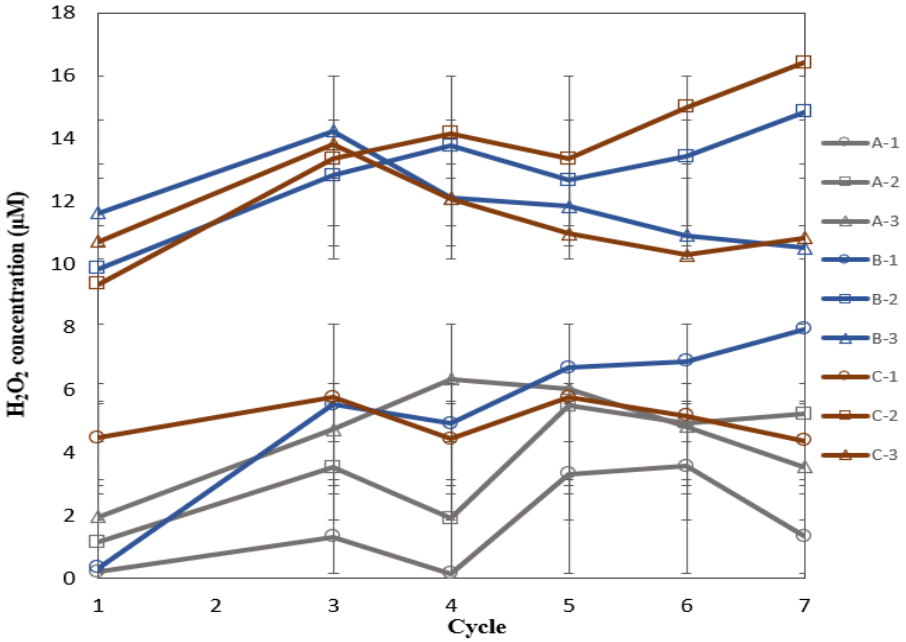

Fig. 1. **Microbial H_2O_2 generation after aerobic period under different sludges and iron catalysts**

3.2. Advanced activated sludge process

3.2.1 H_2O_2 generation in the proposed SBRs

As shown in Figure 2(a), the alternate anaerobic and aerobic conditions resulted in varied H_2O_2 concentrations. There was a tendency that H_2O_2 was produced during aerobic periods in both SBRs. The bulk concentration of H_2O_2 is dependent on the amount of H_2O_2 released into the system as well as the balance between its production and consumption. In the first two cycles, H_2O_2 concentrations in the AS system were slightly higher than those in the AS/Fe_3O_4 system. However, as the experiment continued, the AS/Fe_3O_4 system generated a remarkably increasing amount of H_2O_2 and its bulk concentration reached the highest level of 34.9 μM. Sekar and DiChristina [19] observed 24.5 μM H_2O_2 was produced by a pure culture of facultative IRB, Shewanella oneidensis under aerobic environment without any addition of iron catalyst. It was presumed the generated H_2O_2 was the by-product of microbial aerobic respiration represented by reaction (2), (3), (4) or (5). In the AS/Fe_3O_4 system, microorganisms in the activated sludge were probably adsorbed onto magnetite particles and IRB were considered to be enriched in the presence of magnetite during the dissimilatory Fe (III) reduction [8; 20]. Besides the biological Fenton reaction (reaction (1)), some H_2O_2 may also be consumed due to the H_2O_2-catalyzed Fe (III) reduction reactions, leading to the decrease in H_2O_2 concentration observed in the second aerobic phase of the AS system and the fourth aerobic phase of the AS/Fe_3O_4 system. As the reaction proceeded, the maximum dissolved iron concentration in the AS/Fe_3O_4 system during the whole experiment was

15.4 mg·L^{-1}, see Figure 2(b), which suggested that magnetite was likely maintained under alternative anaerobic and aerobic conditions via the microbial redox process with very low loss of Fe_3O_4.

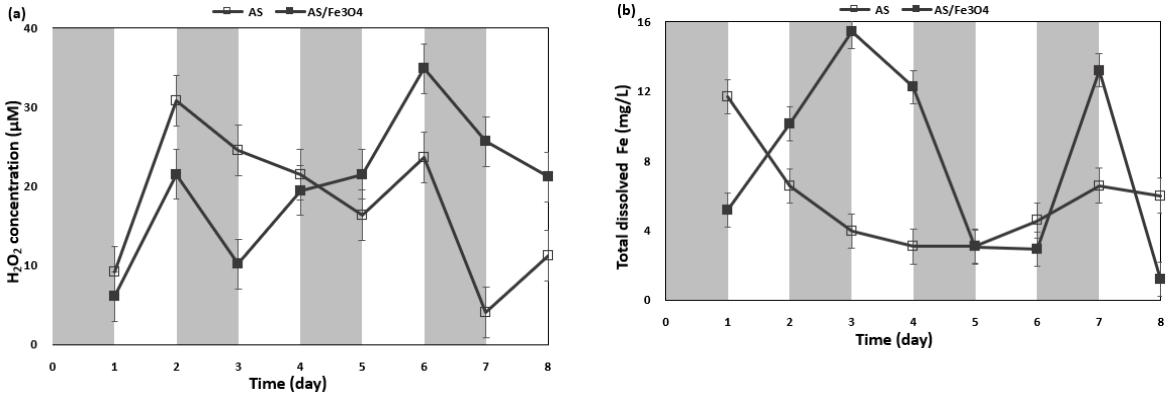

Fig. 2. **Variations of H_2O_2 concentrations (a) and the corresponding dissolved total Fe concentrations (b) in the AS and AS/Fe_3O_4 systems. The grey and white regions represent the anaerobic and aerobic conditions, respectively**

3.2.2 COD and SMX removal

It was found that COD concentrations consistently decreased in both reactors, even though new synthetic wastewater was added to the systems at the end of each cycle [Figure 3(a)]. COD removal in both AS and AS/Fe_3O_4 systems increased toward the end of the experiment, with a removal efficiency of 37.6% and 41.3%, respectively [Figure 3(c)]. The similar COD removal efficiencies in both reactors indicated that organic decomposition reactions proceeded without inhibition of magnetite particles due to the lower toxicity of micro-scale particles, as compared with that of nanoscale particles [21]. The results in Figure 3(b) showed that SMX concentrations increased and decreased during the anaerobic and aerobic periods in both reactors. The increase in SMX concentrations in anaerobic phases was due to the replacement of synthetic wastewater at the end of the aerobic period. The dramatic decrease of SMX concentration from 1.08 to 0.32 mg·L^{-1} in the acclimation period in the AS system was due to the presence of 12 mg·L^{-1} dissolved iron in the initial phase, see Figure 2(b). These iron compounds existed in the form of dissolved Fe (II) under aerobic environment and reacted with H_2O_2, generating hydroxyl radicals via possible Fenton reactions. Concurrently, Fe (II) was oxidized to Fe (III) and precipitated as Fe $(OH)_3$ under neutral conditions. Iron sludge was produced as the anaerobic and aerobic cycle repeated and iron concentration was reduced to approximately 6 mg·L^{-1} as shown in Figure 2(b), which inhibited Fenton reactions in the AS system, leading to a drastic decline in SMX removal efficiency, see Figure 3(d).

In contrast, the AS/Fe_3O_4 system showed a lower SMX removal efficiency in the acclimation period, which can be attributed to the lower concentration of H_2O_2 in the system. It was indicated that to utilize magnetite as an electron acceptor for dissimilatory ferric reduction and the enrichment of IRB needed an acclimation phase, which was also observed in the batch experiment for microbial H_2O_2 production. On the other hand, competitive reactions with H_2O_2 might occur on the surface of magnetite, which is that H_2O_2 was also consumed during the decomposition of organic compounds such as adsorbed microorganisms and extracellular polymer substances. However, as the experiment proceeded, the AS/Fe_3O_4 system performed an enhanced and stable treatment of SMX and had a peak removal efficiency of 60.1%. This was due to the continuous generation of H_2O_2, and biological Fenton reaction may occur on the surface of magnetite particles. The removal efficiencies of SMX during the activated sludge process in WWTPs ranged from -138% to 99%, this is due to that many factors can affect the biological treatment, for example, wastewater components and microorganism species [13; 22].

It is considered that the presence of magnetite caused a change in the kinds of microorganisms and corresponding biological reactions, which also affected the performance of the treatment in removing SMX [13].

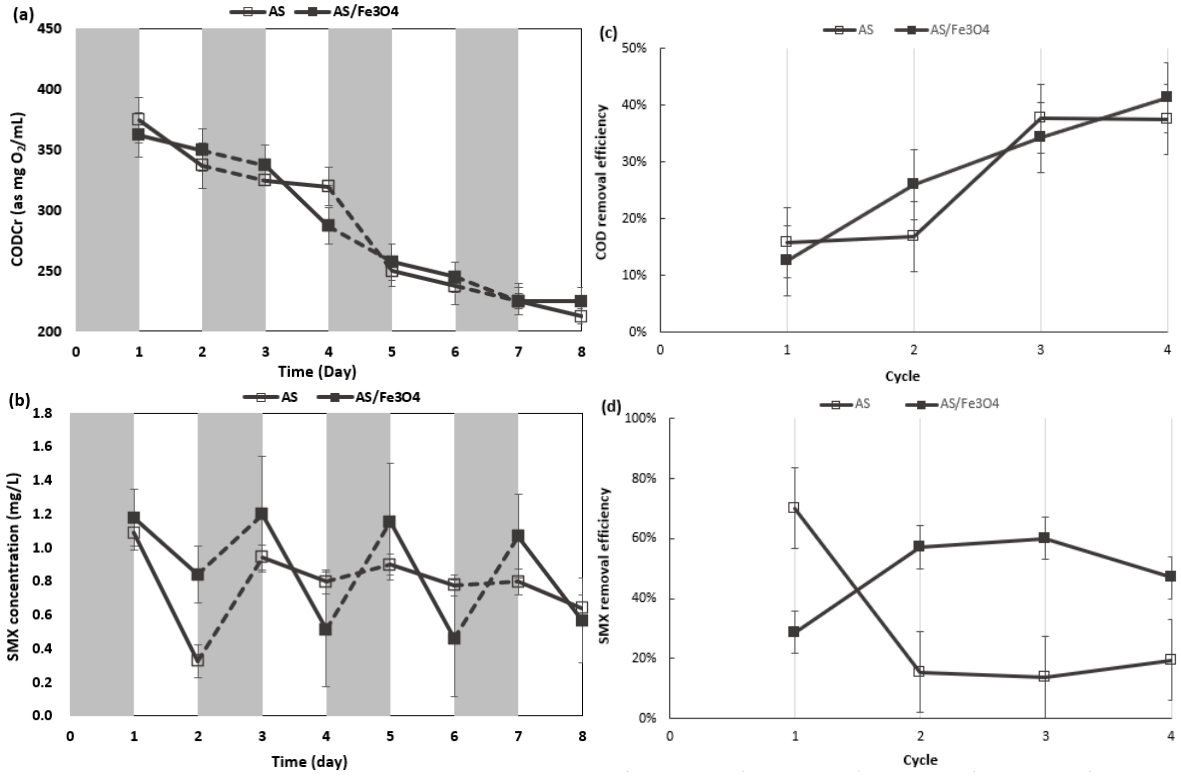

Fig. 3. **Variations of COD concentrations (a), SMX concentrations (b), COD removal efficiencies (c) and SMX removal efficiencies (d) in the AS and AS/Fe_3O_4 systems; the grey and white regions represent the anaerobic and aerobic conditions, respectively**

Conclusions

It was demonstrated that stable and enhanced removal of sulfamethoxazole (SMX) was achieved through adding magnetite powder in an anaerobic/aerobic sequencing batch reactor (SBR). The experimental results showed that, regardless of the presence of magnetite, mixed anaerobic and aerobic sludge could achieve bulk H_2O_2 concentrations of more than 30.0 μM under aerobic conditions. The removal of COD was not affected by the supplemented magnetite and both SBRs performed similar COD removal ability. More than 60% of SMX was treated with addition of magnetite and stable removal was achieved, while the SBR without magnetite showed a decreased SMX removal efficiency and had a final removal efficiency of 20%. Based on the results of this study and previous literature, the microbial H_2O_2 production under aerobic conditions and reduction of magnetite under anaerobic conditions contributed to the enhanced treatment of SMX due to the biological Fenton reaction that can produce hydroxyl radicals. Further studies are needed to investigate the more precise mechanisms, long-term stability and performance, and the optimal operational condition in the proposed SBRs.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors would like to thank Mr. Maehara and Mr. Mitsui for their assistance during the setup and experiments.

Author contributions

Writing – original draft preparation, Tong Shen; writing – review and editing, Yutaka Sakakibara, Yoshihiko Inagaki and Tong Shen; data curation, Tong Shen, Yoshihiko Inagaki and Hiroki Koike; methodology, Ranjusha VaddakePariyarath and Hiroki Koike; validation, Yutaka Sakakibara and Masahito Komori. All authors have read and agreed to the published version of the manuscript.

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