Water Research 160 (2019) 361–370

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Fe^{3+} enhanced degradation of oxytetracycline in water by *pseudomonas*

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ARTICLE INFO

Article history:
Received 17 December 2018
Received in revised form
9 May 2019
Accepted 18 May 2019
Available online 20 May 2019

Keywords:
Oxytetracycline
Biodegradation
Antibiotic resistance genes
Degradation pathways and metabolites

ABSTRACT

The application and fate of antibiotics are closely related to human health and the ecological balance, which has gradually aroused the widespread global concerns. Long-term antibiotic residues can easily induce antibiotic resistance and antibiotic resistance genes (ARGs) in the environment. Although many studies have investigated the metabolic pathways of biosynthesis or degradation of oxytetracycline (OTC) and its influencing factors under laboratory or controlled conditions, the understanding of OTC degradation pathways and influencing factors in the environment is still poor. In the present study, the role of *Pseudomonas* (T4) in OTC biodegradation were investigated with different carbon sources, metal ions, substrate concentrations, temperatures, and pH values, as well as the temporal changes in the relative abundance of OTC ARGs. It was found that OTC could be degraded by T4 as a sole carbon source. Comparison with Cu^{2+}, the addition of Fe^{3+} could significantly promote the growth of T4, and then increased the OTC degradation percentage to 65.3%. The initial concentration of OTC, temperature, and pH had significant impacts on OTC degradation. At the initial OTC concentration of 50 mg L^{-1}, the percentage degradation of OTC by T4 could reach 81.0% at the presence of Fe^{3+}, and at 40 °C and pH = 7. Common tetracycline ARGs were not found during the OTC degradation by T4 in the present study. The eight main putative OTC degradation byproducts were identified by ultra-high definition accurate-mass quadrupole time-of-flight tandem mass spectrometry (QTOF/MS). Six different reaction types and seven possible degradation pathways were proposed, including enol-ketone conversion, hydroxylation, dehydration, deamination, demethylation and decarbonylation. Under optimal conditions, the OTC degradation percentages by T4 could reach to 88.2%, 91.6% and 92.0% in pond water, fish wastewater and industrial wastewater, respectively. These results demonstrate the high effectiveness of T4 at the presence of Fe^{3+} for the enhanced biodegradation of OTC in water environment, without resulting in the occurrence of ARGs. This has important implications for the removal of OTC from aquatic environments by the technology proposed from this study.

1. Introduction

Pharmaceuticals and personal care products (PPCPs), a new class of pollutants, have received increasing attention in recent years. Approximately 3,000 types of PPCPs are now used in medicines such as painkillers and antibiotics, according to the European Union (EU) (Ternes et al., 2004; Ye Dingyi et al., 2006). Veterinary antibiotics (VAs), for example, are frequently used in aquaculture and poultry farming as growth-promoting agents and disease-prevention drugs, as a result, they have gradually entered and accumulated in environments such as water and soils (Chen et al., 2014; Kemper, 2008; Phillips et al., 2004).

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https://doi.org/10.1016/j.watres.2019.05.058
0043-1354/

Oxytetracycline (OTC), a broad-spectrum antibiotic with the formula shown in Fig. 1, is a common tetracycline antibiotic that is widely used in poultry and animal farms around the world. However, OTC cannot be absorbed well by animals, and approximately 90% of antibiotics are released into the environment in the form of their parent or metabolites (Alcock et al., 1999). OTC residues have been detected in environments worldwide (Kumar et al., 2005). In the United States, the residual amount of OTC can reach 0.34 μg L^{-1} in the surface water (Liu et al., 2016c; Yang and Carlson, 2003). OTC residues in Australian urban water and Danish soil can reach 0.35 μg L^{-1} and 2.5–50 mg kg^{-1}, respectively (Kong et al., 2007; Yan et al., 2013). It was also found that the concentration of OTC residues in a sewage treatment plant in China reached 20–800 mg L^{-1}, with the consumption of OTC reaching the high amount of to 1360 t in 2013 (Zhang et al., 2015). Long-term exposure of microorganisms to antibiotics in the environment even at the low level of 0.34 μg L^{-1} is likely to induce resistance and resistance genes in surrounding microorganisms (Zhu et al., 2013). An aquatic ecosystem is a complex system composed of organisms living in a given habitat, therefore, there is no specific provisions and uniform standards worldwide for threshold of antibiotics such as OTC in water environment. In term of the algae, *anabaena cylindrical* is the most sensitive to OTC among all the selected aquatic organisms with the EC50 values of 0.03 mg L^{-1} and *tetraselmis chuii* is the most resistant to OTC with the EC50 values of 11.18 mg L^{-1}(Zhao et al., 2013). As has been previously reported in the literature, the fish is the most resistant to OTC, and daphnia has the middle resistance to OTC, and algae is the most sensitive to OTC in water environment. Now the acceptable OTC level especially in aquatic environment is still not set. Thus, it is very important to effectively degrade and remove OTC and its derivatives in the environment.

There are many proposed methods for removal of the residual OTC in the environment, including advanced oxidation processes, activated carbon adsorption, low temperature plasma technology, membrane bioreactors, and biodegradation processes (Darweesh and Ahmed, 2017; Liu et al., 2016b; Rivera-Utrilla et al., 2018; Sahar et al., 2011; Sokolov and Louhikultanen, 2018). These processes have played a great role in removing OTC residues from environments. The biodegradation process approach has gradually attracted more attention due to its simplicity and low cost (Maki et al., 2006; Chen et al., 2019b). Fe is not only one of the essential trace metal elements for microbial growth, but also an important cofactor of microbial enzyme activity. It exists mainly in the form of oxide in the environment. Fe_2O_3 is a widely used material, and Fe^{3+} plays a significant role in promoting microbial fuel cells. Thus, the application of Fe^{3+} combined with microorganisms may promote the microbial degradation of antibiotics. In addition, biodegradation methods for removing antibiotic residues such as OTC are difficult to apply widely because of the lack of understanding about whether antibiotic resistance genes and metabolites are introduced into the environment. Therefore, the objectives of the present study are: (1) to characterize the positive effect of Fe^{3+} on microbial degradation of OTC, (2) to investigate the dynamic changes of common tetracycline antibiotics resistance genes over time (7 days), such as *tet* (A), *tet* (M), *tet* (O) and *tet* (W), and (3) to understand the potential metabolites of OTC degradation by microorganisms and the associated mechanisms in the presence of Fe^{3+}.

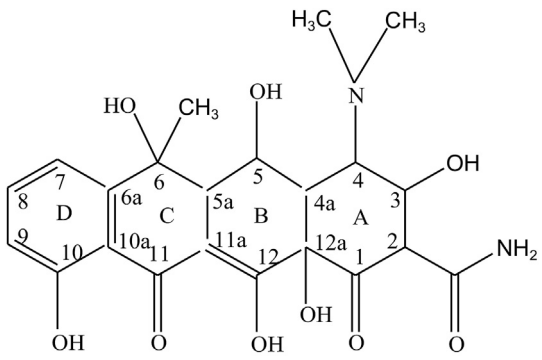


Fig. 1. The molecular structure of OTC.

2. Materials and methods

2.1. Materials

Oxytetracycline hydrochloride (purity > 96%, OTC) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Methanol and acetonitrile (ACN) (HPLC grade) were purchased from Fisher Science Co. The other reagents were of analytical grade. Ultrapure water was obtained with a Millipore Milli-Q system.

2.2. OTC and related antibiotic resistance gene analysis

2.2.1. Quantitative and qualitative analysis of OTC

The concentration of OTC was quantified by HPLC (Agilent 1260 Series) with the method described by Liu (Liu et al., 2016b). The reaction byproducts were detected and identified by an Agilent 6540 ultrahigh definition accurate-mass quadrupole time-of-flight tandem mass spectrometer (QTOF/MS) coupled with an Agilent 1290 Infinity UPLC system (UPLC-QTOF/MS). The Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 μm) used for analysis was supplied by Agilent. The analysis was performed using 0.1% formic acid in Milli-Q water as eluent A and 0.1% formic acid in acetonitrile as eluent B in gradient elution mode at a flow rate of 0.3 mL min^{-1}. The elution gradient started with 15% of eluent B and linearly decreased to 95% B over the first 8 min, was maintained at 95% B for 1 min, and then returned to 15% B over the next 0.1 min. After gradient elution, the column was equilibrated for 2 min before the next injection. An injection volume of 1 μL was used in all analyses. The mass spectrum (m/z 30–500) was analyzed in positive ion mode by electrospray ionization (ESI) with a drying gas temperature of 320 °C, drying gas flow of 8 L min^{-1} and collision energy of 25 eV. The QTOF scan data were analyzed using Agilent MassHunter B.07.00 workstation software.

The degradation percentage (D%) of OTC was calculated according to the following equation:

$$D\% = (C_0 - C_t)/C_0 \times 100\%$$

where C_0 is the initial concentration of OTC, and C_t is the concentration of OTC at time t.

2.2.2. Bacterial concentration determination

Bacterial concentration changes were monitored using a UV-VIS spectrophotometer at 600 nm and presented as OD value.

2.2.3. Determination of ARGs by real-time fluorescent quantitative PCR

Real-time fluorescent quantitative PCR (qPCR) was used to determine the abundance of ARGs related common tetracycline antibiotics, including *tet* (A), *tet* (M), *tet* (O), and *tet* (W). The qPCR reaction system (10 μL) comprised 5 μL of TB Green Premix Ex Taq II (Tli RNaseH Plus), 0.4 μL of both forward and reverse primers (Wcgene Biotech, Shanghai, China), 1 μL of template DNA (or control), and 3 μL of double distilled water. The qPCR conditions were as follows: initial hold for 30 s at 95 °C, followed by 40 cycles of 95 °C for 30 s and 40 cycles of 60 °C for 30 s. The qPCRs were

conducted on StepOnePlus™ Real-Time PCR system (Wcgene Biotechnology, Shanghai).

2.3. Experiments on the optimization of OTC biodegradation

2.3.1. Degradation of OTC by T4 bacteria

T4 bacteria were isolated from soils contaminated by OTC in our laboratory before the experiment. The bacteria were identified as *Pseudomonas* via 16S rDNA gene sequence analysis (Meng et al., 2018). The colony morphology of T4 is round, smooth and opaque milky white colonies with a diameter of 0.2–0.4 cm at 30 °C on beef extract peptone medium in three days. There were two treatments set up in the experiment including (1) OTC: only OTC was added to the inorganic salt medium, and (2) OTC + T4: both OTC and T4 bacteria were added to the inorganic salt medium. Each treatment was performed in triplicate. The 100 mL of inorganic salt medium (ISM) (NH_4Cl 1g, NaCl 1g, K_2HPO_4 1.5g, KH_2PO_4 0.5g, $MgSO_4$ 0.2g, distilled water 1L and pH = 7) was added to a 250 mL tube which was wrapped by foil to avoid the photodecomposition reaction occurrence of OTC, and was sterilized at 121 °C for 30 min. Then 1.0 mL of OTC water solution with a concentration of 5 mg mL^{-1} was added to the tube with ISM, yielding a final OTC concentration of 50 mg L^{-1}, after its temperature decreased to room temperature (25 °C). For OTC + T4 treatment, a total of 1.0 mL of T4 bacteria solution (3.0×10^8 CFU mL^{-1}) at the logarithmic growth stage was added to the tube. All the above operations were carried out in the ultraclean workbench with no microorganisms and darkness conditions. All the tubes containing the solution mixture were incubated at 30 °C in a constant temperature and light-proof shaker with a rotational speed of 150 r min^{-1}. During the incubation period, the samples were taken on 0, 1, 3, 5, and 7 days after treatment and 5.0 mL of liquid mixture was sampled each time.

2.3.2. Different carbon sources

Four types of carbon sources including starch, maltose, beef extract peptone, and yeast extract, and inorganic salt liquid medium were used to investigate the efficiency of OTC degradation by T4 with different carbon sources. Each carbon source was replicated 3 times. The liquid medium was prepared and sterilized, OTC, T4 were added according to the methods described in 2.3.1. In addition, the only OTC adding without T4 adding treatment were set for each carbon sources to eliminate the difference in OTC spontaneous degradation at different carbon source. During the incubation period, the samples were taken on 0, 1, 3, 5 and 7 days after treatment and 5.0 mL of liquid mixture was sampled each time.

2.3.3. Different metal ions

To investigate the enhanced efficiency of OTC degradation by T4 in the presence of common metal ion pairs, Cu^{2+} and Fe^{3+} were selected in the present study. Two treatments were set: (1) OTC, T4 (1%), and Cu^{2+} (0.1%) were adding together; (2) OTC, T4 (1%), and Fe^{3+} (0.1%) were adding together. Fe^{3+} and Cu^{2+} were selected based on the following facts. 1) Copper is an essential microelement for domestic animals such as pig, chicken, and cow and is often used as a feed additive to promote domestic animal growth in livestock farm. 2) It has been reported that lots of organic compounds including antibiotics can be removed by Cu and Fe_3O_4 nanomaterials (Pham et al., 2018a, 2018b). The liquid medium was prepared and sterilized, OTC, T4 were added according to the methods described in 2.3.1. Cu^{2+} and Fe^{3+} were added in the form of $CuSO_4 \cdot 5H_2O$, and $FeCl_3 \cdot 6H_2O$, respectively. During the incubation period, the samples were taken on 0, 1, 3, 5 and 7 days after treatment and 5.0 mL of liquid mixture was sampled each time.

2.4. Influencing factor experiments

There were three influencing factors: substrate concentration, temperature and pH. There were five different substrate concentrations including 5, 10, 25, 50, and 100 mg L^{-1}, and four different temperatures including 25, 30, 35, and 40 °C. In addition, seven different pH values were set as 3, 4, 5, 6, 7, 8 and 9. Four OTC systems were set such as OTC, OTC + T4, OTC + T4+Fe^{3+}, and OTC + Fe^{3+} in the present experiment. Each factor at each level was performed in triplicate. The liquid medium was prepared and sterilized, OTC, T4 and Fe^{3+} were added by the same methods described in 2.3.1. During the incubation period, the samples were taken on 0, 1, 3, 5, and 7 days after treatment and 5.0 ml of liquid mixture was sampled each time.

2.5. Detection of ARGs related to common tetracycline antibiotics in OTC removal solution

The ARGs monitoring experiment was conducted by using a real-time sampling and detection by real-time fluorescent quantitative PCR under optimal degradation conditions. Common tetracycline antibiotic resistance genes including *tet* (A), *tet* (M), *tet* (O), and *tet* (W), were selected for monitoring over 7 days. There were two treatments: (1) OTC and (2) OTC/T4 (1%)/Fe^{3+} (0.1%). The sampling times were 1, 3, 5, and 7 days. OTC, T4 and Fe^{3+} were added by the same methods described in 2.3.3.

2.6. Application of T4 in the presence of Fe^{3+} in real water matrix

There were two treatments including (1) OTC + T4 (1%) and (2) OTC + T4 (1%) + Fe^{3+} (0.1%) in the present experiment. Three different water matrices were selected such as sewage plant wastewater, aquaculture water and pond water. The OTC concentration was 50 mg L^{-1}. The samples were collected on 3 days after treatment.

2.7. Statistical analysis

SPSS Statistics version 19 software was used for statistical analyses. One-way analysis of variance (ANOVA) was used to determine if treatment means were significantly different, and Duncan's multiple range tests was performed to determine if the individual means were different from each another. Mass Hunter B.07.00 (Agilent) was used to further determine the OTC metabolites under the action of microorganisms. Chem Draw Ultra 8.0 was used to visualize molecular structures.

3. Results and discussion

3.1. Degradation efficiency of OTC

3.1.1. Biodegradation of OTC over time

As shown in Fig. 2, the percentage degradation of OTC increased with time, with a value of nearly 34.1% in the T4 treatment and only 17.4% in the treatment without T4 on day 7. Furthermore, the residual amount of OTC was reduced for all four sampling times, i.e., on day 1, 3, 5, and 7. The degradation rates with bacteria T4 were 14.3% and 16.7% higher than those withoutT4 for days 5 and 7, respectively. In fact, the degradation rates with T4 were significantly different from those at the treatment without bacteria on both days 5 and 7. The results indicate that OTC could undergo spontaneous degradation reactions, but the spontaneous degradation is slow (Xuan et al., 2010). Our findings are consistent with previous reports that OTC is a special antibiotic that is more difficult than other antibiotics to degrade in the environment (Pouliquen et al., 2007).

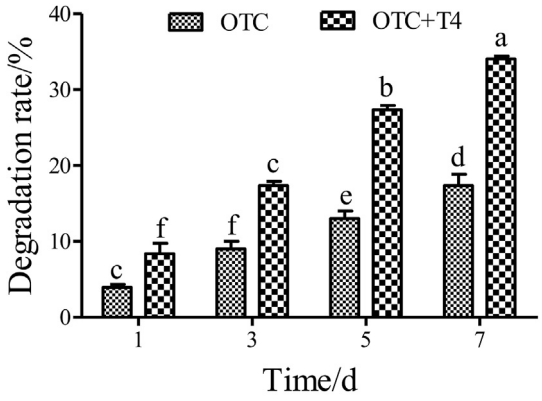


Fig. 2. Biodegradation of OTC with time.

3.1.2. Role of different carbon sources and metal ions in the biodegradation of OTC

Biodegradation is the process of converting organic compounds to a lower complexity or simple inorganic molecules such as water and carbon dioxide (CO_2) (Nzila, 2013). Organic pollutants are eliminated by microorganisms mainly through co-metabolism as a carbon source. Co-metabolism is a process in which organic compounds are biodegraded in the presence of only primary energy materials. The degradation of OTC by T4 with different carbon sources, namely, starch, maltose, beef extract peptone, yeast extract, and ISM, is shown in Fig. 3 (A). Among the first four carbon sources, starch had the best effect on degradation of OTC, while beef extract peptone the worst effect. Compared with these carbon sources, the percentage degradation was nearly 34.1% in inorganic salts, and this weak degradation was most likely caused by hydrolysis in the other carbon sources for 7 days. Beef extract peptone can provide rich carbon sources, nitrogen sources and vitamin C to meet the requirements of microbial growth. The T4 competes with the beef extract peptone at a disadvantage, which resulted in OTC degradation being not effective in the carbon source material solution. The experimental results agree with the principle of screening bacteria for pollutant degradation, in which antibiotics are consumed by microbes as the only carbon source, that is, OTC was degraded as a carbon source by T4. Some similar results have been reported for *Sphingomonas paucimobilis*, which could utilize fluoranthene as the sole source of carbon and energy for growth and degraded a variety of high molecular weight polycyclic aromatic hydrocarbons (PAHs) (Ye et al., 1996). On the one hand, organic pollutants are used as carbon sources to promote the growth of microorganisms, which can secrete enzymes to catalyze the biodegradation of OTC. On the other hand, if there is competition between OTC and energy substances in the same reaction system, microorganisms will preferentially consume the additional carbon sources, and pollution is easily produced by the excess energy substances, which is not conducive to the reaction.

Metal ions play important roles in microbial growth. In this study, 0.1% Fe^{3+} and 0.1% Cu^{2+} were added into the reaction system to promote the biodegradation of OTC. The results revealed that Fe^{3+} can promote the percentage degradation of OTC up to 65.3%, as shown in Fig. 3 (B). The effect of Cu^{2+} was not as obvious as that of Fe^{3+}, and the highest percentage degradation only reached to 34.0% within 7 days. The OTC degradation rate was slow from day 3 to day 5 after Fe^{3+} was added. The difference was found for the OTC degradation in response to Fe^{3+} and Cu^{2+} additions on both day 3 and 5.The degradation rate of OTC under the addition of Fe^{3+} was 30.7% higher than that under the addition of Cu^{2+} on day 3. T4 secretes a low molecular weight active polypeptide that chelates the vitro of iron, which promotes the growth of T4 (Ratledge and Dover, 2000), thus increasing the biodegradation of OTC. Since Fe is an essential microelement for organisms, the addition of Fe^{3+} is conducive to the microbial degradation reaction.

3.2. Other factors influencing OTC biodegradation

3.2.1. Effect of substrate concentration on the biodegradation of OTC

The initial OTC concentrations (5 mg L^{-1} to 100 mg L^{-1}) significantly affected the degradation of OTC by T4. (Fig. 4). The effects of different initial OTC concentration on the degradation of OTC followed the order of 50 > 25>10 > 5>100 mg L^{-1}. The percentage degradation of OTC at 50 mg L^{-1} reached 32.0% when treated with T4 only (Figs. 4 A), and 65.5% treated with the combination of T4 and Fe^{3+} (Fig. 4 B), respectively. The OD values varied from 0.062 to 0.123 in the presence of T4, and from 0.062 to 0.736 in the presence of T4 and Fe^{3+}, respectively. It indicates that Fe^{3+} can promote the growth of T4, then resulting in enhanced biodegradation of OTC. This could be explained by the fact that low concentrations of OTC may provide insufficient energy to maintain microbial metabolism, but excessive OTC can also inhibit the growth of microorganisms, possibly because OTC itself is a bactericidal substance or might be toxic to T4 at a high concentration. In this study, the higher the initial concentration of OTC led to the lower the degradation rate of OTC. This is consistent with the gentamicin biodegradation by FZC3 (Liu et al., 2016a). Nevertheless, some relevant experimental results are contrary to the conclusions of our experiment, for example, the degradation of cephalexin by *Pseudomonas* was not significantly affected by the increase in the cefalexin concentration (Lin et al., 2015). This indicated that the responses of bacteria depend on the types of the antibiotics.

3.2.2. Effect of temperature on the biodegradation of OTC

It has been found that temperature has a significant effect on the degradation of OTC in the aquatic environment (Kummerer, 2009b;

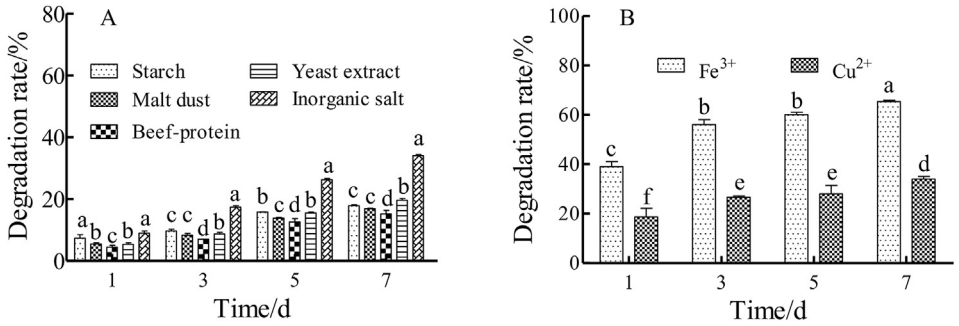


Fig. 3. The percentage degradation of OTC by T4 in different conditions with (A) different carbon sources and (B) 0.1% Fe^{3+} and 0.1% Cu^{2+}. Data bars with the same letter indicate not significantly different as determined by Duncan's test ($p < 0.05$).

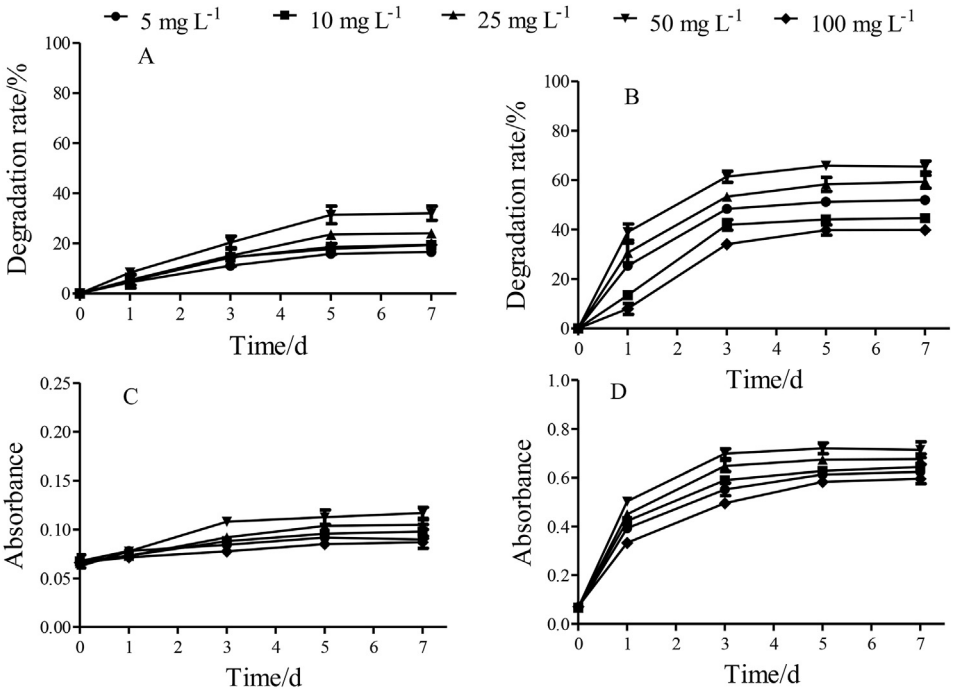


Fig. 4. Degradation rates of OTC by T4 at different initial concentrations. (A) OTC degradation by T4 at different initial concentrations. (B) OTC degradation by T4 with Fe^{3+} at different initial concentrations. (C) and (D) are the absorbance changes of T4 with or without presence of Fe^{3+} at 600 nm determined by UV-VIS spectrophotometery.

Loftin et al., 2008; Xuan et al., 2009). The changes in the degradation rate of OTC and the bacterial growth at different temperatures are shown in Fig. 5. The highest degradation of OTC was found at 40 °C, with the value of 54.8% at the treatment of T4 (Figs. 5A) and 80.7% at the treatment of T4 and Fe^{3+} (Fig. 5B), respectively. In short, the degradation of OTC at different temperatures followed the order of 40 °C>35 °C>30 °C>25 °C. The bacterial growth increased with increasing temperature. At a given temperature, the bacterial growth in the treatment of T4 and Fe^{3+} was significantly higher than that in the treatment of T4. The results proved that elevated temperatures can promote the degradation of OTC in the range of 25 °C to 40 °C (Wang and Yates, 2008). OTC was shown to be heat-labile by Hassani et al. (Mounir et al., 2008). Antibiotics in the same class have also been reported to show different heat stabilities depending on different matrices and temperature treatments (Abou-Raya et al., 2013; Franje et al., 2010). It has also been

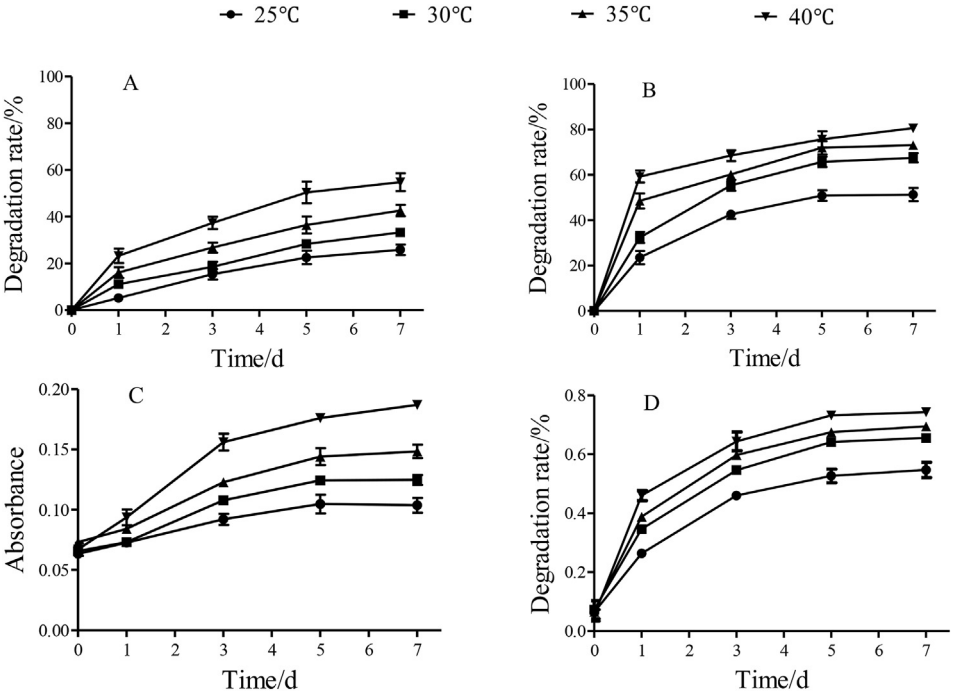


Fig. 5. Degradation of OTC by T4 at different temperatures. (A) $C_0 = 50$ mg L^{-1}, and T4 degraded OTC at different temperature. (B) $C_0 = 50$ mg L^{-1}, T4 degraded OTC with adding Fe^{3+} at different temperatures. (C) and (D) are the absorbance changes of (A) and (B) by UV-VIS spectrophotometer at 600 nm.

reported that increasing temperatures increase the rate of OTC hydrolysis (Kummerer, 2009a). In addition, temperature has a great influence on microbial activity. It has been reported that microbial activity and temperature are key factors in the degradation of tetracyclines during manure composting (Wu et al., 2011). Based on the published information and Fig. 5, we can deduce that Fe^{3+} could promote T4 growth and its activities, which then promote the OTC biodegradation. In addition, there is an optimum temperature for the formation of iron carriers. Few iron carriers are secreted when the temperature is below 25 °C, but a large number of iron carriers can be produced in the range of 25–40 °C. Therefore, with increasing temperature in the range of 25–40 °C, the ability of T4 bacteria to produce iron carriers is gradually enhanced, which is beneficial to the degradation of OTC. This result coincides with the results for animal manure compost. There are some similar reports for composting that increasing the temperature can promote OTC degradation, which the highest degradation rates occurring at high composting temperatures (Arikan et al., 2007; Wang and Yates, 2008). The above result also conforms to the molecular collision theory that higher temperatures cause more collisions between particles, thereby increasing the reaction rate.

3.2.3. Effect of pH on the biodegradation of OTC

pH values have a significant influence on the growth and physiological activities of microorganisms. As shown in Fig. 6(A) and (B), the degradation efficiencies at different pH values are showing in the following order: pH 7 > 6 > 8 > 9 > 5 > 4 > 3. The percentage degradation values were the highest at pH 7 and reached 54.1% and 81.0% without and with presence of Fe^{3+}, respectively. At pH 3, the two groups had the lowest degradation rates of 14.2% and 27.3%, respectively. The degradation rates of OTC under strongly acidic, medium-strongly acidic and strongly alkaline conditions were slower than those under neutral conditions. It is consistent with the previous reports that OTC degradation in neutral solution appeared to be much faster than that in both acidic and alkaline solutions. (Xuan et al., 2010). It has been reported that pH can not only affect the form of OTC (Doi and Stoskopf, 2000; Loftin et al., 2008) but also affect the growth of T4 bacteria. This microorganism can grow under neutral conditions but can't grow well under strongly acidic, alkaline conditions. These results are consistent with previous reports, in which cell growth was depend on the initial pH (Solieri and Giudici, 2008). The obviously decreased dissolution capacity of CO_2 could affect the growth of a strain at a lower pH in culture medium (Wang et al., 2018a). Furthermore, a low pH usually results in a lower level of ATP in cells and the inhibition of bacterial growth. Therefore, both the degradation rates of OTC and T4 the OD600 value of the reaction were low at pH 3, 4 and 5. pH and temperature can promote T4 growth and make it more active but can't change the generation of OTC metabolites as shown in the C, and D of Figs. 5-6, and Table S6, and Table S7.

3.2.4. Effect of EDTA on the biodegradation of OTC

As shown in Fig. 7, OTC was degraded by 28.1%, and 67.7% at the treatments of OTC, and OTC plus Fe^{3+}, respectively. However, with the addition of EDTA buffer as the extracting agent, OTC degradation rate decreased to 26.3%. This indicates that Fe^{3+} could promote OTC hydrolysis and then result in the degradation of OTC. However, with the addition of EDTA buffer solution, Fe^{3+} and EDTA maybe form a more stable complex. It has been reported that the stability constant of EDTA and Fe^{3+} complex is 25.1 (Shan et al., 2018). It also indicated that the enhanced degradation of OTC in the presence of Fe^{3+} is mainly attributed to the promotion of T4 growth. In addition, It is also reported that OTC has complexing ability which can react with different metal ions (Chen et al., 2011).

3.3. Real-time monitoring of ARGs during the biodegradation of OTC

Resistance genes are easily produced in environmental media contaminated by OTC, which destroys the diversity of the biological community. The rapid and efficient degradation of OTC can

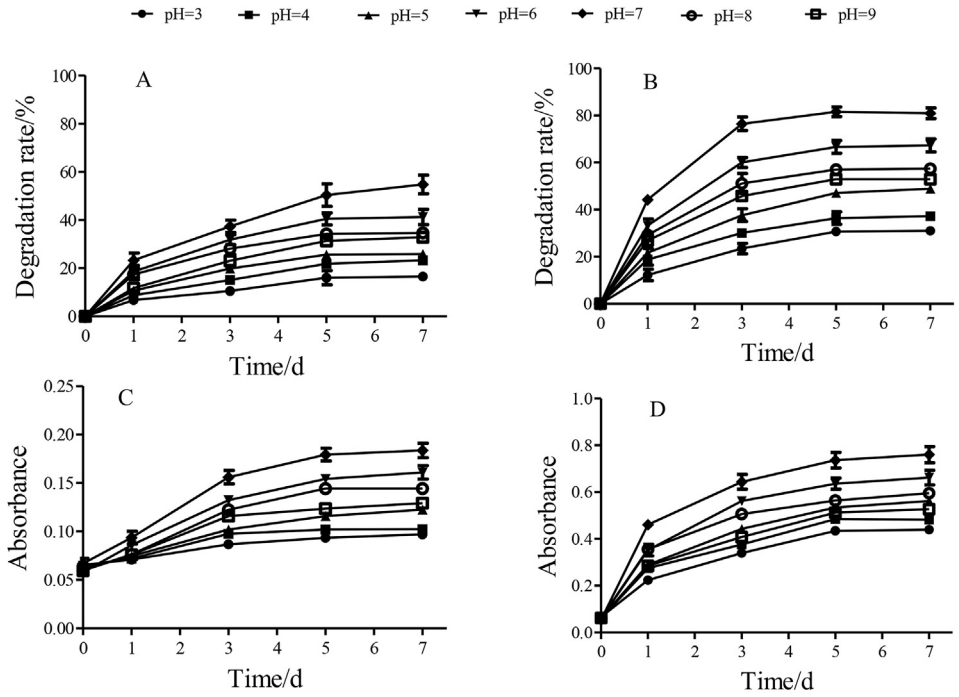


Fig. 6. Degradation rates of OTC by T4 at different Ph. (A) $C_0 = 50\ mg\ L^{-1}$, T = 40 °C, and T4-degraded OTC at different pH values. (B) $C_0 = 50\ mg\ L^{-1}$, T = 40 °C, T4-degraded OTC with adding Fe^{3+} at different pH values. (C) and (D) are the absorbance changes of (A) and (B) at 600 nm by UV-VIS spectrophotometer.

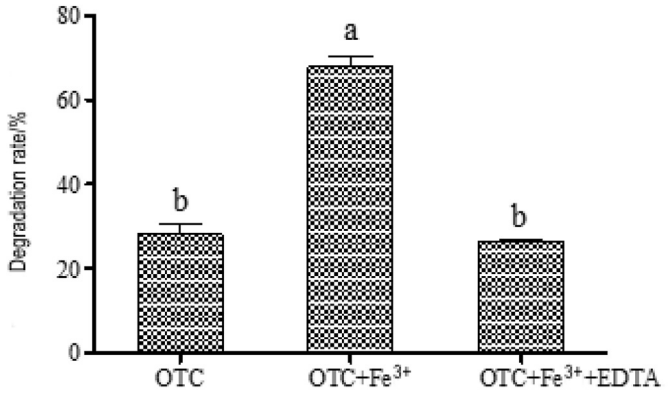


Fig. 7. Effect of EDTA on the biodegradation of OTC. $C_0 = 50$ mg L^{-1}, T = 40 °C, and pH 7, the mean values and standard deviations (error bars) are presented (n = 3). Data bars with the same letter are not significantly different, as determined by Duncan's test ($p < 0.05$). The figure was created by Graph Pad Prism 5.

effectively reduce the occurrence of resistance genes in the environment. Four common tetracycline ARGs such as *tet* (M), *tet* (A), *tet* (O) and *tet* (W), were not detected (Table 1) in this study. This indicates that ARGs could not be introduced into the environment when T4 was used to degrade OTC. It is inspiring that this new finding can provide a new way to degrade the antibiotics in short time, which may decrease the spreading of ARGs in environments due to the reduced antibiotic stress. It has been reported that *tet* (W) and *tet* (O) were present in treated drinking water and recycled wastewater (Pruden et al., 2006). ARGs of high-use tetracycline antibiotics had significantly higher detected resistance gene levels than those of mixed-use and no-use antibiotics in lagoons. Although *tet* (M) was the most commonly detected gene, both in absolute number and after normalization 16S-rRNA, *tet* (O), *tet* (Q) and *tet* (W) levels were also high in the mixed and high-use lagoons (Peak et al., 2007). Some ARGs such as *tet* (M), *tet* (O), *tet* (Q), *tet* (W), *tet* (C), *tet* (H) and *tet* (Z), were detected in groundwater near pig farms over three years (Koike et al., 2007). These results suggest that the potential pathways for the spread of ARGs are widely variable. Therefore, it is vital to find efficient and rapid degradation methods for humans to avoid the spread of ARGs. In dual graphene modified bioelectrode microbial fuel cell (O-D-GM-BE MFC), OTC could be quickly degraded by the group of microorganisms including *Moheibacter, Comamonas, Pseudomonas, Dechloromonas, Nitrospira, Methylomicrobium, Pseudorhodoferax, Thiobacillus, Mycobacterium,* but the coding resistance genes of efflux pump, ribosome protective protein and modifying or passivating were all found in O-GM-BE (Chen et al., 2019a). In the present study, OTC is rapidly degraded without the application of electrical energy with the merit of the four common resistance genes, *tet* (M), *tet* (A), *tet* (O) and *tet* (W) being not detected. This implies that the new way to eliminate the spreading of the ARGs in the environment may be developed.

Table 1
Detection of ARGs in this experiment on day 7 after application.

Treatment	Time (d)	*tet* (A)	*tet* (M)	*tet* (O)	*tet* (W)
T4	1	undetected	undetected	undetected	undetected
T4+Fe^{3+}	1	undetected	undetected	undetected	undetected
T4	3	undetected	undetected	undetected	undetected
T4+Fe^{3+}	3	undetected	undetected	undetected	undetected
T4	5	undetected	undetected	undetected	undetected
T4+Fe^{3+}	5	undetected	undetected	undetected	undetected
T4	7	undetected	undetected	undetected	undetected
T4+Fe^{3+}	7	undetected	undetected	undetected	undetected

3.4. The degradation products of OTC by T4 with the presence of Fe^{3+}

OTC degradation processes mainly include demethylation, dehydration, decarbonylation, deamination hydroxylation and enol-ketone isomerization. The first degradation pathway is the hydrolysis of OTC. Because the microbial degradation of OTC is completed with a hydration system, the hydrolysis reaction of OTC will inevitably occur when the bacteria acts on OTC. The second degradation pathway is biodegradation, which is mainly caused by degrading enzymes in T4 bacteria. The degradation products analyzed by UPLC/Q-TOT/MS predicted that there were three proposed hydrolysis pathways (Fig. S1) and four biodegradation pathways (Fig. S2). The relative abundance of products was used as a reference because the byproducts lacked standard products and could not be quantitatively analyzed (Liu et al., 2016c). As shown in Fig. S3 (A) and (B), mass spectrometry was used to further identify prominent byproducts to derive their evolutionary pathways.

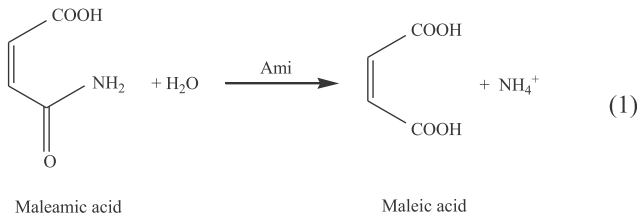
Dehydration mainly occurred on the B and C rings. The mass-to-charge ratios of these rings differed by 18, due to the parent compound at m/z 461 to m/z 443. On the one hand, the methyl of the link on C6 is an electronic group, which makes the hydrogen on C5a and the hydroxyl sites of C6 prone to eliminate the reaction and lose a water molecule, thus becoming a stable ring structure (Hasan et al., 1985; Liu et al., 2016c). On the other hand, there is dehydration in the thermal pyrolysis of enolic acetylacetone (Choudhury and Lin, 1990). Heat and light both provide energy that causes degradation reactions to occur (Liu et al., 2016b). In the presence of microorganisms, the microorganisms may also provide energy or specific enzymes to generate dehydration products. Cazes et al. (2014) discovered that immobilized laccase synergistic redox agents can produce dehydrated products of OTC.

Decarbonylation occurs in the ring A. It has been reported that OTC undergoes α cleavage at C1—C12a to become an intermediate in the form of a double radical. Due to instability, the product subsequently loses the carbonyl to form another double radical (Liu et al., 2016c). In terms of energy, the energy of C1—C12a (sp^3 hybrid) is lower than that of C1—C2 (sp^2 hybrid), and cracking of C1—C12a produces double free radical intermediates by CO removal and a closed loop operation. Further induction by hydroxylase causes the hydroxylation of C2 to form m/z 451.

Demethylation occurs in the part of dimethylamine at C4 position, from m/z 461 to m/z 447and m/z 433. The secondary mass spectrometry details of m/z 437 and 443 are shown in Fig. S3 (A) and (B), respectively. Ultraviolet irradiation or hydroxyl radicals have been reported to promote demethylation (Fang et al., 2011; Khan et al., 2014). With the action of microorganisms, it is possible to induce hydroxylase to cause demethylation. Similar report had shown that tetracycline also occurred demethylation in the process of laccase degradation (Llorca et al., 2015).

The microbial degradation of OTC mainly involves deamination, hydroxylation and enol-ketone isomerization. The isomerization of enol-ketone is due to the higher activity and instability of enol-type structures, which are easy to convert into more stable ketone-type structures.

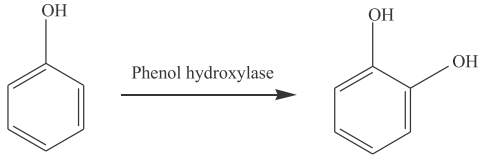
Deamination occurs at the position of the amide group attached to C2. Deamination causes the C2 position to connect to the amide group. It loses its amino group and becomes a carboxyl group and then react with the hydroxyl group is connected to the C3 position in the process of esterification to produce a derivative of m/z 443. Maleamate amidase Ami plays an important role in the microbial degradation of nicotine by fetor *Pseudomonas aeruginosa*, and its role is to degrade maleic acid into maleic acid and ammonia, as shown in Eq. (1) (Wu et al., 2014).



(1)

Phenol hydroxylase can be identified in the *Pseudomonas* gene organization, causing catalytic reactions that hydroxylate some organic substances (Wang et al., 2018b). Bacterial multicomponent monooxygenases (BMMs), a type of hybrid hydroxylase, can hydroxylate a variety of alkanes, olefins and aromatic compounds with high regional stereoselectivity (Sazinsky et al., 2004). These two types of enzymes play important roles in the degradation of aromatic hydrocarbons (Arenghi et al., 2001; Cafaro et al., 2004). There were similar reports that *Pseudomonas* has the function of hydroxylation (Koshimura et al., 2010; Tamm and Gubler, 1959).

The parent molecule generates m/z 433 by decarbonylation, and then the hydroxylation of C2 by hydroxylase forms m/z 450. The o-hydroxylation of C10 obtained m/z 467 under the action of phenolic hydroxylase. Phenol hydroxylase can hydroxylate the ortho position of phenol. It was reported that the phenol hydroxylase of *Pseudomonas stutzeri OX1* was able to hydroxylate ortho-hydroxylate phenol and cresol isomers of the corresponding catechols. The reaction formula is shown in Eq. (2) (Fang et al., 2011). The structure of the C12a enol was transformed into a more stable ketone structure. C11a was hydroxylated to form m/z 483 under the action of hydroxylase.



(2)

C6 was dehydrated to produce m/z 449 dehydrated products, and m/z 465 was generated under the action of hydroxylase. C-11a is easily hydroxylated because the structure of diketone is unstable, which is beneficial for generating m/z 465 and m/z 481 under the action of hydroxyl enzymes. *Pseudomonas* can secrete hydrogenase, which may generate m/z 463 and m/z 437 under the action of hydrogenase. In addition, m/z 435 is formed by decarbonylation from m/z 463. Hydrogenase is a type of metal enzyme in microorganisms. The main function of hydrogenase is the reversible catalytic reaction of $H_2 = 2H^+ + 2e^-$, which is closely related to the energy metabolism of microorganisms.

3.5. OTC degradation by T4 in natural water matrices

As shown in Fig. 8, the percentage degradation of the first three groups in 3 days exceeds 45.0%. The percentage degradation of the last three groups, which exceeds 88.0%, was greater than that of the first three groups. As described in 3.1 above, OTC is a carbon source that supports bacterial growth. It is known that the propensity of bacterial cells for attachment is related to nutrient availability and that cell starvation often leads to increased adhesiveness (Marshall et al., 2000). In the different water matrices, OTC was adsorbed on the surface of T4 bacteria and then being biodegraded. T4 bacteria secreted iron carriers, which increased the OTC degradation rate with the addition of Fe^{3+}. Fe^{3+} is the most important factor to affect the degradation of OTC by T4. Fe_2O_3 can be added to control the Fe^{3+} concentration in the water environment polluted by OTC to

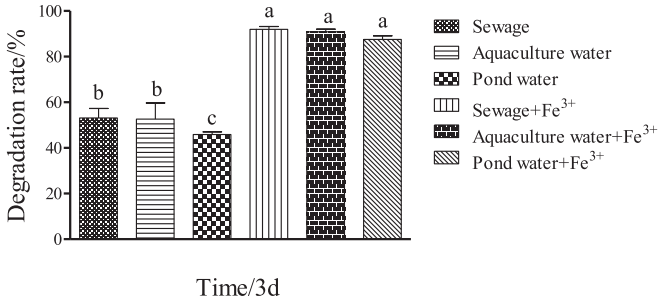


Fig. 8. OTC degradation by T4 in different water matrices. The mean values and standard deviations (error bars) are presented (n = 3). Data bars with the same letter are not significantly different, as determined by Duncan's test ($p < 0.05$). The figure was created by Graph Pad Prism 5.

enhance the degradation of OTC by T4 bacteria. These results imply that this method was suitable for the removal of OTC pollutants with a high concentration in the waters of this experiment. The benefits of using *Pseudomonas* in degrading OTC include its high efficiency and low cost, and no production and accumulation *tet* (A), *tet* (M), *tet* (O) and *tet* (W) in the environment. This new finding can also dispel the doubt that antibiotic biodegradable bacteria will cause the spreading of some ARGs in environments when they are applied to degrade antibiotics. In addition, the degradation rate of OTC by T4 is higher than that reported by (Shao et al., 2018) with *Ochrobactrum* sp. Compared with the results of Sun et al. (2019), we only gave the facts that OTC can be quickly degraded by T4, but did not conversed the biological energy to electric energy. We think this gives us inspiration to make our efforts to realize the conversion of biological energy to electric energy in the future research.

4. Conclusions

This study systematically investigated the degradation mechanisms and influencing factors of OTC by T4. Our results demonstrated that Fe^{3+} had a significant positive effect on the biodegradation of OTC by T4, with the greatest degradation rate at 50 mg L^{-1} of OTC in this experiment. Temperature and pH were important factors influencing the biodegradation of OTC. Increasing the temperature can not only promote the growth of T4 and its activity, but also facilitate the degradation of OTC. The increase in temperature helps T4 secrete iron carriers to promote the degradation of OTC. In addition, no resistance genes were detected during the microbial degradation of OTC. There were six different reaction types and seven possible degradation pathways, including enol-ketone conversion, hydroxylation, dehydration, deamination, demethylation and decarboxylation. The degradation rates of OTC in different water matrices including sewage, aquaculture water and pond water, were found all exceeding 88.0%. This study indicates that T4 is highly effective for removing OTC from contaminated water in the presence of Fe^{3+}, which provides valuable information on the removal of OTC in various aquatic environments.

5. Declaration of interest statement

- The authors declared that they have no conflicts of interest to this work.
- We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted

Conflicts of interest

The authors declared that they have no conflicts of interest to

this work.

Acknowledgments

This work was financially supported by the National Key Technology R&D Program of China (No. 2018YFD0500206), the National Natural Science Foundation of China (31772395), and the scientific base research program of Guizhou province (2019 -1238). The author is grateful for the constructive comments of the editor and reviewers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.05.058.

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