Ozonation of oxytetracycline and toxicological assessment of its oxidation by-products

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Abstract

Antibiotic formulation effluents are well known for their difficult elimination by traditional bio-treatment methods and their important contribution to environmental pollution due to its fluctuating and recalcitrant nature. In the present study the effect of ozonation on the degradation of oxytetracycline (OTC) aqueous solution (100 mg l⁻¹) at different pH values (3, 7 and 11) was investigated. Ozone (11 mg l⁻¹ corresponds the concentration of ozone in gas phase) was chosen considering its rapid reaction and decomposition rate. The concentration of oxytetracycline, chemical oxygen demand (COD), biochemical oxygen demand (BOD) and BOD₅/COD ratio were the parameters to evaluate the efficiency of the ozonation process. In addition, the toxic potential of the OTC degradation was investigated by the bioluminescence test using the LUMISTox 300 instrument and results were expressed as the percentage inhibition of the luminescence of the marine bacteria *Vibrio fischeri*. The results demonstrate that ozonation as a partial step of a combined treatment concept is a potential technique for biodegradability enhancement of effluents from pharmaceutical industries containing high concentration of oxytetracycline provided that the appropriate ozonation period is selected. At pH 11 and after 60 min of ozonation of oxytetracycline aqueous solutions (100 and 200 mg l⁻¹) the BOD₅/COD ratios were 0.69 and 0.52, respectively. It was also shown that COD removal rates increase with increasing pH as a consequence of enhanced ozone decomposition rates at elevated pH values. The results of bioluminescence data indicate that first by-products after partial ozonation (5–30 min) of OTC were more toxic than the parent compound.

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1. Introduction

Oxytetracycline (OTC), a member of tetracyclines, is one of the widely used pharmaceuticals in today's human and veterinary medicine (Zhu et al., 2001; Kim et al., 2005; Sar-mah et al., 2006). Residues of antibiotics have been detected in surface water resources that receive effluents of municipal wastewater treatment plants (WWTPs), agricultural runoff and discharges of pharmaceutical manufacturers (Kolpin

et al., 2002, 2004; Miao et al., 2004; Simon, 2005). The presence of low levels of antibiotics and their transformation products in the environment is suspected to provide conditions for the transfer and spread of antibiotic resistance bacteria and it clearly shows that elimination in municipal sewage treatment plants is often incomplete (Boxall et al., 2003). The immediate concern is the potential toxicity of these compounds to aquatic organisms and humans through drinking water or the consumption of vegetables and crops irrigated by polluted water. Data on the occurrence of pharmaceuticals as contaminants in wastewater effluents and in the aquatic environment in China is scarce.

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Larsson et al. (2007) report about the abundance of high concentration of pharmaceuticals in the effluent from a wastewater treatment plant serving about 90 drug manufacturers in Patancheru, near Hyderabad, India, a major production site of generic drugs for the world market. Among the top 11 active pharmaceutical ingredients analyzed the concentration of the most abundant drug, ciprofloxacin ranged from 28 to 31 mg l⁻¹, exceeding levels toxic to some bacteria more than 1000-fold. Even the authors state that the investigated samples contained by far the highest level of pharmaceuticals reported in any effluent. There are some cases in China showing the urgent need of appropriate treatment for the wastewater effluents of pharmaceutical industry. Qiting and Xiheng (1988) quoted an average sewage treatment plant effluent concentration of an oxytetracycline production facility in China of about 50 mg l⁻¹, which could be considered as a point discharge affecting a limited area but with a huge potential of contamination when used for irrigation. It is known that many waterways throughout China which serve as the supply for plant treatment are contaminated by industrial effluent discharges without proper treatment. This pollution load makes it difficult to treat the water to levels suitable for drinking, other potable uses or even irrigation. The preliminary results of our recent investigations at one of the biggest OTC producers worldwide located in Hebei Province, China, with an annual OTC output of 10000 tons (2004), show that the effluent of the wastewater treatment plant of the factory still contains extremely high concentrations of OTC (20–800 mg l⁻¹), depending on the performance of the WWTP. It could be shown that the receiving surface water still had an OTC concentration of 0.38–2.0 mg l⁻¹ (personal communication).

Considering the water scarcity in China and the use of sewage and wastewater effluents even from pharmaceutical industries for irrigation of fields in urban and semi-urban areas for growing fruit and vegetables, it is important to enhance the efficiency of the WWTP of pharmaceutical and chemical industries to prevent further pollution. One potential method could be the combination of traditional wastewater treatment (biological) with an advanced oxidation treatment method such as O₃, O₃/UV, UV/H₂O₂, O₃/UV/H₂O₂, Fe(II)/UV/H₂O₂ and O₃/Fe(II)/UV/H₂O₂.

In this study, ozonation, having a high potential for the oxidation of harmful chemicals in drinking and wastewater treatment plant effluents, has been chosen to determine the degradation rate (BOD₅/COD) of high amounts of oxytetracycline (100 and 200 mg l⁻¹) in synthetic aqueous solutions (Koch et al., 2002; Huber et al., 2003; Ternes et al., 2003; Wang et al., 2003). Additionally, the toxic potential of the reaction intermediates and products formed during ozonation have been investigated by the bioluminescence test (*Vibrio fischeri*). This information is of particular importance to highlight whether ozone oxidation is capable to remove antibiotics from aqueous media and to improve the biocompatibility of oxytetracycline containing wastewater effluents.

2. Materials and methods

2.1. Material

Oxytetracycline hydrochloride (CAS No. 2058-46-0) was purchased from Sigma. Main characteristics of oxytetracycline are given in Fig. 1. Sodium hydroxide and hydrochloric acid were obtained from Merck (Darmstadt, Germany). The other reagents used were of analytical grade. All aqueous solutions were prepared with ultra pure water by a Millipore Waters Milli-Q purification water unit (Millipore, Watford, UK).

2.2. Methods

Ozone was generated from dried air by an ozone generator (Erwin Sander Elektroapparatebau, Uelzen, Germany, Model 1992; 24 g h⁻¹). The ozone concentration was determined by an ozone measuring device model Ozon Meßgerät (D 3162). Ozonation was performed in a cylindrical glass reactor (volume 1.4 l) by bubbling the ozone/air mixture through a sintered glass filter (pore size 50–80 µm) fixed at the bottom of the reactor into the solution at a flow rate of 20 l h⁻¹. Samples (20 ml) were withdrawn at defined time intervals and nitrogen gas was used to remove the residual ozone.

The oxytetracycline concentration in all samples were analyzed by an Agilent 1100 series HPLC system (Agilent Technologies, Germany) consisting of a low-pressure degasser, a binary high-pressure pump, an auto sampler with an automated injection system, and a diode array multiple wavelength detector. Samples were analyzed by using a ZORBAX SB-C18 reverse phase liquid chromatography column (4.6 mm ID × 150 mm, packing type: monomeric without endcapped, particle size: 5 µm, pore size: 80 Å, Agilent Technologies) equipped with an ODS, Octadecyl guard column (Phenomenex).

Oxytetracycline (OTC)

Molecular formula: C₂₂H₂₄N₂O₉;

Molecular weight: 460.4 g mol⁻¹

Water solubility >100 mg ml⁻¹ (21 °C)

Fig. 1. The structure and characteristics of oxytetracycline.

Isocratic elution was carried out with aqueous oxalic acid dihydrate (0.01 M) and a mixture of MeOH/acetonitrile (1:1) at a flow rate of 1 ml min^{-1} and 26°C with an injection volume of $20 \mu\text{l}$. The acquisition wavelengths were 355 nm. Sample pH was measured with WTW-pH-Meter Multiline P4 (Germany). Chemical oxygen demand (COD) was determined with commercially available test kits (Macherey&Nagel, Düren, Germany). Biodegradability test (BOD_5) of the samples based on the standard method of OECD (1992) 301D (closed bottle test) was conducted before and after ozonation of the aqueous oxytetracycline solutions. Secondary effluent of the municipal wastewater treatment plant of Munich City, Germany was used as inoculum for the BOD_5 measurements.

For the assessment of toxicity of the parent compound and its oxidation by-products the standardized bioluminescence assay with *V. fischeri* was applied to the samples before and after the ozone treatment at different time intervals. Relatively high oxytetracycline concentrations were used (100 and 200 mg l^{-1}) considering the fact that point discharges with high amount of pharmaceuticals may still occur in developing countries due to the lack of proper wastewater treatment plants. All tests were run as duplicates. The test utilizes the inhibition of the bioluminescence of the marine photobacteria, *V. fischeri*, as an indication of acute toxicity (Froehner et al., 2000; Jennings et al., 2001). All tests were conducted according to the German standard method DIN 38412; L34; L341, Germany and ISO DIS 11348 on a Dr. LANGE LUMISTox 300 photometer (Düsseldorf, Germany). The marine photobacteria were added to different dilutions of a sample and all vials were allowed to stabilize at 15°C for 15 min. After 30 min incubation luminescence of the solutions was measured. In this study, the results are expressed as the percentage inhibition of luminescence in the test solutions relative to a control solution. The higher the inhibition, the more toxic is the sample. Luminescence bacteria and all reagents required were also obtained commercially by Dr. LANGE. Care has been taken to conduct the experiments under dim light conditions because it is known that oxytetracycline is sensitive to light exposure (Doi and Stoskopf, 2000).

3. Results and discussion

3.1. Effect of pH

Since pH is one of the important factors for many processes and can affect ozonation pathways, this parameter was selected as the main process variable. Ozonation experiments were performed for one hour at three different initial pH values (3, 7 and 11) and samples were adjusted with sodium hydroxide and hydrochloric acid. In general, the rate of ozone decomposition increases with increasing solution pH since the hydroxyl ions catalyse the decay of ozone to form radicals serving as reactive species. In Fig. 2a the decrease of oxytetracycline concentration at varying initial pH values as a function of ozonation time in an aqueous OTC solution without buffer is given. At pH 3 the decomposition of OTC is slower than under neutral and basic conditions most probably due to the fact that only the molecular ozone is reacting directly with the organic molecule since the formation of hydroxyl radicals are suppressed. Surprisingly, the loss and degradation rate of OTC at pH 7 is fastest. Almost 90% OTC decomposition appears after 5 min of ozonation, most probably as a result of a higher $\text{HO}^\bullet$ radical attack than by ozone molecules. In general, it is expected and also confirmed by numerous authors (Esplugas et al., 2002; Vogna et al., 2004; Dantas et al., 2007) that the removal of concerned chemicals in aquatic environment is fastest at pH value higher than 7, because both the ozone molecule and hydroxyl radicals are the oxidizing agents. At pH 11 the OTC decomposition is slower than at pH 7. This result could be explained by the fact that the generated degradation products and the parent compound (OTC) itself are attacked at the same time, both becoming important scavengers of hydroxyl radicals. Akme Mehmet Balcioglu and Ötör (2003) report the similar phenomena for so called “human antibiotic I” when they investigated the effect of different pH values (3, 7 and 11) on the overall COD and the aromaticity removal rates (UV_{254}) of a synthetic wastewater. Authors describe that after one hour ozonation the decomposition rate was highest at pH 7.

Fig. 2. (a) Effect of pH (3, 7, 11) on the oxidation of oxytetracycline and (b) COD removal during ozonation ($C_{\text{OTC}} = 100 \text{ mg l}^{-1}$; $\text{CO}_3 = 11 \text{ mg l}^{-1}$; 25°C).

In Fig. 2b the COD degradation rates are illustrated. The COD removal rates increase with increasing pH as a consequence of enhanced ozone decomposition rates at elevated pH values. This is because at high pH values the solution can adsorb more ozone (Fig. 3). Ozone decomposition increases with increasing solution pH since the hydroxyl ions catalyse the decay of ozone to form radicals which are powerful and non-selective oxidants. The decomposition of ozone yielding free radicals could also be confirmed by simple comparison of the absorbed ozone rates: only 30% of the applied ozone dose is absorbed at pH 3, whereas this rate increases to 58% and 79% at pH 7 and pH 11, respectively.

Fig. 4 shows the alteration of pH value during ozonation process. As it can be seen the initial pH of 7 and 11 decreases gradually most probably as a result of carboxylic acid accumulation in the process water. Under acidic conditions the ozonation does not measurably affect the solution pH.

In general, the decrease of pH may considerably affect the oxidation rate, reaction mechanism and ozone absorption rate during ozonation. For that reason, additional experiments were performed where the pH of the solution was adjusted with different kind of buffers ($\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ for pH 3; $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ for pH 7 and $\text{Na}_2\text{HPO}_4/\text{Na}_3\text{PO}_4$ for pH 11). The oxytetracycline concen-

tration of the aqueous solution was 100 mg l^{-1} , ozonation time was 60 min. Phosphates are known to react with $\text{OH}^\cdot$ radicals at relatively slow rates ($k_{\text{OH}^\cdot}$ of $\text{PO}_4^{3-} = 2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) compared with other inorganic salts (Arslan-Alaton and Dogruel, 2004). Hence, they have been selected as the most suitable pH buffering agents. As expected, the pH of the buffered reaction solutions remained stable (maximum changes in pH <0.2 units) while the phosphates react with $\text{OH}^\cdot$ radicals at relatively slow rates. It can be concluded that the present of buffer does not influence the OTC degradation significantly. COD removal rates also did not slow down but increased appreciably as compared to the non-buffered effluent samples.

3.2. Changes in toxicity

To obtain detailed information in which way the parent compound and its degradation by-products would affect the aquatic microbial activity, the standardized bioluminescence assay with *V. fischeri* was applied to the samples (100 and 200 mg l^{-1} oxytetracycline) before and after the ozone treatment at different time intervals. This information is of particular importance in order to highlight if, during the treatment, the transformation of OTC results in more toxic species or the toxic effect of the parent compound lessens. As shown in Table 1, the inhibitory effect on the bioluminescence of the bacteria is decreasing as a function of ozonation time and pH, caused most probably due to the destruction of oxytetracycline and its degradation by-products. At an oxytetracycline concentration of 100 mg l^{-1} , the inhibition of the bioluminescence is fastest at pH 11 after 30 min of ozonation and remains at the same level (20%) until the end of the treatment. The inhibitory effect occurs to be much lower under neutral and acidic conditions.

In Fig. 5a the results of bioluminescence tests are presented after 120 min of ozonation of relatively high OTC concentration (200 mg l^{-1}). As it can be seen, the inhibitory effect on the bioluminescence of the bacteria is increasing from 68% to 99.5% as a function of ozonation time for ozonation periods of 15–60 min. A drastic decrease in toxicity from 99.5% to 32% with prolonged ozonation time of 120 min reflects the disappearance of toxic compounds. The increase of the inhibition demonstrates a considerable high toxic effect of the solution for ozonation periods of 15–60 min. This is most likely due to the formation of first by-products with a high toxic potential which seems to be

Fig. 3. Influence of pH (3, 7 and 11) on the ozone consumption. RO_3 = residual ozone.

Fig. 4. Alteration of pH value during ozonation process ($C_{\text{OTC}} = 100 \text{ mg l}^{-1}$; $C_{\text{O}_3} = 11 \text{ mg l}^{-1}$; 25°C).

Table 1

Effect of ozonation time and pH values on the BOD_5/COD ratio and the percentage inhibition of the bioluminescence of marine photobacteria *Vibrio fischeri* ($C_{\text{OTC}} = 100 \text{ mg l}^{-1}$; $C_{\text{O}_3} = 11 \text{ mg l}^{-1}$; 25°C)

Ozonation time (min)	BOD_5/COD			Inhibition (%)		
	pH 3	pH 7	pH 11	pH 3	pH 7	pH 11
0	0	0	0	68	67	68
30	0.33	0.39	0.45	45	51	20
60	0.57	0.61	0.69	26	25	18

Fig. 5. (a) Effect of ozonation time and pH values on the percentage inhibition of the marine photobacteria *Vibrio fischeri* and (b) the BOD₅/COD ratio ($C_{OTC} = 200 \text{ mg l}^{-1}$; $C_{O_3} = 11 \text{ mg l}^{-1}$; 25°C).

destroyed by further ozonation. The results of the assay confirm the assumption that partial oxidative treatment of aqueous oxytetracycline solutions yields toxic intermediates with harmful effects on organisms.

Glaze (1986) has noted that the classes of organic by-products that might be formed during ozonation are of special interest in water treatment because of their potential health effects. These ozonation products are suspected to contribute to the principal toxicity of the intermediates. Halling-Sorensen et al. (2002) reported that the degradation products of OTC such as EOTC, α -Aop-OTC and β -Aop-OTC exhibit a higher toxicity (EC_{50}) than the parent compound. In the present study, none of the previously described degradation products, such as the epi- and anhydro-compounds, could be identified by HPLC as degradation products. Identification of ozone by-products and the toxic intermediates of OTC by HPLC–MS–MS is a subject of further studies.

3.3. Changes in biodegradability

Ozonation may enhance the biocompatibility of industrial wastewater significantly (Alvares et al., 2001). In the present study the changes of the BOD₅/COD ratio have been measured in ozonated samples to evaluate their biodegradability. BOD₅ was determined in accordance with the test procedure described in OECD (1992) method 301D and a mixed bacterial culture adapted to municipal wastewater was used as inoculum. The BOD₅ of 100 and 200 mg l^{-1} OTC solutions was zero, indicating that oxytetracycline is not readily biodegradable. It has been generally accepted that a BOD₅/COD ratio higher than 0.3 represents a “readily biodegradable” waste water effluent (Ledakowicz and Gonera, 1999; Benitez et al., 2001). For an OTC concentration of 100 mg l^{-1} , as shown in Table 1, the BOD₅/COD ratio increases from 0 to 0.33, 0.39 and to 0.45 after 30 min of ozonation depending on the solution’s pH (3, 7, and 11). The BOD₅/COD ratio is lowered when the drug concentration increases (200 mg l^{-1}), emphasizing the toxicity of the parent compound as given in Fig. 5b. Dantas et al. (2007) report an enhancement of the biodegradability (BOD₅/COD) from 0 to 0.28 after

60 min ozonation of sulfamethoxazole (200 mg l^{-1}), whereas a short ozonation period seems to induce an increase of toxic effects due to the production of toxic intermediates.

Considering these results, it is obvious that ozonation can be recommended as a partial oxidation step of a combined treatment (ozonation/biological) concept in which ozone can be employed as a pretreatment method to reduce refractory substances since ozone is able to transform parts of the organic fraction into biodegradable matter. It should be emphasized that the ozonation time and the ozone concentration should be kept as low as possible in order to reduce the wastewater treatment costs and make the ozonation suitable for industrial application.

4. Conclusions

The results of COD, BOD, BOD₅/COD ratio and the bioluminescence test after ozonation of aqueous oxytetracycline solutions demonstrate the potential of the ozone treatment for reduction measures of highly loaded pharmaceutical wastewater. Ozonation, as an integrative part of an oxidative–biological treatment system in order to remove recalcitrant substances from pharmaceutical wastewater, can be recommended for industrial application. Considering the economic and ecological benefits of the suggested method ozonation should convert rather than eliminate recalcitrant substrates for subsequent mineralization by the cheaper biological process.

The results of the present study have clearly shown that ozonation at three different pH values provides a promising technique for the treatment of wastewater containing antibiotic agents. Results revealed that pH control was essential to obtain efficient COD and oxytetracycline removal. Before the industrial application it is recommended to evaluate the required ozonation time resulting in destruction not only of the antibiotics but also of the toxic primary by-products.

Therefore, a combined treatment involving oxidative and biological methods of either the total antibiotic effluents or pre-selected process lines suitable for re-use purposes containing less antibiotic and COD, should be

employed to achieve the best economic and ecological result and to ensure the acceptance of the methods by the industry.

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