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Characterization of microbial degradation of oxytetracycline in river water and sediment using reversed phase high performance liquid chromatography

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The fate of oxytetracycline (OTC) in river water and sediment and control experiments was investigated. A high-performance liquid chromatography (HPLC) separation and identification method was used to separate, identify and quantify OTC and its major degradation products. Minimum degradation of OTC was observed in control experiments. Non microbial degradation observed up to day 26 contributed slightly above 20% of the degradation in exposed distilled water experiment. Increase in degradation of OTC after day 26 for both the covered and exposed distilled water experiments were attributed to microbial degradation due to contamination through the openings left in the set ups. Microbial degradation was observed in the river water and sediment experiment and two major degradation products were identified: 4-epi-oxytetracycline (4-epi-OTC) and β-apo-oxytetracycline (β-apo-OTC). β-apo-OTC was found to be the most stable degradation product as compared to the other main degradation products 4-epi-oxytetracycline (4-epi-OTC) and α-apo-oxytetracycline, (α-apo-OTC). The present results have shown that microbial degradation plays a major role in the removal of OTC in natural environments.

Key words: Oxytetracycline, microbial degradation, rephrased phase HPLC, river water and sediment.

INTRODUCTION

Contamination of aquatic environment with antibacterial agents has been a subject of discussion by many authors (Winckler and Grafe, 2001; Zhou et al., 2011, 2013). Tetracycline antibacterials are the widely applied antibiotics worldwide. More than 2500 tonnes are used annually in Europe and 21000 tonnes in China (Zhou et al., 2013) with oxytetracycline being the most used antibiotic. In Africa, tetracycline antibacterials are the widely applied antibiotics because they can be used to treat diseases caused by both Gram positive and Gram negative bacteria. They are also cheap. Oxytetracycline (OTC) has been detected widely in surface waters and soil with concentration reaching up to 2 mg L^{-1} (Ooishi and Tosa, 2010). Recently, Zhou et al. (2013) and Yang et al. (2011)

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monitored different types of environmental samples and detected frequently tetracyclines than any other antibiotics. Oxytetracycline has been reported to degrade abiotically in solution therefore many studies that have been done so far concentrated on photodegradation and hydrolysis of oxytetracycline in buffered and humic acid added distilled water or filtered surface waters (Xuan et al., 2010; Harring-SØrensen et al., 2003). Photodegradation products either in distilled water or filtered environmental waters that have been identified include, 4-epi oxytetracycline (4-epi-OTC), α-apo-oxytetracycline, (α-apo-OTC), β-apo-oxytetracycline (β-apo-OTC) (Xuan et al., 2010; Kuhne et al., 2001) and in addition 4-epi-N-desmethyl-OTC, N-desmethyl-OTC, N-didesmethyl-OTC and 4-epi- N-didesmethyl-OTC (Halling-SØrensen et al., 2003). Insights emanating from these studies are now being applied to treat drinking water of pharmaceuticals. Effectiveness of UV, ozone and other advanced oxidation techniques to degrade tetracyclines have been reported in literature (Wu and Chen, 2010; Chung et al., 2009). These techniques have some limitations because they can only be applied to treat clear water and they are expensive. If effluents from farms, hospitals and municipal sewage works are to be rendered free of pharmaceuticals more cost effective strategies still need to be sort. Pharmaceuticals have been assumed not to degrade microbiologically because they are bacteriostatic (Halling-SØrensen et al., 2003). Recent development has shown that micro-organisms in the environment have the potential to degrade tetracyclines and many other antibiotic types (Maki et al., 2006). A review of literature show limited information on the microbiological degradation of tetracyclines in the aquatic environment where photolysis is expected to be hampered by presence of radiation attenuating materials (Xuan et al., 2010). Therefore this study was aimed at studying microbial degradation of oxytetracycline in river water and sediment. Microcosm experiments were setup so as to resemble the real aquatic environment. High performance liquid chromatography has been the widely chosen method for analysis of environmental samples (Halling-SØrensen et al., 2003; Xuan et al., 2010). This is because there is a need to separate the parent compound from its degradation products. A previously reported high-performance liquid chromatography (HPLC) method (Xuan et al., 2010; Chinese pharmacopeia, 2005) was modified and used to separate oxytetracycline and its degradation products from river water and sediment.

MATERIALS AND METHODS

Chemicals

Oxytetracycline hydrochloride M_w, 496.9 g (OTC), 4-epi-oxytetracycline M_w, 460, 4 g (4-apo-OTC) α-apo-oxytetracycline M_w, 442.4 g (α-apo-OTC), β-apo-oxytetracycline M_w, 442.4 g (β-apo-OTC) were purchased from Sigma Aldrich, Darmstadt, Germany. All were of 95-98% purity. Methanol (HPLC grade), primary and secondary amine sorbent material (57738-U-SUPELCO supelclean PSA), acetonitrile (HPLC grade) and nylon disposable filter units (MILLPORE 0.45 µm) were also obtained from Sigma Aldrich. Oxalic acid, orthophosphoric acid, nitric acid, sodium hydrogen phosphate, citric acid and disodium ethylenediamine tetraacetate (Na_2EDTA) were of analytical grade and were obtained from SKYLABS, Gauteng, South Africa. River water (80 L) and sediment (2 kg) was collected from Wayerera River, Bindura, Zimbabwe (19° 19' 52'' South, 42° 21' 52'' East).

High-performance liquid chromatography (HPLC) system

OTC and its metabolites were analyzed according to a procedure reported in the Chinese pharmacopeia (2005) and by Xuan et al. (2010). A Varian HPLC UV Prostar 325 equipped with a Rodyne manual injector, a 20 mL loop and a Prostar 325 UV detector was used to analyze OTC and its metabolites. The detector was controlled remotely by the Varian Star/ Galaxie Chromatography Workstation software version 6. All HPLC separations were carried out using C18 (Varian Microsorb MV 1005 packed columns (250 × 4.6 mm id, 5 µm SPELCO). A mixture of methanol, acetonitrile and 0.01 M aqueous oxalic acid in the ratio of 1:1.5:7.5, pH 3.0 offered the best resolution of parent compound and its metabolites Figure 1. The flow rate was maintained at 1.0 $mlmin^{-1}$ in the isocratic mode, at ambient temperature. A sonicator was used to mix and remove air bubbles from the mobile phase prior to HPLC analysis. The detector was set at 360 nm (the absorbance at maximum wavelength was determined using a UV-Vis instrument, GENESYS 10S UV-Vis v4.003 2L9Q129001, Thermofisher). Sample injection volume was 10 µL.

Preparation of standard samples

Stock solutions of OTC, 4-apo-OTC, α-apo-OTC and β-apo-OTC of concentration 1×10^{-3} g mL^{-1} were prepared in methanol and kept in refrigerator in amber bottles. Duplicate calibration curves were made by diluting the stock solutions in the range 0.01-1 µg mL^{-1}.

Microbial degradation experiments

Microcosm experiments were set up following a previous method described by Zaranyika and Nyoni (2013) with some modifications. Volumes of 1 × 80 L of river and 2 × 80 L each of distilled water were added into separate 80 L white plastic tanks (Mega Pak Zimbabwe (Pvt), Harare), and levels marked. Two kilograms of sediment was added in the vessel with river water. Control experiments consisting of distilled water and heat sterilized river water and sediment were also set as follows: one vessel containing distilled water was covered with aluminium foil to prevent light penetration, but making sure that air was not excluded. The other tank with distilled water was left exposed to sunlight. The last tank consisted of heat sterilized river water and sediment. Heat was applied cautiously so that only microbes would be destroyed without destroying humus. The tanks were spiked with standard oxytetracycline dissolved in methanol so as to obtain a final concentration of 1.2 µg mL^{-1}. The contents were then mixed thoroughly. The system was left to settle for 1 h and samples taken immediately thereafter. Containers were covered with perforated transparent polythene and left outside in a safe place near the Bindura University laboratory. Thereafter samples were collected periodically for a period of 90 days, each time compensating for evaporation by adding distilled water 24 h prior to sampling. Sediment samples were collected from the bottom of tanks with minimum agitation using a stainless scoop. The temperature and

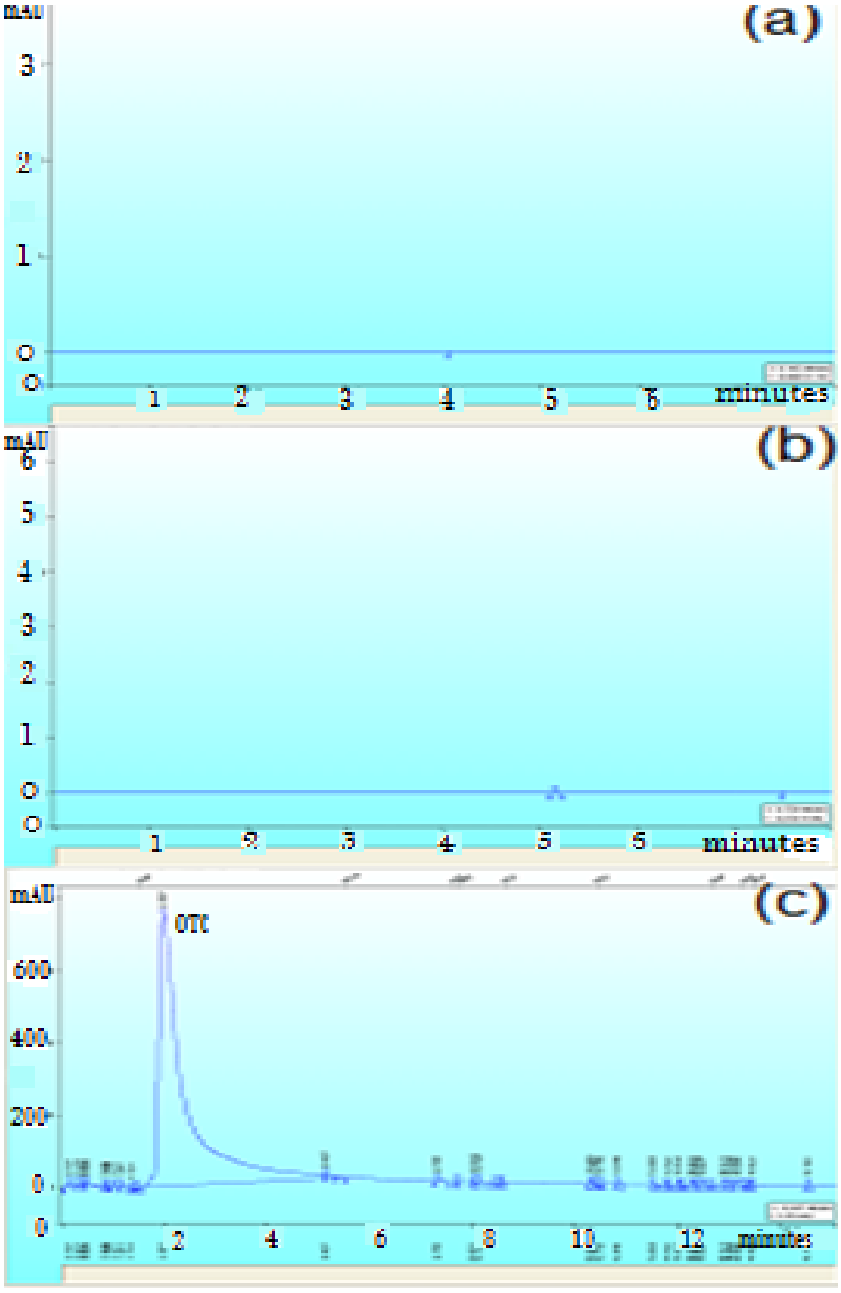

Figure 1. Selected chromatograms for river water, (a) blank river water sample, (b) blank sediment sample, (c) chromatogram for day 34, (d) 64, (e) and (f) 90.

pH of each tank were recorded before each sampling. The following data were obtained: temperature of 27 ± 5°C; pH, 7.2 ± 0.4 (river water); temperature 28 ± 3°C and 5.3 ± 0.8 (distilled water). Once collected, all samples were stored in a freezer in plastic bottles with screw caps until required for analysis.

Sample extraction, clean up and concentration

OTC was extracted from water and sediment samples using ultrasonic assisted dispersive solid phase extraction (UA-DSPE). Dispersive solid phase extraction was previously used to extract pharmaceuticals from food samples (Cruz-Vera et al., 2011).

Water samples

Water samples were analyzed in triplicate. One hundred milliliters of water samples were centrifuged at 3000 rpm. The supernatant was collected and vigorously shaken with 10 mL of acetonitrile in a separating funnel. Five milliliters of 0.1 M Na_2EDTA, and 10 mL of McIlvaine buffer (pH 4) were also added to chelate any metals present. Magnesium sulphate and sodium chloride 0.5 g each were then added to displace the extraction equilibrium towards the

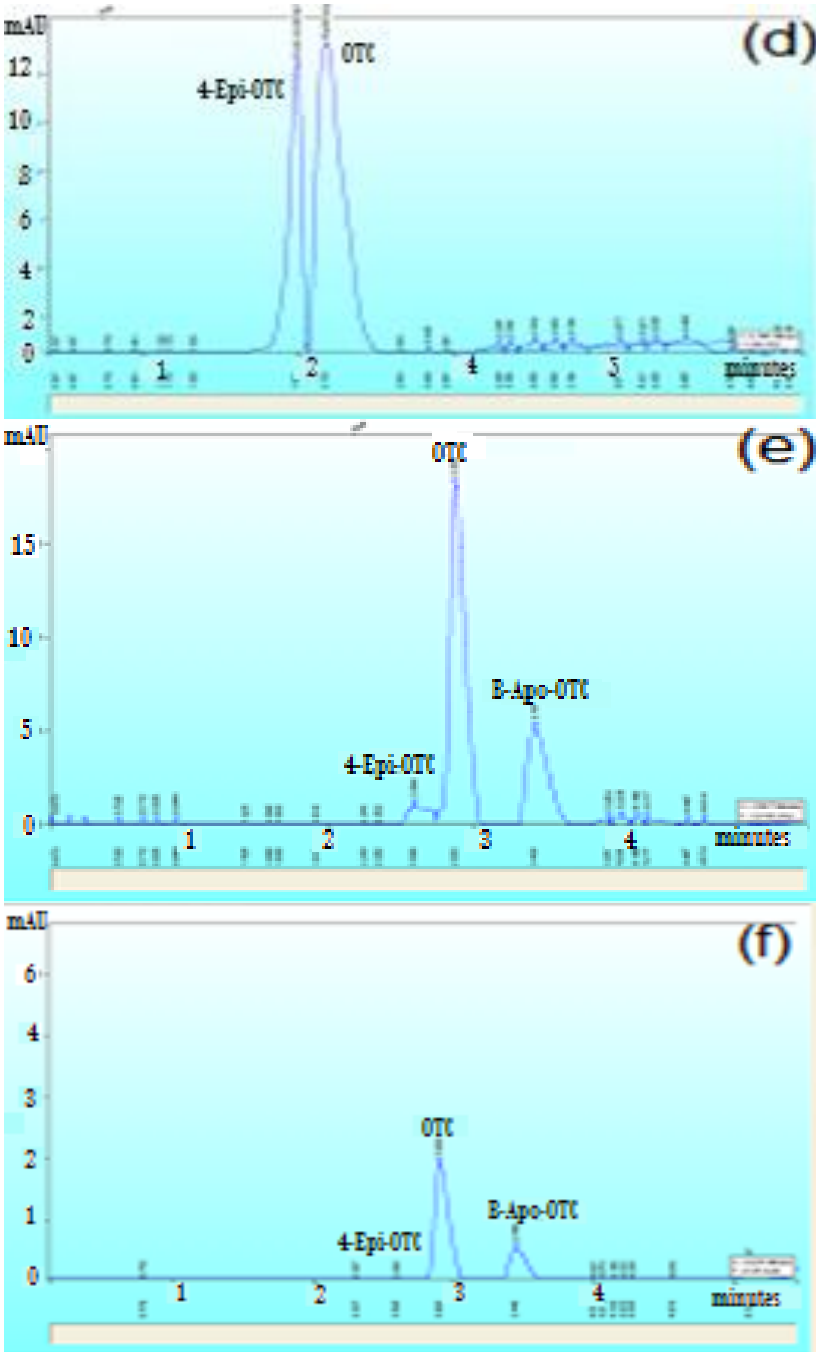

Figure 1. Contd

organic phase. The contents were centrifuged at 3000 rpm for 10 min and the organic supernatants were transferred to a conical flask followed by addition of 40 mg of primary secondary amine sorbent material (57738-U-SUPELCO Supelclean PSA) to remove interferences such as humic acid (Cruz-Vera et al., 2011). The analyte of interest remained in the organic phase. The mixture was ultrasonicated for 15 min and centrifuged at 3000 rpm for 10 min. The supernatants were collected and evaporated to almost dryness under vacuum and then redissolved in 500 µL of methanol. The contents were filtered through a 0.45 µm glass Millipore filters to remove any particulate matter and then placed into amber vials and stored in a fridge until HPLC-UV analysis (Zhou et al., 2011).

Sediment samples

Two grams of sediment samples were extracted after removing excess water by centrifugation. Ten milliliters of McIlvaine buffer (pH 4) were added into each glass tube and mixed for 1 min, then centrifuged at 3000 rpm for 10 min. The supernatants from each

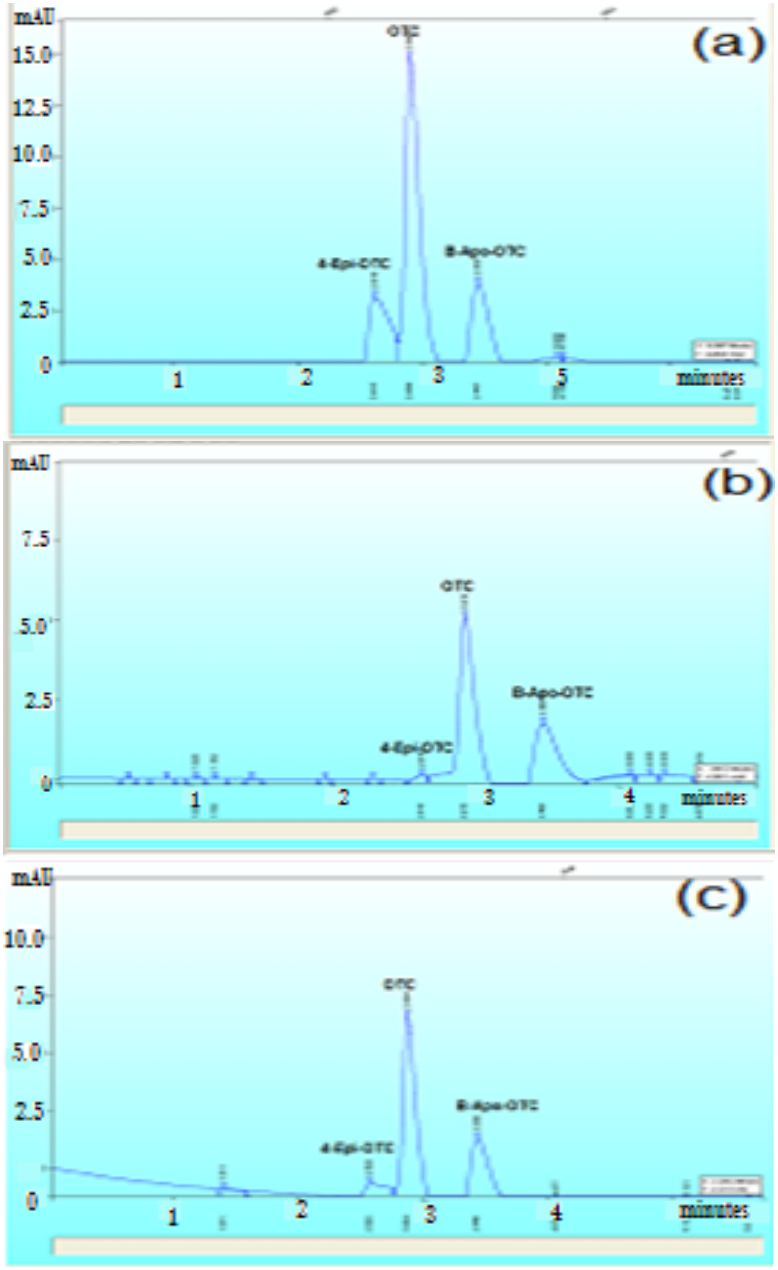

Figure 2. Sediment sample selected chromatograms for days: (a) 26, (b) 34, (c) 64, (d) 72, (e) 90.

tube were placed into 250 mL flasks. The extraction process was repeated twice and the supernatants from the two extractions were combined and diluted to 100 mL with ultrapure water and vigorously shaken with 10 mL of acetonitrile in a separating funnel. Five milliliters of 0.1 M Na_2EDTA, and 10 mL of McIlvaine buffer (pH 4) were also added to chelate any remaining metals, after which the extraction was carried out as described above for water samples.

RESULTS AND DISCUSSION

Degradation products

In the present study degradation products peaks (Figures 1 and 2) t_R(4-apo-OTC) = 2.38 ± 0.34 min and (β-apo-OTC) = 3.26 ± 0.32 min were only visible when the concentration of the parent peak had reduced to 0.06 µg/mL such that they were not visible in the covered and exposed distilled water experiments since the concentration did not drop to such levels. Degradation product α-apo-OTC) was not detected. The reasons might be that it was in very small quantities or it degraded quickly as it was formed. In an almost similar study that was conducted by Halling-SØrensen et al. (2003) α-apo-OTC was found to degrade faster in light than its counterpart epimer β-apo-OTC. Degradation products

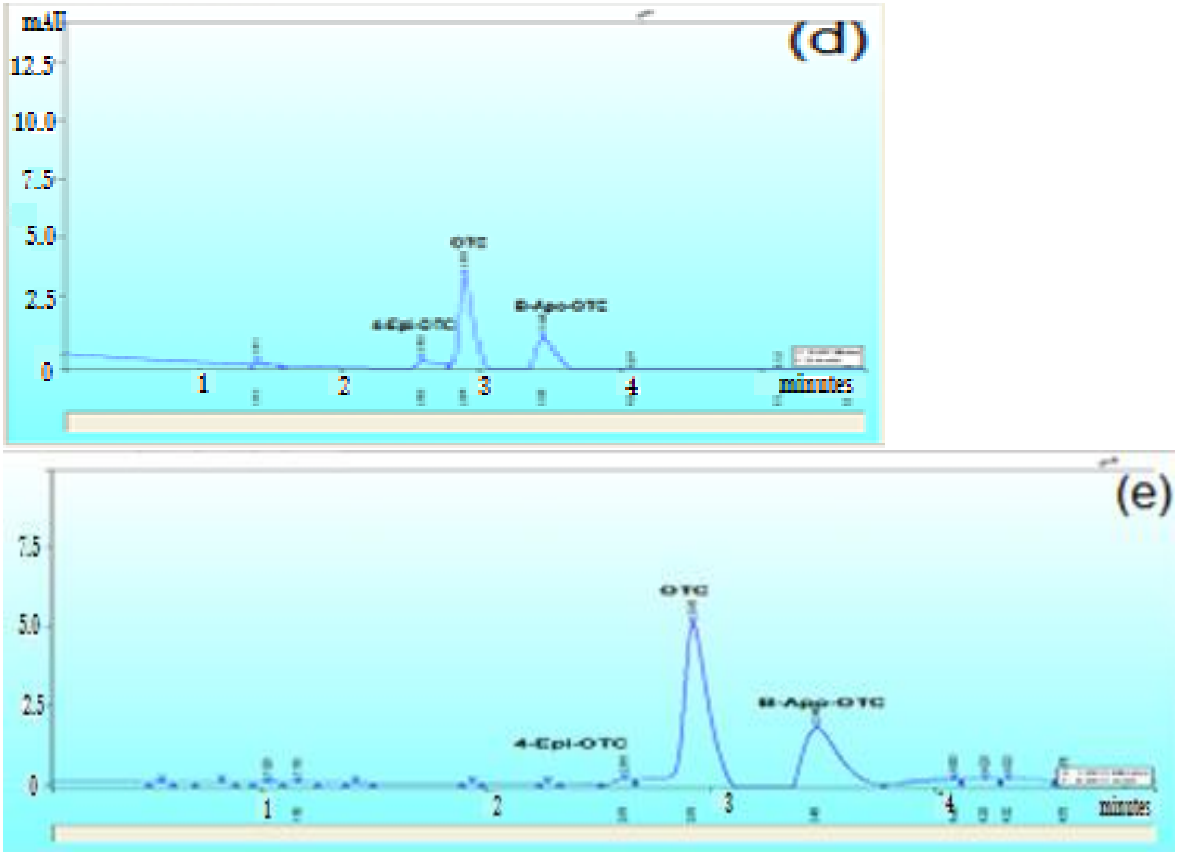

Figure 2. Contd

such as 4-epi-N-desmethyl-OTC, N-desmethyl-OTC, N-didesmethyl-OTC and 4-epi- N-didesmethyl-OTC that were reported in Halling-SØrensen et al. (2003) study was not detected in this study. The major reason might be the different spiking concentrations that were applied. For this study very low concentrations were chosen so as to mirror as close as possible concentrations in the real environment while Halling-SØrensen and coworkers used a higher spiking level that ensured detection of very small fractions of degradation products as compared to parent compound.

Degradation of OTC in microcosm and control experiments

Degradation experiments were performed over 90 days and followed the transformation of OTC in distilled water both exposed to light and in the dark, river water and sediment sterilized and unsterilized. Figures 3 and 4 show the concentration of OTC and its metabolites respectively versus time over the period of degradation study. Slow degradation of OTC (Figure 3a) was initially observed in the covered distilled water experiment however slight increase in degradation was observed from day 26. This was attributed to microbial degradation since the experiment consisted of openings to allow exchange with the environment. Furthermore no significant difference was observed between the results obtained from the covered distilled water set up and sterilized river water and sediment showing that no other transformation process other than hydrolysis was significant. Tetracyclines have been reported to degrade abiotically by Fenton reactions involving Fe^{2+} and by oxidation in the presence of metal oxides (Chen and Huang, 2011). Also traces of algae were observed at the end of the experiment. Xuan et al. (2010) performed similar experiments with modified distilled water in the dark and also observed slow degradation of OTC. Similar profiles were observed by Doi and Stoskopf (2000) in their study of the degradation of OTC in deionized water however they did not discuss the issue. Much faster degradation as compared to the covered distilled water experiment was observed in the exposed distilled water setup Figure 3(b). This can be attributed to photo-degradation. OTC has been found to degrade readily in distilled water under UV light irradiation (Doi and Stoskopf, 2000). A change in degradation speed was also observed from day 26 and this was attributed to microbial degradation. Contamination by micro- organisms in the atmosphere

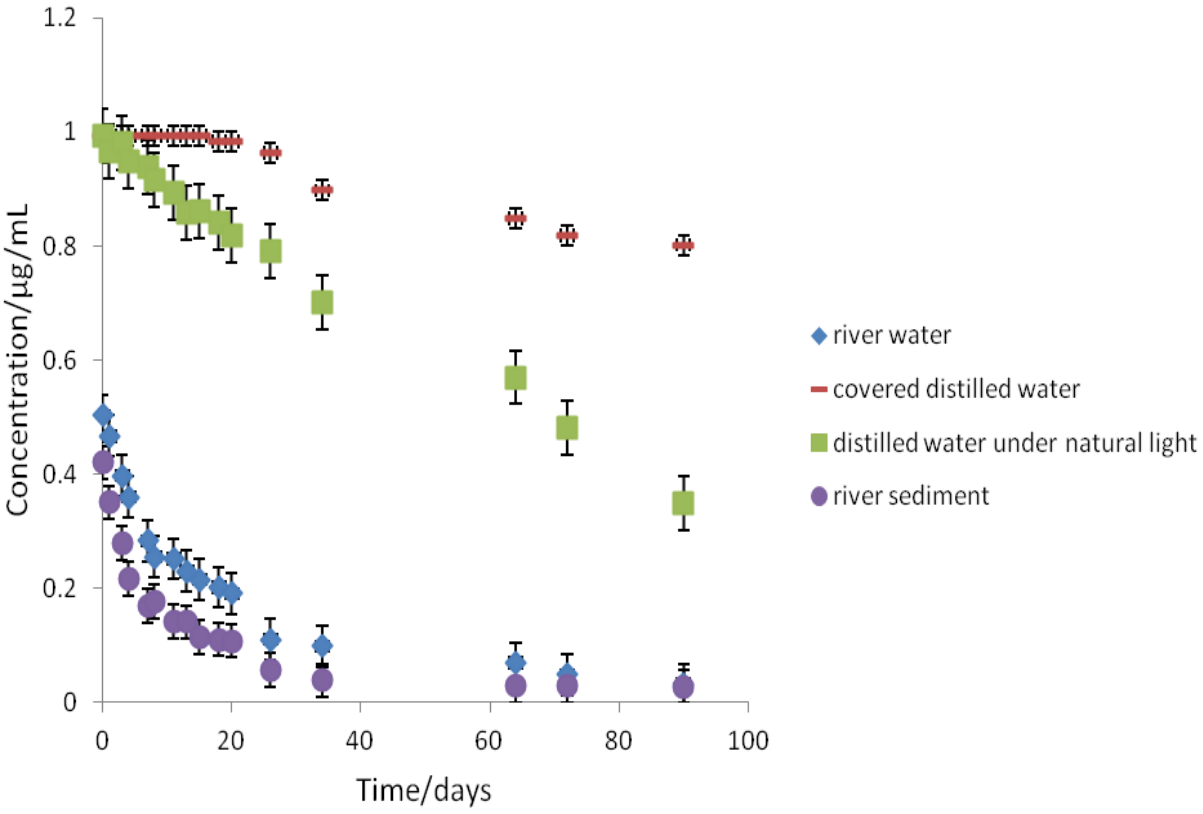

Figure 3. Concentration of oxytetracycline (OTC) in river water, covered distilled water and distilled water exposed to sunlight, river sediment.

may have occurred through the transparent plastic perforations. Abiotic degradation under natural conditions contributed to slightly above 20% degradation in the exposed distilled water experi-ment over the 90 day period showing that OTC can persist under natural conditions if microbes that degrade the antibiotic are absent. In filtered and autoclaved soil, interstitial water Halling-SØrensen and coworkers observed a general stability of OTC molecules with half-lives up to 270 days.

Significant degradation was observed in the river water and sediment Figure 3(c) and (d) and this can be attributed to microbial degradation. Photodegradation was not expected to be significant in the study by looking at results from the control experiments. Photodegradation is only significant in shallow clear water and is expected to be hampered by presence of soil and organic particulate matter in the naturally relevant conditions as employed in this study (López-Peñalver et al., 2010). Traces of algae were also observed in the microcosm experiments. It has been reported in previous studies that algae can degrade organic molecules such as tetracyclines by using them as a source of carbon by releasing enzymes into the solution (Migliore et al., 2012).

Figure 4 a-d shows the disappearance of the main degradation products in river water and sediment spiked with OTC. The Figures depict β-apo-OTC to be the dominant degradation product whereas in similar studies α-apo-OTC was the dominant product (Loke et al. (2003). Halling-SØrensen et al. (2003) also observed β-apo-OTC to be the predominant degradation product. 4-epi-OTC reached the highest concentration in this study as compared to the other degradation products. Formation of 4-epi-OTC reached a mole fraction of 0.2-0.3 to the parent compound at day 64 in river water Figure 1 (d). These results are almost in agreement with what was reported by Halling-SØrensen et al. (2003). In their study more than 60% of OTC was converted to 4-epi-OTC after 100 days. Conversion from OTC to β-apo-OTC Figure 5 involves loss of a water molecule.

The degradation therefore most likely involves dehydrolase enzymes released from micro-organisms and algae (Nnenna et al., 2011). These enzymes can be extracted, incubated, optimized and applied in the remediation of contaminated sites and to treat effluents from farms, pharmaceutical industry, hospital and municipal effluents before they can be released into the environment. Very few studies have been devoted to this regard. Meyers and Smith (1962) investigated the application of *Xylaria digitata*, a fungi to degrade tetracycline antibiotics and found out that they were effective. Other investigations involving the use of microbial degradation are reported by Maki et al. (2006).

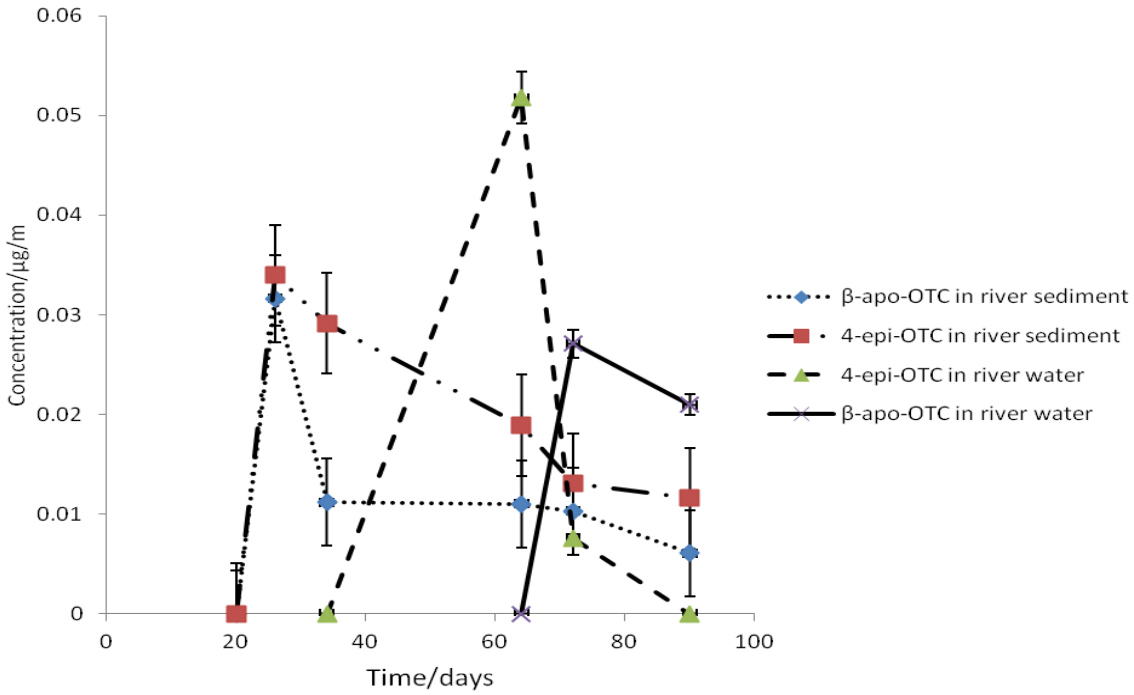

Figure 4. Concentration of, 4-epi-oxytetracycline (OTC), β-apo- oxytetracycline (OTC) in river sediment and water.

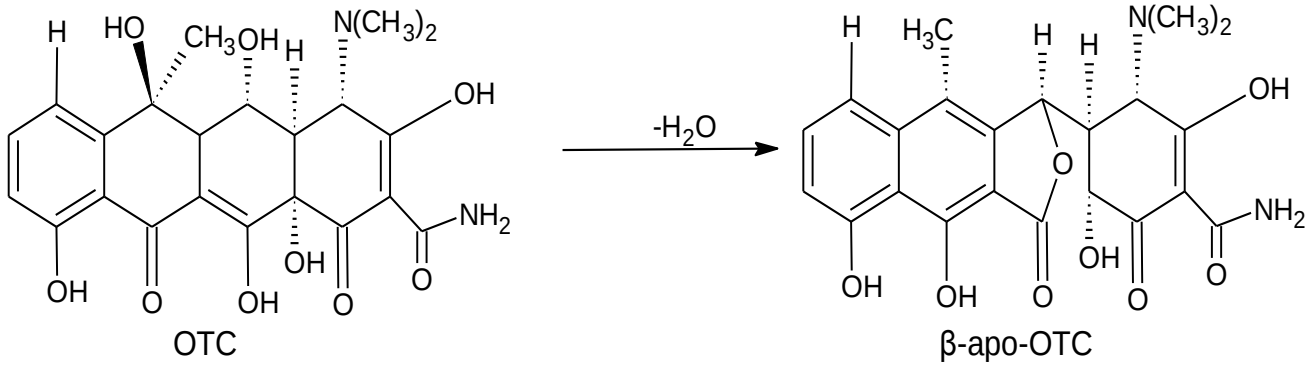

Figure 5. Hydrolysis of oxytetracycline (OTC) to β-apo-oxytetracycline (OTC).

and Wen et al. (2009).

Conclusion

Results of the present study show that microbial degradation plays an important role in the removal of OTC in the aquatic environment. All degradation products were present in trace levels. The study found out that in river water and sediment β-apo-OTC is the most stable degradation product. Microbial degradation of OTC to 4-epi-OTC reached a higher mole ratio, 0.2:0.3 as compared to the parent compound as other compounds.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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