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Biodegradation of tetracycline antibacterial using green algal species collected from municipal and hospital effluents

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ABSTRACT

In the present study biodegradation of oxytetracycline (OTC), doxycycline (DC), chlortetracycline (CTC) and tetracycline (TC) using algal species collected from municipal and hospital waste water was studied using microcosm experiments. Changes in antibiotic concentration were determined using reversed phase HPLC. The results show that algal species, Haematoloccus pluvialis, Chlorella sp,(hospital waste water) Selenastrum capricornutum and Pseudokirchnerilla subcapitata (municipal waste water) degrade tetracycline antibiotics. The order of degradation was DC > OTC > CTC > TC. Results are explained in terms of a two compartmental model consisting of free and adsorbed form with initial fast degradation rate constants ranging from 1.1 x 10^{-3} to 3.7 x 10^{-2} mg L^{-1} day^{-1}. The corresponding slow degradation rate attributed to degradation of the bound form was not significantly different from zero, t-test p = 0.05 showing non degradability. TC results could not be explained using a two compartmental model. The results followed monophasic kinetics with slow degradation rates ranging from 1.3 x 10^{-4} to 9.0 x 10^{-3} mg L^{-1} day^{-1}. H. pluvialis, S. capricornutum and P. subcapitata were better candidates than chlorella sp.

Key words: biodegradation, tetracyclines, algae, biphasic kinetics, monophasic kinetics

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INTRODUCTION

Persistence of pharmaceuticals in the aquatic environment is becoming a great concern. This is because they have a potential to induce drug resistance to microbes in the environment which can be transferred to humans through drinking water and the food chain [1] and this complicates treatment of diseases. Other important concerns include development of allergic conditions and discoloration of secondary teeth in children [2]. Presence of antibiotics in the environment is not only fatal to humans but can also disturb important ecological roles such as nutrient recycling [3].

Pharmaceuticals enter the environment through effluent discharge from municipal treatment works as these are not designed to look for or remove pharmaceuticals [4]. Another route of contamination includes runoff and seepage from fields and vegetable gardens applied with animal manure that has not been treated adequately by composting [5]. Tetracyclines antibiotics merit attention because they are the mostly applied antibiotics to treat diseases in humans and farm animals worldwide [6]. They are also added in micrograms per kilogram level in animal feeds to act as growth promoters and prophylactics [7]. Oxytetracycline OTC, tetracycline TC, chlortetracycline CTC and

doxycycline DC are among the widely applied antibiotics and because of this they were selected for this study. Significant to low levels have been found in surface waters, municipal, hospital and swine waste water [8-10].

In the last decades a lot of studies have been carried out to address the problem of antibiotic contamination in the environment. A number of physical and chemical degradation technologies such as chemical or Fenton oxidation, adsorption, ozonation, use of membrane filters, activated sludge, membrane and fixed bed reactors have been tested [11, 12]. The overall aim has been to degrade the antibiotics from their environmental matrix. The major draw backs of these technologies are that they are to a great extent condition based and potential expense [13]. Photodegradation has been reported to be the only major removal mode of tetracyclines from the aqueous phase on the assumption that they are bacteriostatic [14] however recent studies have shown that microbial degradation plays an important role in the dissipation of antibiotics from the real environment [15, 16] where photodegradation is expected to be hampered by presence of radiation attenuating materials [17]. Phytoremediation coupled to biodegradation does not require specific conditions such as pH, temperature and molar ratio therefore can be easily adopted for pharmaceutical remediation of contaminated areas.

Algae has been selected in the present study as model organisms for decontamination of tetracycline antibiotics from municipal and hospital waste waters as they have been reported to be suitable candidates for bioremediation of organic molecules [18]. Algal species have the advantage not only of being the major players in fixation and turnover of carbon but they are also ubiquitous distributed. While some tetracycline antibiotics have been reported to be toxic to algae recent studies have shown that certain algal species can degrade them [19]. Reviews of scientific studies show limited knowledge on the application of algae to degrade pharmaceuticals in municipal and hospital waste waters. Previous studies [20-22] involving bioremediation identified several types of enzymes produced by plants, algae and bacteria that play a significant role in biodegradation of organic molecules. Enzymes such asphosphatases, hydrolases and laccase have been reported [23] to be major players in the degradation of organic molecules consisting of benzenoid rings. Therefore the goal of this study was to investigate biodegradation of OTC, TC, CTC and DC using algal species isolated from municipal and hospital waste waters.

MATERIALS AND METHODS

OTC, TC, CTC and DC standard 96-99% purity, methanol, acetonitrile (HPLC solvents), primary secondary and amine sorbent material (57738-U-SUPELCO supelclean (PSA) and nylon disposable filter units (0.45 µm) were purchased from Sigma Aldrich (Germany). Analytical grade chemicals, oxalic acid, orthophosphoric acid, nitric acid, sodium hydrogen phosphate, citric acid and disodium ethylenediaminetetraacetate (Na_2EDTA)) were bought from SKYLABS, Gauteng, South Africa. Municipal and hospital waste waters 10 L each were collected in August 2014 from discharge points located in and around city of Bindura, Zimbabwe. Background levels of OTC, CTC, DC were measured and values of 23-89 ng/L were detected while no TC was detected. Total suspended and volatile suspended solids measured using a standard method [24] were 12 and 16 g/L on average. The pHs of the solutions were in the range 6.9-7.1. Individual standards, 1000 mg/L were prepared in methanol and were later used to prepare working solutions. Sock solutions were stored in a fridge, 0-5^{o}C before use.

Collection of algae, growth conditions and batch experiments

Algal species were collected from Bindura town municipal and hospital waste waters. Species were taxonomically identified as *Haematoloccus pluvialis*, *Chlorella sp*,(hospital waste water) *Selenastrum capricornutum and Pseudokirchnerilla subcapitata* (municipal waste water).Unicellular algal species were maintained each in an axenic culture as recommended by OECD [25]. Cultures were grown in 500 mL flasks sealed with cotton bungs and containing heat sterilized waste water medium. The flasks were incubated at room temperature and manually shaken twice per day. Cultures of grown algae were judged to be continually axenic when they fail to indicate the presence of bacteria and fungi when spread onto nutrient agar. The flasks were then doped with a mixture of 2 mg/L the highest concentration detected in surface waters currently [26] of each antibiotic. Control experiments consisting of sterilized and unsterilized waste water but with no algal cultures were also set up in 500 mL flasks. Changes in antibiotic concentrations were then monitored for 30 days. Samples were collected on days 0, 1, 2, 3, 5, 7, 10, 15 20, 30. On day 0 the samples were collected 2 hours after the doping to make sure that the antibiotics were distributed evenly in the flasks.

Sample preparation and analysis

Samples, 2 mL aliquots were analyzed in triplicate according to a slightly modified procedure reported in the Chinese pharmacopeia [27]. 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 the antibiotics and their metabolites. The detector was controlled remotely by the Varian Star or Galaxie Chromatography Workstation software version 6. All HPLC separations were carried out using C18 (Varian Microsorb MV 1005 packed columns (250 x 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 was used as the mobile phase. 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. Typical chromatograms recorded at day zerofor non-fortified and fortified municipal waste water sample are shown in Fig 1. Retention times for OTC, TC, TC and DC were 2.1, 2.8, 3.3 and 7.2 respectively. Percentage recovery was 88.65 ± 5. 21 % and 89.23 ± 3. 66 % of waste water spiked with 0.05 and 2 mg/L of antibiotic respectively.

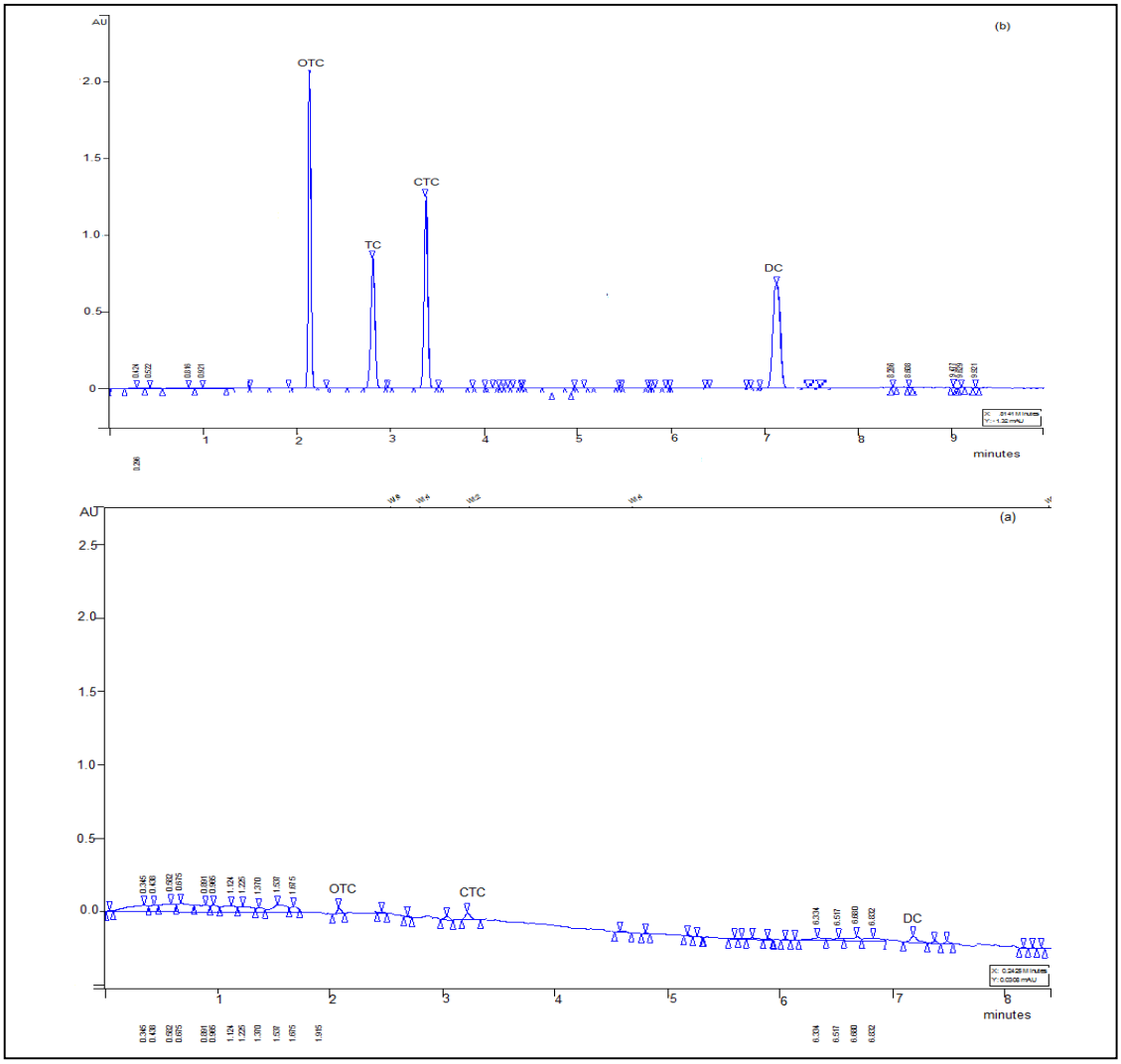

Fig 1. Typical chromatographs for standard antibiotics non-supplemented (a) and supplemented waste water samples (b), OTC = oxytetracycline, CTC = chlortetracycline, DC = doxycycline

RESULTS AND DISCUSSION

The percentage residual antibiotics over the 30 day period are shown in Figure 2 (a), (b), (c), (d), (e), and (f).Antibiotics in control experiments consisting of sterilized waste water with no algae Fig 2 (a) remained almost unchanged. Significant declines were observed after day 20 that can be attributed to recolonization by microbes from the surrounding. Noticeable degradation in unsterilized waste water control Fig 2 (b) can be attributed to biodegradationby microbes in the waster waters [28]. Antibiotic resistance bacteria and fungi have been observed to degrade tetracyclines in environmental samples [29]. In waste water consisting of algal cultures Fig 2 (c) (*H. pluvialis*), (d) (*Chlorella sp*), (e) (*S.capricornutum*) and (f)*P. subcapitata* significant degradation was observed for DC, OTC and CTC while TC remained almost unchanged until the 20th day. A possible explanation for these results is that enzymes capable of degrading DC, OTC, and CTC had evolved already in the algae species collected from the waste waters. DC was the mostly degraded antibiotic in both hospital and municipal effluents implying greater adaptation of the algae species to use DC as a source of carbon and nitrogen. A slight increase in TC degradation after the 20th day is as a result of adaptation of enzymes to degrade it or due to recolonization. Antibiotic degradability was in the order DC > OTC > CTC > TC.

A two compartmental model was used to analyze the degradation kinetics of the antibiotics:

$$\frac{dp}{dt} = k_1[C_1]$$

$$\frac{dp}{dt} = k_2[C_2]$$

Where $\frac{dp}{dt}$ is the rate of formation of products while k_1 is the rate constant for the initial fast degradation and k_2 is the rate constant of the subsequent slow degradation rate while C_1 and C_2 are the concentration of the free and particulate bound antibiotic respectively. C_t-C_o[30] was computed by regression analysis as a function of time where (C_o is the initial concentration at day zero, while C_t is concentration at any time t). The slopes of the linear regression lines give the rates of degradation of the two forms. The computed rates of degradation obtained are summarized in Table 1. Degradation of OTC,CTC and DC showed biphasic pattern with a fast degradation rate in the first 10 days and the rate stabilized thereafter. Degradation of TC followed monophasic pattern with no subsequent slow degradation rate. Fast degradation rate correspond to degradation of the free antibiotic form while the slow degradation correspond to degradation of particulate matter bound form. In the subsequent slow degradation component the rate was found not to be significantly different from zero (t-test, p>0.05) implying that microbes could not degrade the bound form. Tetracyclines antibiotics have been reported to sorb onto particulate matter [31].

Table 3. Biodegradation rates of OTC, TC, CTC and DC(µg/g/day)

Experiment	Phase	Initial rate	R^2	Final rate	R^2
(*H.pluvialis*)	OTC	1.2×10^{-2}	0.98	NS	0.98
	TC	9.0×10^{-3}	0.99	-	-
	CTC	2.1×10^{-2}	0.97	NS	0.99
	DC	2.6×10^{-2}	0.99	NS	0.99
(*Chlorella sp*),	OTC	1.1×10^{-3}	0.99	1.0×10^{-4}	0.99
	TC	1.3×10^{-4}	0.99	-	0.99
	CTC	1.7×10^{-3}	0.98	1.4×10^{-4}	0.99
	DC	1.5×10^{-3}	0.99	1.6×10^{-4}	0.99
S.capricornutum	OTC	2.9×10^{-2}	0.99	NS	0.98
	TC	1.3×10^{-4}	0.99	-	-
	CTC	3.1×10^{-2}	0.99	NS	0.98
	DC	3.6×10^{-2}	0.99	NS	0.99
P. subcapitata	OTC	2.7×10^{-2}	0.99	NS	0.98
	TC	2.2×10^{-4}	0.99	-	-
	CTC	3.3×10^{-2}	0.99	NS	0.97
	DC	3.7×10^{-2}	0.99	NS	0.99

NS = not significant from zero, p > 0.05

- = The degradation could not be explained using biphasic kinetics

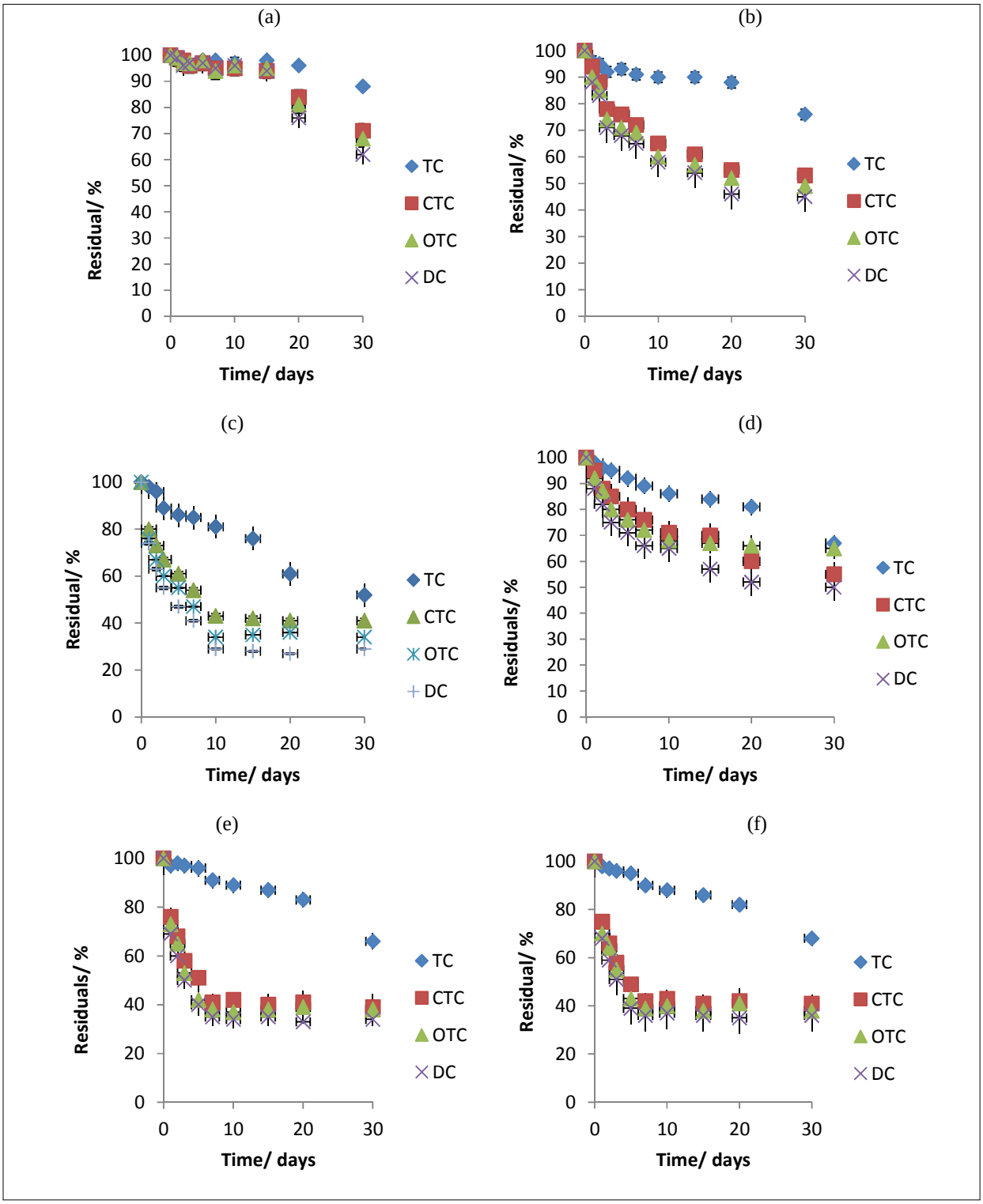

Fig 2.Percentage residual antibiotics over the 30 day period

The rate of this compartment is no longer dependent on concentration of the antibiotic but on the nature of adsorption. Sorbed compound are relatively less accessible to microorganisms. In the real environment such as employed in the microcosm experiments adsorption/desorption equilibria is a dynamic process. As the free form degrades the bound form can desorb from the particulate matter and achieve a new equilibrium. If the adsorption process is reversible then the antibiotic can eventually be completely mineralized however if it is irreversible the antibiotic can persist in the environment. In the present study results show that certain proportion of the antibiotic remained adsorbed onto particulate matter. This proportion could persist thus cannot be accounted for in the half-life concept.

CONCLUSION

Algae harvested from municipal and hospital effluents managed to degrade significantly OTC, CTC and DC. Degradation of the antibiotics followed the following order DC > OTC > CTC > TC. Almost no degradation was observed in sterile experiment while slight degradation was observed in non-sterile experiment consisting of no algae. Biphasic degradation was observed for OTC, CTC and DC while TC exhibited monophasic degradation. The fast degradation compartment was attributed to degradation of the free form while the slow degradation corresponds to degradation of the particulate adsorbed form.

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