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Toxicity of chlortetracycline and its metal complexes to model microorganisms in wastewater sludge

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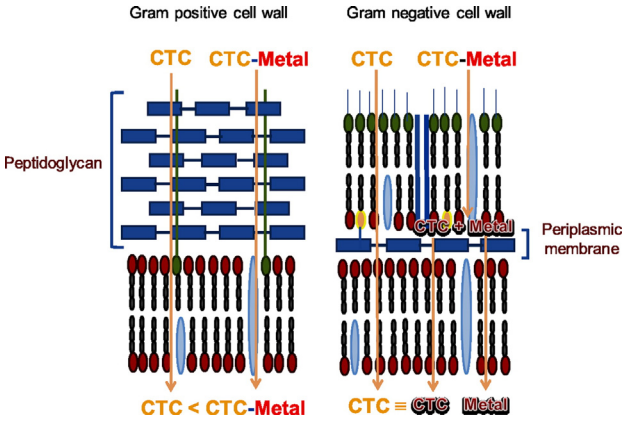
HIGHLIGHTS

- Chelation affinity of CTC with metals has an order of Mg(II) > Ca(II) > Cu(II) > Cr(III).
- Discrepancy in the uptake of CTC in gram-positive and gram negative bacteria.
- Uptake of CTC–metal complex is mainly depending on the role of corresponding metal.
- Adsorption affinity of CTC towards sludge shows less toxicity to the microflora.
- CTC affects the metabolic path of bacteria.

GRAPHICAL ABSTRACT



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ABSTRACT

Complexation of antibiotics with metals is a well-known phenomenon. Wastewater treatment plants contain metals and antibiotics, thus it is essential to know the effect of these complexes on toxicity towards microorganisms, typically present in secondary treatment processes. In this study, stability constants and toxicity of chlortetracycline (CTC) and metal (Ca, Mg, Cu and Cr) complexes were investigated. The calculated stability constants of CTC–metal complexes followed the order: Mg–CTC > Ca–CTC > Cu–CTC > Cr–CTC. Gram positive *Bacillus thuringiensis* (Bt) and Gram negative *Enterobacter aerogenes* (Ea) bacteria were used as model microorganisms to evaluate the toxicity of CTC and its metal complexes. CTC–metal complexes were more toxic than the CTC itself for Bt whereas for Ea, CTC and its metal complexes showed similar toxicity. In contrast, CTC spiked wastewater sludge (WWS) did not show any toxic effect compared to synthetic sewage. This study provides evidence that CTC and its metal complexes are toxic to bacteria when they are biologically available. As for WWS, CTC was adsorbed to solid part and was not biologically available to show measurable toxic effects.

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1. Introduction

Veterinary usage of antibiotics for growth promotion and feed efficiency enhancement by providing subtherapeutic doses is a flourishing market. Although there is a considerable controversy regarding the necessity of antibiotic use at subtherapeutic doses, still their usage continues. On the other hand, the continued usage of antibiotics has led to emergence of antibiotic resistance (Hayes et al., 2004; Levy, 1978; Libby and Schaible, 1955). Currently, there are no alternatives for antibiotics to control infectious diseases.

Chlortetracycline (CTC) is a broad-spectrum antibiotic against wide range of aerobic and anaerobic Gram-positive and Gram-negative bacteria. CTC is the first antibiotic to show antibacterial (bacteriostatic) activity by inhibiting protein synthesis through the binding of amino acyl-tRNA to the acceptor site on the mRNA-ribosome (Chopra et al., 1992; Schnappinger and Hillen, 1996). Among all tetracycline antibiotics, CTC has lowest absorption (25–30%) with maximum excretion varying from 30–90% among different living organisms (Agwuh and MacGowan, 2006; Elmund et al., 1971; Feinman and Matheson, 1978). Hence, maximum unmetabolized CTC ultimately ends up in the environment (wastewater (WW), agriculture field, surface water). CTC is soluble in water but its ability to form complexes with metals results in the formation of less soluble CTC–metal complexes. Further, chelation of CTC with metals changes the chemical properties, such as solubility and partition coefficient. Apart from chelation, its high adsorption capacity leads to retention of CTC in the WWS instead of going into WW. Pulicharla et al. (2014) reported higher concentration of CTC in WWS (0.4–0.8 mg/L). Authors correlated higher metals concentration in sludge to CTC accumulation due to its chelation property.

Due to continuous application of biosolids in agriculture or for landfilling, the accumulated CTC in WWS is transported to the soil. In soil, there is a co-existence of CTC and metal ions and their complexes. Hence, the accumulated CTC and its metal complexes in soil may influence the soil microbial community due to their broad-spectrum antibacterial activity. Further, CTC may also leach into groundwater aquifers from soil.

Despite wide application and detection of CTC in different environment (soil, WW, WWS, and surface water), knowledge of metal complexation and adsorption behavior on fate of CTC in WW is limited. Chlortetracycline is known to form complexes with metal ions and shows higher adsorption on sediments, soils and clay materials (Pils and Laird, 2007; Rivera-Utrilla et al., 2013; Sassman and Lee, 2005; Wang et al., 2008). Extensive investigation has been carried out on the complexation of tetracyclines with metals (Orth et al., 1999; White and Cantor, 1971). Although numerous studies have focused on the chemistry of the tetracyclines-metal complexation, solution conditions complicate the stoichiometry equilibrium between metals and tetracyclines (Wessels et al., 1998). Complex matrix, such as WW contains macro and micro elements and heavy metals having different ionic potentials, thus exhibiting varied complexation with organic ligands. However, little is known about as to how such a complex matrix affects the CTC–metal equilibrium and further influences their adsorption behavior. Unfortunately, the reported complexation constants of CTC with metals (Ca(II), Mg(II), Cu(II)) and stoichiometry ratios are in strong disagreement. As solution conditions (pH, metal type, and temperature) complicate the stoichiometry equilibrium between metals and tetracyclines (Brion et al., 1986; Couto et al., 2000; Lambs et al., 1988). Hence, it is difficult to use reported stoichiometric CTC–metal equilibrium values for further toxicity evaluation in the bacteria.

Owing to inherent differences in cell wall composition of Gram-positive and Gram-negative bacteria, the toxicity of CTC and its metal complexes may be different. However, this possibility has not been investigated as yet. In this study, *Bacillus thuringiensis* (Bt), an insecticidal Gram positive soil-dwelling bacterium has been selected as model microorganism. Likewise, *Enterobacter aerogenes* (Ea) was selected as model Gram negative bacteria. Toxicity of CTC on microorganisms was evaluated using a rapid, short-term and sensitive biochemical toxicity test at environmentally relevant concentrations.

Overall, the objectives of this study were to: 1) determine the stability constant of CTC–metal complexes of four metal ions Ca(II), Mg(II), Cu(II) and Cr(III); 2) estimation of toxicity of CTC and CTC–metal complexes towards Gram positive and Gram negative bacteria; 3) effect of total solids on the toxicity of CTC towards bacteria grown in synthetic growth media (GM), synthetic sewage (SS), secondary sludge 1 (SS1) and settled secondary sludge 2 (SS2); 4) uptake study of CTC and its metal complexes and; 5) effect of CTC on the metabolic products of bacteria.

2. Materials and methods

2.1. Chemical and reagents

Chlortetracycline was purchased from Toronto Rsearch Chemicals (Toronto, Canada). Calcium chloride anhydrous ($CaCl_2$, >99%), chromium nitrate ($(CrNO_3)_3 \cdot 9H_2O$), cupric chloride anhydrous ($CuCl_2$, >99%) and magnesium perchlorate anhydrous ($Mg(ClO_4)_2$, >99%) were obtained from Fisher Scientific (New Jersey, USA). Calcium chloride hydrate ($CaCl_2 \cdot 2H_2O$), potassium dibasic phosphate (K_2HPO_4) and potassium monobasic phosphate (KH_2PO_4) was supplied by Laboratoire MAT Inc. (Quebec, Canada). Glucose, hydrochloric acid (HCl, 36.5–38%) magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), sodium chloride (NaCl), sodium hydroxide (NaOH, >98%) and urea (>99%) were obtained from Fisher Scientific (USA). Tryptic soya broth (TSB) was purchased from EMD chemicals Inc. (Darmstadt, Germany). Disodium ethylenediamine tetraacetate (Na_2H_2EDTA, 99%) was purchased from E-bay (Tokyo, Japan). Methanol (HPLC grade, purity >99.8%) and ammonium hydroxide (NH_4OH, 28–30% w/w) were purchased from Fisher Scientific (Ontario, Canada).

2.2. Potentiometric measurements

In this study, four metals were selected; two macro-elements, Ca(II) and Mg(II) having higher importance for the cellular activities and higher abundance in WWS and two heavy metal ions, Cu(II) as trace element for growth of microorganisms and Cr(III) as a toxic metal. The stability constants and stoichiometric equilibrium for binding of the CTC to Ca(II), Mg(II), Cu(II) and Cr(III) metal ions was studied by potentiometric titrations using an automatic potentiometric titrator (T870, Radiometer Analytical, ON, Canada). Experiments were conducted in milliQ water having pH 6.0 ± 0.5 at room temperature similar to WWS pH conditions. The metal ion solutions of Ca(II), Mg(II), Cu(II) and Cr(III) were gravimetrically prepared using anhydrous chloride salts of Ca, Mg and Cu and chromium nitrate in water, respectively. Errors in metal ion concentrations were minimized by using the fresh anhydrous salts and they were completely dissolved in water. Prior to the experiments, CTC was freshly prepared by dissolving it in water. The ionic strength of all working solutions was adjusted to 0.1 mol/L using sodium chloride to minimize potential shifts in pH upon ion binding. All solutions were prepared in Milli-Q water containing residual metal ion concentrations less than 1.0 μg/L. The final pH of all working solutions was adjusted to 6.0 ± 0.5 with NaOH or HCl solutions. The stability constants of CTC were determined by titration of the appropriate solutions of CTC (1×10^{-3} mol/L) in water, using metal solutions as titrant (0.5×10^{-3} mol/L), in 0.1 mol/L NaCl (Şanli et al., 2009). All solutions were purged with nitrogen gas to remove dissolved carbon dioxide in titrant solutions and they were later completely mixed using a magnetic stirrer. The stabilization criterion for the potential readings was 0.2 mV within 60 s. About 0.5 mL/min addition of metal solution was performed by calibrated burette and the titrations were continued, until a constant potential was reached.