Influence of montmorillonite on antimicrobial activity of tetracycline and ciprofloxacin

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ABSTRACT

Antibiotics are used not only to fight infections and inhibit bacterial growth, but also as growth promoters in farm livestock. Farm runoff and other farm-linked waste have led to increased antibiotic levels in farm livestock. Antibiotics are used not only to fight infections and inhibit bacterial growth, but also as growth promoters in farm livestock. Farm runoff and other farm-linked waste have led to increased antibiotic levels in farm livestock. In this study a swelling clay mineral montmorillonite was preloaded with antibiotics tetracycline and ciprofloxacin at varying concentrations and bioassays were conducted to examine whether the antibiotics still inhibited bacterial growth in the presence of montmorillonite. Escherichia coli was incubated with montmorillonite or antibiotic-adsorbed montmorillonite, and then the number of viable bacteria per mL was determined. The antimicrobial activity of tetracycline was affected in the presence of montmorillonite, as the growth of non-resistant bacteria was still found even when extremely high TC doses were used. Conversely, in the presence of montmorillonite, ciprofloxacin did inhibit E. coli bacterial growth at high concentrations. These results suggest that the effectiveness of antimicrobial agents in clayey soils depends on the amount of antibiotic substance present, and on the interactions between the antibiotic and the clays in the soil, as well.

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1. Introduction

Antibiotics are used extensively for both human and veterinary medicine purposes. Human use of antibiotics is limited to medicinal therapeutic purposes, while the widespread use of antibiotics in the agricultural industry involves use not only as disease-fighting agents, but as prophylactics and growth promotants as well – termed subtherapeutic use. In the United States, between 40% and 84% of all antibiotics produced are used in agriculture, with the majority of that amount used for a subtherapeutic purpose (Shea, 2003). An estimated 132 million metric tons of manure is generated in the United States annually, and is applied to 9.2 million hectares of farmland as a source of nutrients for crop production (USDA, 2005). As antibiotics are only partially metabolized by the livestock that receive them, their manure often contains high levels of antibiotics – as much as 70–90% of the initial antibiotic dose passes through the animal unused (Marshall and Levy, 2011). While doses given to livestock are relatively small (<200 g/ton feed), the fact that so much of that dose was not metabolized allowed for antibiotic concentrations in manure to range from trace levels to >200 mg/L (Kumar et al., 2005). An investigation on the fate of three antibiotics from the sulfonamide, tetracycline, and macrolide groups conducted in two consecutive years after pig slurry was applied to a field in arable production showed a tetracycline (TC) content of up to 1691 µg/kg in soils (Kay et al., 2004). Concentration levels of chlortetracycline and oxytetracycline (OTC) remain constant for over 150 days in four types of microcosms with pig manure slurry amendment (Agersø et al., 2006).

In addition to elevated soil antibiotic concentrations, common antibiotics were also detected in surface water and wastewater. Ciprofloxacin (CIP) and norfloxacin with an initial concentration of 255–568 ng/L in raw sewage and 36–106 ng/L in final wastewater effluents were detected in Switzerland (Golet et al., 2002). Among the 31 antimicrobials from the macrolide, quinolone, quinolxine dioxide, sulfonamide, and TC classes in the final (treated) effluents of eight wastewater treatment plants (WWTPs), located in five Canadian cities, CIP and TC among others were frequently detected (Miao et al., 2004). In the state of Wisconsin, TC and CIP accounted for 80% and 40% detection among the 21 antibiotic compounds screened in several WWTPs with the average TC and OTC concentrations as high as 48 and 47 µg/L in the influent (Karthikeyan and Meyer, 2006). Even worse, in the effluent of the Hospital of the Federal University of Santa Maria in Brazil, the measured
environmental concentrations of CIP were 19–155 μg/L and 32–99 μg/L before and after filter system treatment, respectively (Martin et al., 2008). These concentrations were on par or even greater than the minimum inhibition concentration (MIC) of 0.016 and 0.128 mg/L for a CIP sensitive and a CIP resistance E. coli strain (Lecomte et al., 1994).

In addition to soil and water contamination, the presence of antibiotics in the environment would also increase bacterial resistances to antibiotics (Sarmah et al., 2006). While the antibiotics that leak into soils from manure are not present at high enough concentrations to therapeutically inhibit bacteria, they do still influence bacterial populations in such a way that selects for antibiotic-resistant bacteria in the environment (USEPA, 2002). For montmorillonite, both external and interlayer were the most important soil clay minerals. Investigations of interactions between CIP or TC and these clay minerals have already been thoroughly conducted and cation exchange was attributed to the major mechanism of TC and CIP uptake (Li et al., 2010; Wu et al., 2010). There is a concern that this may be contributing to the spread of antibiotic-resistant bacterial strains in the environment. Moreover, the effects of antibiotics on ecosystem processes, which are highly dependent of antibiotic bioavailability, were not comprehensively addressed (Kong et al., 2006).

Antibiotics have been shown to have a strong tendency to bind with particles in soil (Kumar et al., 2005). While their presence in soil after the application of manure is known, it is not well known how binding with soil particles affects the antimicrobial activity of these antibiotics. It was demonstrated that clay type is a big factor impacting antibiotic bioavailability and its antimicrobial activity in soils (Kong et al., 2012). Montmorillonite and kaolinite are two of the most important soil clay minerals. Investigations of interactions between CIP or TC and these clay minerals have already been thoroughly conducted and cation exchange was attributed to the major mechanism of TC and CIP uptake (Li et al., 2010; Wu et al., 2010). For montmorillonite, both external and interlayer were available for TC or CIP uptake, resulting in extremely high TC or CIP adsorption capacities (Li et al., 2010; Wu et al., 2010). However, whether the adsorbed or intercalated antibiotics still maintained their antimicrobial activities or not was subject to some debate. A previous study showed that some antibiotics did retain antimicrobial activity after sorption by soil and that this activity was potent enough to select for antibiotic resistant mutants (Chander et al., 2005). A separate study also confirmed that the antimicrobial activity of TC, measured by inhibition tests, was maintained after adsorption on montmorillonite (Parolo et al., 2010). Many bacterial isolates from sow, nursery, and finisher farms in the southeastern United States were resistant to penicillin, cephalosporin, and TC class antibiotics, while nearly all were susceptible to quinolone antibiotics (Brooks and McLaughlin, 2009). The same TC resistance genes and frequency of detection were found in the manure and lagoon samples for each commercial farm and the levels of TC resistance remained high throughout the waste treatment systems, suggesting that the potential impact of land application of treated wastes and waste treatment by-products on environmental levels of resistance should be investigated further (Jindal et al., 2006). High levels of bacterial antibiotic resistance were detected in both freshwater- and treated wastewater-irrigated soils, indicating that the high number of resistant bacteria that enter the soils from the treated wastewater did not significantly contribute antibiotic resistance gene to soil bacteria (Negreanu et al., 2012).

In this study the efficacy of clay-bound antibiotics tetracycline and ciprofloxacin in inhibiting the growth of Escherichia coli bacteria was assessed. It was anticipated that the results would help to elucidate the effects of antibiotics in soils with significant swelling clay component on microbial susceptibility and resistance to these antibiotics.

2. Materials and methods

2.1. Materials

The standard montmorillonite (SAz-1, here abbreviated as mmt) obtained from the Source Clays Repository of The Clay Minerals Society, was used as received without further purification. The antibiotics (Fig. 1) were TC obtained from Calbiochem (Darmstadt, Germany) and CIP purchased from Hangzhou Minsheng Pharmaceutical Group Co., Ltd. (China). TC from Sigma–Aldrich (Saint Louis, Missouri, USA) was also employed for the antimicrobial comparison analysis (see below). Both were used as received and were in hydrochloride form. The molecular weight and solubility for TC HCl and CIP HCl were 480.9 and 367.8 g/mol and 12.7 and 30 g/L, respectively (Varanda et al., 2006).

2.2. TC or CIP adsorption on mmt

For TC or CIP adsorption experiment, 0.1 g of mmt and 20 mL of TC or CIP solutions with varying concentrations (100–2000 mg/L) were mixed in 50-mL centrifuge tubes and the mixtures were shaken at a speed of 150 rpm for 24 h at 22 °C. The mixtures were then centrifuged, and the supernatant filtered through 0.45 μm syringe filters before being analyzed for equilibrium TC or CIP concentrations. The amounts of TC or CIP adsorbed were determined by the difference between the initial and equilibrium solution concentrations.

2.3. TC or CIP desorption from mmt by DI water

Although solution ionic strength would greatly affect the processes of antibiotic adsorption/desorption, extensive studies of TC and CIP desorption under different pH and ionic strength conditions were studied elsewhere (Wu et al., 2013; Chang et al., 2013). Thus, DI water was used for desorption of TC or CIP to mimic natural water under a lower ionic strength condition. For desorption study, 2 g of mmt were mixed with 400 mL of 2000 mg/L TC solution for 24 h. The mixture was centrifuged and the supernatant analyzed for equilibrium solution concentration, and the amount of TC sorbed was calculated from the difference between the initial and equilibrium TC concentrations. After decanting the supernatant, 400 mL of DI water was added, and 5 mL aliquot of the mixture was taken out at varying amounts of time. After centrifugation, the supernatant was analyzed for TC concentration.

![Fig. 1. Molecular structure of tetracycline (a) and ciprofloxacin (b).](image-url)
and the amount of TC desorbed was calculated from the different between initial TC sorption and equilibrium TC desorption concentrations. A similar procedure was followed for CIP desorption.

2.4. Bacterial strains, reagents, MIC determination

Standard aseptic microbiology protocols were used for all experiments. Two *E. coli* isogenic strains were used: XL1Blue (XLFTc) was the TC-resistant control strain and XL1Blue MR F’Kan (XLFKn), with selectable antibiotic resistance to kanamycin, served as the TC-sensitive strain. They were purchased from Stratagene (La Jolla, California). The bacteria were cultured on Luria broth (LB) and Luria agar (LA) (Lennox recipe; Fisher Scientific).

TC and CIP minimum inhibitory concentrations were determined for the XL1 lab strains using a variation of the microtiter plate method described previously (Andrews, 2001). The MIC values were determined with log-phase starter cultures in LB broth so as to be comparable to the clay-antibiotic liquid culture studies.

2.5. Antimicrobial activity tests

The antimicrobial activities of TC preparations from two different sources (Calbiochem or Sigma–Aldrich) were tested against the XLFTc and XLFKn, strains in the absence of mmt first. Bacteria in log phase growth were subcultured in varying concentrations of TC (0, 10, 50, and 500 μg/mL) and incubated at 37 °C for 3 h in a shaking incubator. Ten-fold serial dilutions were then prepared from each culture; these dilutions were spotted (5 μL each) on LA plates that contained the antibiotic to which the strain was resistant (10 μg/mL Kn for XLFKn and 10 μg/mL for XLFTc). These plates were incubated overnight at 37 °C, and the number of colony forming units (CFU) per milliliter of culture was calculated using the following formula:

\[
\text{CFU/mL} = \frac{\text{Colonies present}}{\text{Volume spotted (mL)} \times \text{Dilution factor}}
\]

To investigate the effect of mmt on TC antimicrobial activity, 0.10 g clay was added to each of 12 microcentrifuge tubes and then autoclaved. TC (Calbiochem) solutions of 10, 50, and 500 μg/mL were prepared. The test tubes containing mmt were then loaded with either sterile water or one of the TC solutions. The solutions were then incubated, with agitation, at 25 °C (simulating an average, ambient outdoor temperature) for 2 h. Subsequently, the mixture was allowed to settle and the liquid was pipetted out. Half of the samples were refrigerated immediately, while the other half received 1 mL LB before refrigeration (“prior”). After 1 week of refrigeration, 1 mL LB was added to the tubes containing only mmt + TC (“day 0”), and then each tube (“prior” and “day 0” LB clay samples) was inoculated with 110 μL of log-phase *E. coli* XLFTc or XLFKn to give a 1:10 dilution of cells to total volume. These tubes were incubated (37 °C for 3 h with agitation), and then serial dilutions were prepared, plated, and incubated as described earlier. CFU/mL was then calculated.

The effect of mmt on CIP antimicrobial activity was investigated using mmt loaded with 0, 3, 30, or 100 μg/mL CIP (mmt + CIP). 0.10 g of each mmt + CIP concentration was mixed with 1.8 mL LB in a sterile test tube, for a total of 8 tubes (4 concentrations, 2 samples per concentration). Each tube was then inoculated with 0.10 mL of bacteria in log-phase XLFKn and then the procedures for TC bioassays followed.

In a separate experiment, 0.1 g sterile mmt aliquots were incubated with 30 mg/L CIP as described above except that LB replaced the water. After 2 h, the clay was allowed to settle and the supernatant was removed from half the samples. An equivalent volume of fresh LB was added to the clay. The supernatant was retained for further study. The remaining samples were kept in the LB liquid used to load the CIP. These samples were mixed with XLFKn as described above. After the 2 h incubation period, these samples were plated to determine CFU/mL as previously described.

2.6. Methods of analyses

The equilibrium TC and CIP were analyzed using a UV–Vis spectrophotometer (Milton Roy Spectronic 601, Ivyleand, PA) at a detection wavelength of 254 and 275 nm, respectively. The detection limit was 1 mg/L and the linear response range was from 2 to 40 mg/L with the coefficient of determination \(r^2 = 0.9997\). For all experiment, the equilibrium solution pH was the range of 6–7.

3. Results and discussion

3.1. TC and CIP adsorption

As the mechanism of adsorption of cationic drugs on charged mineral surfaces was via a cation exchange mechanism, the TC and CIP adsorption on mmt was fitted to the Langmuir adsorption isotherm (Fig. 2). At an initial TC concentration of 2000 mg/L, the equilibrium concentration was 290 mg/L, resulting in a TC adsorption of 342 mg/g on the clays. Similarly for CIP at an initial concentration of 2000 mg/L, the equilibrium concentration was 315 mg/L, resulting in a CIP adsorption of 337 mg/g on the clays. These results agree well with previous results, suggesting that both the external surfaces and interlayer spaces are available for TC or CIP uptake (Li et al., 2010; Wu et al., 2010).

3.2. TC and CIP desorption by DI water

Desorption of TC or CIP from mmt surface was almost instantaneous. The data were fitted to several kinetic models and the pseudo-second order kinetic model fitted the results best (Fig. 3). However, the amounts of TC or CIP desorbed were extremely low. The average amounts of TC and CIP desorbed from initial loadings of 342 and 337 mg/g were only 8 and 5 mg/g, respectively, resulting in equilibrium concentrations of 30–40, and 24–26 mg/L for TC and CIP, respectively. In contrast the average of TC and CIP desorption from an initial loading of 40 mg/g was only 0.5 and 0.4 mg/g, resulting in an equilibrium TC and CIP concentrations of about 2–3 and 1–2 mg/L, respectively. Considering that the environmental TC or CIP concentrations are much lower than these initial loadings, the TC and CIP adsorbed on the clay are extremely resistant to desorption when DI water was used as the desorbing reagent. The amounts of TC or CIP desorbed by DI water were much lower in comparison to those desorbed by 50 mM Ca\(^{2+}\) and Al\(^{3+}\) (Wu et al., 2013; Chang et al., 2013). The extremely low desorption of TC and CIP under low ionic strength conditions suggests that the
adsorbed TC or CIP in the interlayer of mmt was highly stable. Even though, low but persistent desorption of TC and CIP may result in slight elevation in aqueous TC or CIP concentration, hence, inducing potential antibiotic resistance for microbes in contact with such low concentration over prolonged time period.

3.3. Antimicrobial activity of TC and CIP

TC is a broad-spectrum antibiotic, classified as bacteriostatic, that is produced by the *Streptomyces* genus of Actinobacteria. It works by binding the 30S ribosomal subunit through an interaction with 16S rRNA, to prevent the docking of amino-acylated tRNA (essentially stopping the assembly of polypeptides, or proteins). Resistance to TC can arise through drug efflux pumps, ribosomal protection proteins, 16S rRNA mutations, and drug inactivation by a monoxygenase.

CIP is a broad-spectrum, synthetic antibiotic that belongs to the fluoroquinolone class of bactericidal antibiotics. It is active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, both of which are enzymes necessary to separate double-stranded bacterial DNA. Therefore, it kills bacteria by interfering with the enzymes that allow DNA to unwind during replication, thus stopping DNA and protein synthesis.

Bacteriostatic antibiotics such as TC inhibit growth and reproduction of bacteria without killing them, while bactericidal agents (e.g., CIP) kill bacteria. Bacteriostatic antibiotics must work with the immune system to kill and remove the bacteria from the body. However, there is not always a precise distinction between bacteriostatic and bactericidal compounds. High concentrations of some bacteriostatic agents can be bactericidal, whereas low concentrations of some bactericidal agents appear to be bacteriostatic.

The antibiotic MICs were determined for each of the *E. coli* strains. XLF'Kn had a TC MIC of $1 \times 10^{-3}$ mg/mL and a CIP MIC of $2.5 \times 10^{-4}$ mg/mL. The XLF'Tc, with an MIC of $1.25 \times 10^{-4}$ mg/mL, was slightly more sensitive to CIP. Both bacterial strains contain the Nal A mutation which renders them resistant to nalidixic acid and CIP. The XLF'Tc displayed a high level of resistance to TC, as to be expected of a strain carrying a TC resistance gene. The MIC was 0.25 mg/mL but XLF'Tc produced visible growth when incubated in medium containing 0.128 mg/mL TC which is over ten times the amount used to inhibit sensitive organisms in the laboratory.

3.4. Montmorillonite decreases the effectiveness of TC and CIP

The impact of mmt on antibiotic growth inhibition profile was examined by measuring the cell number after a three hour exposure to antibiotic in the presence or absence of the clay. Cell number was monitored by plating on solid growth medium. The assumption was that one bacterium led to one colony on an LA plate, so that the CFU values were a representation of the number of living bacteria present. When neither clay nor antibiotic was present, XLF'Tc and XLF'Kn reached a similar cell density (Fig. 4); however, increasing concentrations of TC slowed the growth of the strains as indicated by decreased CFU/mL. For the XLF'Kn strain, cell growth was slowed in the presence of 0.05 mg/mL such that the resulting density after 3 h incubation was only one third that of the antibiotic-free culture. At the highest concentration tested (0.5 mg/mL), no colonies resulted. As stated above, bacteriostatic antibiotics can become bactericidal when present in high concentrations. Alternatively, the amount of TC present in the 5 μL of culture that was applied to the Luria agar plate was enough to inhibit growth even after diffusion.

XLF'Tc also showed a decrease in growth rate albeit not as rapidly as was seen for XLF'Kn; the highest TC concentration (0.5 mg/mL) led to four log reduction in CFU/mL ($5.0 \times 10^7$ CFU/mL vs. $5.5 \times 10^4$ CFU/mL). Thus even the growth of the TC-resistant strain was inhibited greatly at this high level of TC.

In the aforementioned bacterial sensitivity experiments, TC preparations from two different sources were compared (Fig. 4). The potencies of the samples were equivalent. Thus all subsequent microbiology experiments were performed with the Sigma–Aldrich material.

Cell growth was monitored in the presence of mmt with and without preloaded TC. With mmt alone, both the XLF'Tc and XLF'Kn strains reached cell densities comparable to the no clay controls (Compare Figs. 4 and 5). For the XLF'Tc strain, the presence of the mmt-TC complex did not inhibit cell growth regardless of whether the growth medium was added to the mmt-TC prior to or on the day of the bacterial exposure. Like the samples containing no clays, growth inhibition was seen in the XLF'Kn cultures exposed to the mmt-0.05 mg/mL TC; however, at 0.5 mg/mL TC, the clay presence was able to prevent the cell death seen in the 0.5 mg/mL TC only control. These results suggest that TC absorbed on mmt is greatly decreased in its antimicrobial efficacy.

The effects of present mmt on antimicrobial activity of CIP were tested for only XLF'Kn, as similar results would be expected for XLF'Tc. The CIP MIC for the XLF'Kn was $2.5 \times 10^{-4}$ mg/mL. In the presence of mmt, the antimicrobial activity of CIP was similar to that of TC. At 0.03 mg/mL of CIP, the CFU of XLF'Kn was $10^5$ CFU/mL, similar to that of a TC concentration of 0.5 mg/mL (Fig. 6). However, a complete inhibition is seen at 0.1 mg/mL, showing that the antibiotic is still active even in the presence of mmt. Part of the inhibition may be due to CIP that was not sorbed on the clay but the major inhibition was associated with bound CIP (Fig. 7).
The MIC was 0.016 and 0.128 mg/L for a CIP sensitive and a CIP resistant strain, respectively (Lecomte et al., 1994). The \( \text{EC}_{50} \) to \( E. \text{coli} \) aeruginosa (algae) (Halling-Sørensen et al., 2000). An \( \text{EC}_{50} \) of 0.25 mg/L was reported for \textit{Pseudomonas putida} mt-2 (Girardi et al., 1994). The XLF'Kn strain used in the present work was inhibited to CIP up to 20 mg/L (Bryan et al., 2004). In comparison to CIP, the reduced antimicrobial activity in the presence of mmt may create optimal conditions to select for antibiotic resistance rather than bacterial inhibition or death due to a net reduction in effective concentration. If the environmental equilibrium antibiotic concentration in the presence of mmt is lower than the MIC, it would yield more antibiotic resistant microbes. In support of this hypothesis, greater numbers of TC-resistant bacteria have been found in areas where farm manure is used as fertilizer (Sengeløv et al., 2003).

4. Conclusions

Higher amounts, up to 342 mg/g of TC or 337 mg/g of CIP, could be adsorbed on both external surfaces and interlayer spaces of montmorillonite. The adsorbed TC or CIP was stable against desorption using DI water. The strong adsorption of TC or CIP on mmt resulted in a significant decrease in antibiotic activity of TC or CIP at input concentrations significantly higher than the MICs. The decrease in antibiotic activity was displayed for both TC-sensitive and TC-resistant strains. On the other hand, the slow but persistent desorption of TC or CIP from mmt surfaces at sub-MIC or \( \text{EC}_{50} \) levels may induce bacteria resistance to the antibiotics adsorbed.
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References


