INFLUENCE OF ADSORBED ANTIBIOTICS ON WATER QUALITY AND SOIL MICROBES

Dr. Zhaohui Li
Department of Geosciences
University of Wisconsin – Parkside

Dr. Maria MacWilliams
Department of Biological Sciences
University of Wisconsin – Parkside

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Project Completion Report

By

Zhaohui Li, Professor

Department of Geosciences

University of Wisconsin – Parkside

Kenosha, WI 53141

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Project Summary

Title: Influence of Adsorbed Antibiotics on Water Quality and Soil Microbes

Project ID: WR10R006

Investigators: Dr. Zhaohui Li, Professor of Geosciences, Department of Geosciences, University of Wisconsin – Parkside

Dr. Maria P. MacWilliams, Department of Biological Sciences, University of Wisconsin – Parkside

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Background/Need: The use of antibiotics and growth hormones in human and veterinary medicine has had a significant effect on water quality. Many pharmaceutical compounds were even frequently detected in the treated effluent from many wastewater treatment plants located in different countries. Thus, the occurrence and biological impacts of pharmaceutically active compounds in the environment is becoming an emerging issue. Contradictory results have been reported on the antibacterial activity and bioavailability of antibiotics adsorbed on soils and sediments due to binding or complex formation. Furthermore, the mobility of ionizable antibiotics in soil environments has not been fully elucidated. Thus, there is an urgent need to investigate the fate and transport of antibiotics in soils and groundwater. In addition, the influence of adsorbed antibiotics on the antimicrobial activity of bacteria needs to be fully studied, too.

Objectives: The following objectives were focused during this period of study: (1) What are the adsorption and desorption kinetics of selected antibiotics onto or from the external surfaces of non-swelling clays and intercalated in the interlayer spaces of swelling clays? (2) How will desorption of antibiotics affect water quality? (3) What are the antimicrobial activities of the antibiotics adsorbed on external surfaces or intercalated into the interlayer spaces of clay minerals? (4) Will soil microbes develop resistance to the antibiotics adsorbed on external surfaces or intercalated into the interlayer spaces of clay minerals?

Methods: Kaolinite and illite representing non-swelling clays and montmorillonite representing swelling clays were preloaded with antibiotics on the external surface and in the interlayer space. Desorption of antibiotics from the external and interlayer spaces were conducted together with the tests on the influence of adsorbed antibiotics on their antimicrobial activities. Tetracycline (TC) and ciprofloxacin (CIP) were used as the representative antibiotics and *Salmonella enterica serotype typhimurium* and *Escherichia coli* as the test bacteria.

Results and Discussion: It was found that desorption of TC and CIP was minimal under normal low ionic strength conditions. However, the charges and types of desorbing cations had significant influence on TC and CIP desorption. The following trend was observed: hexadecyltrimethyallmonium, a cationic surfactant > Al^{3+} > Ca^{2+} > Na^{+} > H_2O. The XRD patterns before and after TC and CIP desorption revealed no changes in basal spacing, even after five desorption cycles, suggesting that the removal of TC or CIP from swelling clays was largely from the external surfaces.

Maximum desorption was achieved at alkaline condition, particularly when solution pH was higher than the pK_{a2} of CIP. Cation exchange was the major mechanism of TC and CIP desorption from clay mineral surfaces. The FTIR analyses provided an evidence of the formation of complexes between Al^{3+} and CIP, suggesting that the detachment of Al-CIP complex may contribute to the enhanced CIP desorption by Al^{3+}.

The effectiveness of the antimicrobial treatment was inferred from the relative number of colonies on the experimental plates to the no-antibiotic controls using efficiency of plating (EOP), which is defined as CFU/mL for experimental sample divided by CFU/mL for antibiotic only control.

In the absence of clay, even the lowest TC concentration (5 mg/L) tested inhibited growth of the TC-sensitive strains. Higher TC concentrations resulted in greater inhibition of cell growth as demonstrated by the lower EOP values. The high TC concentrations tested inhibited both the TC-sensitive and TC-resistant strains. At 500 mg/L TC, few if any colonies resulted. The standard lab inhibitory concentration was 10 mg/L. Binding of the TC to kaolinite partially ameliorated TC activity. The TC-resistant strains attained cell numbers close to the controls while the TC-sensitive samples were at least 20% of the untreated controls. The swelling SAz-1 clay was extremely effective at preventing TC activity. Initial studies showed no decrease in CFU/mL compared to the clay only controls for the TC range from 25 to 500 mg/L. Subsequent results determined that effective inhibition was demonstrated only above 5000 mg/L TC.

Conclusions/ Implications/ Recommendations:

The present results imply that much antimicrobial activities of the bound TC was sequestered and was unable to disrupt normal bacterial functions. As for whether or not exposure to TC-clay complexes increased the antibiotic resistance profile of the TC-sensitive strain, TC gradient plate screens did not reveal the clay-exposed cultures to be any more resistant than the unexposed control culture.

Related Publications:

Lv, G., Pearce*, C.W., Gleason*, A., Liao, L., MacWilliams, M.P., Li, Z. (2013) Influence of montmorillonite on antimicrobial activity of tetracycline and ciprofloxacin, *J. Asian Earth Sci.*, in press. http://dx.doi.org/10.1016/j.jseaes.2013.04.025

Chang, P.-H., Li, Z., Jean, J.-S., Jiang, W.-T., Wu, Q., Lin, K.-H., Kraus*, J. (2013) Desorption of tetracycline from montmorillonite by aluminum, calcium and sodium: an indication of intercalation stability, *Int. J. Environ. Sci. Technol.*, in press. http://dx.doi.org/10.1007/s13762-013-0215-2

Wu, Q., Li, Z., Hong, H., Li, R., Jiang, W.-T. (2013) Desorption of ciprofloxacin from clay mineral surfaces. *Water Res.*, **47**, 259-268. http://dx.doi.org/10.1016/j.watres.2012.10.010

Key Words: adsorption, antibiotics, antimicrobial activity, clays, desorption, resistance

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Introduction

Antibiotics are used for the therapeutic treatment of bacterial diseases in humans and animals. They are also commonly employed as growth promotants for cattle, swine, poultry, and fish. In 2000, more than 22.7×10^7 kg of antibiotics were produced in the United States and more than 40% of them were used as feed supplement to enhance animal growth [1]. The extensive use of pharmaceuticals resulted in frequent detection of their residues in final effluents of wastewater treatment plants (WWTPs) in Canada [2], Europe [3], the United States [4], and Australia [5]. An 80% frequency of detection with average influent concentrations of 48±3 and 47±4 μg/L and average effluent concentrations of 3.6±0.3 and 4.2±0.4 μg/L were reported for tetracycline (TC) and oxytetracycline (OTC), respectively, from several WWTPs in Wisconsin [6].

Although the measured concentrations were generally low and rarely exceeded drinking-water guidelines and advisories, many compounds do not have such guidelines established [7]. In addition, too little is known about the effects of these compounds, their metabolites, and degradation products in natural environments [8]. Moreover, because of the high excretion rates of bioactive compounds, there is a growing concern that the use of antibiotics in livestock production will promote the evolution of microbial populations resistant to the antibiotics used by humans [9]. Thus, the occurrence and biological impact of pharmaceutically-active compounds in the environment is becoming an emerging issue.

Some antibiotics may be deactivated or reduced in their activity due to sorption. Antibiotics present in soil and sediment can lose their antimicrobial activity as a result of binding to sediment particles or complex formation [10]. A strong reduction in the antibacterial effect of OTC due to formation of complexes in sea water was also noticed [11]. More recently, a link between the rroutine used of antibiotics in healthy farm animals and the rise in drug-resistant bacteria in humans was observed [12]. The controversy could be caused by the differences in adsorption sites (internal vs. external) for antibiotics and/or kinetics of desorption of antibiotics from soil or sediment surfaces.

In this study, the adsorption and desorption of antibiotics TC and ciprofloxacin (CIP) on and from different types of soil minerals were studied. The central goal was to assess the water quality and antimicrobial activity of clay-bound antibiotics and to evaluate any potential development of antibiotic resistance by soil microbes exposed to clay-bound antibiotics.

Procedures and Methods

Materials

All the soil minerals, except the montmorillonite (MMT) for CIP adsorption study, were obtained from the Source Clay Minerals Repository (Purdue University, West Lafayette, IN). They were used as received without further purification. SAz-1 is a high charge Ca-MMT containing 98% smectite, 1% quartz, 1% other; SAz-2 is a substitute for SAz-1. SWy-2 is a low charge Na-MMT containing 95% smectite, 4% quartz, 1% feldspar + gypsum; SHCa-1 is a Li-bearing trioctahedral smectite containing 97% smectite, 2% calcite, 1% dolomite + kaolinite; while SYn-1 is a synthetic mica-MMT containing 95% mica-MMT and 5% boehmite. Their reported cation exchange capacity (CEC) values were 1230, 850, 64 and 700-1400 $mmol_c/kg$, respectively, and their specific surface area (SSA) were 65, 23, 36 and 118 m^2/g, respectively. The kaolinite used was KGa-1b, a low defect kaolinite, and KGa-2, a highly defected kaolinite. KGa-1b contains 96% of kaolinite, 3% of anatase, 1% of crandallite, and trace amount of dickite. The CEC is 30±1 $mmol_c/kg$. The SSA is 13.1 m^2/g measured by the BET method.

TC was obtained from either Calbiochem (Darmstadt, Germany) or Alfa Aesar (Ward Hill, MA). It has a molecular weight of 444.43 g/mol. With different functional groups (Fig. 1), its pK_{a1}, pK_{a2}, pK_{a3} values were 3.3, 7.7, 9.7, respectively [13]. It had a $logK_{ow}$ value of -2.2 – -1.3 [13, 14]. CIP was obtained from Hangzhou Minsheng Pharmaceutical Group Co. Ltd (China). Its pK_{a1}, pK_{a2} values are 6.1 and 8.7, respectively [15]. The cationic form CIP^+ due to protonation of the amine group in the piperazine moiety predominates when solution pH is below 6.1 (Fig. 2). As the solution pH is above 8.7, the anionic form CIP^- due to loss of a proton from the carboxylic group prevails. When solution pH is between 6.1 and 8.7,

the zwitterionic form CIP^0 is the dominant species resulted from the charge balance of the two groups mentioned above.

Batch TC and CIP adsorption experiments

To each 50 mL centrifuge tube a fixed amount of clay minerals (normally 1.0 g of kaolinite or 0.1 g of MMT) and a fixed volume of solutions (either 10 or 20 mL) of different initial concentrations of up to 4000 mg/L for TC and CIP were combined and mixed on a reciprocal shaker at 150 rpm at room temperature for varying amount of time (e.g. 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 24.0 h) for kinetic study and 24 hours for all other studies. The centrifuge tubes were wrapped with aluminum foils to prevent light induced decomposition. After mixing, samples were centrifuged at 4000 rpm for 20 min or 15000 rpm for 5 min and the supernatant analyzed for equilibrium TC or CIP concentrations by a UV-Vis spectrophotometric method or an HPLC method. For the pH adsorption edge experiment, the solution pH was adjusted by adding 1 M HCl or 1 M NaOH several times over the course of equilibrium till the final desired pH values were reached. The amount of TC or CIP adsorbed was determined by the difference between initial and equilibrium concentrations. All experiments were run in duplicate.

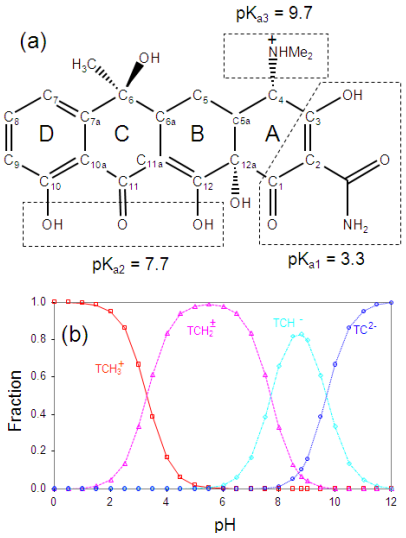

Fig. 1. Molecular structure (a) and speciation of TC under different pHs (b).

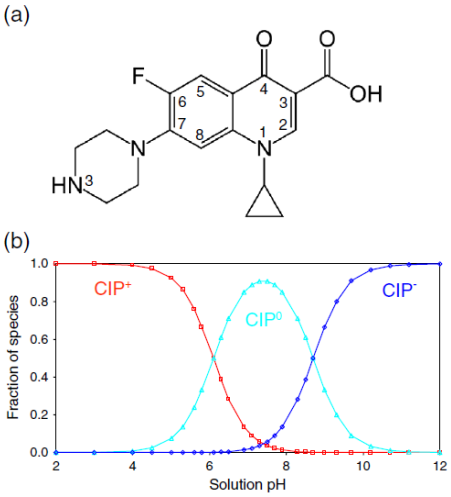

Fig. 2. Molecular structure (a) and speciation of CIP under different pH conditions (b).

Batch TC and CIP desorption experiments

To each 50 mL centrifuge tube, 0.1 g of SAz-2 and 20 mL of TC solution with varying concentrations were combined and shaken for 24 h. After being centrifuged, the supernatants were removed and analyzed for equilibrium TC concentration. After the supernatants were decanted, 20 mL of desorbing solution at an initial concentration of 0.05 M were added and the mixture shaken for varying amount of time for the kinetic study, and at different pH conditions for pH-dependent desorption. For kinetic desorption studies, the initial TC loadings were 37, 183, and 356 mg/g. As the solution was not buffered, the equilibrium solution pH varied from 3.5 to 4.6 under the desorption of 0.05 M Al^{3+}, Ca^{2+}, and Na^+. In this pH range, the TC will be mainly in its zwitterionic form TCH_2^0 (Fig. 1). The mixing time was 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 24.0 h. For pH desorption edge experiments, the initial TC loadings were 187 and 400 mg/g, corresponding to 35 and 75 % of the CEC of SAz-2. The equilibrium solution pH was adjusted by adding HCl or NaOH from 2 to 11 with an increment of 1. In addition, a five cycle desorption experiment was also performed, each with a concentration of 0.05 M of desorbing reagent or distilled water. The time for each desorption cycle was 6 h.

For CIP desorption experiments, 0.1 g CIP-preloaded MMT or 0.4 g CIP-preloaded kaolinite was combined with 20 mL of deionized (DI) water, or NaCl, $CaCl_2$, $AlCl_3$, or hexadecyltrimethylammonium

(HDTMA) solution of varying concentrations in each 50 ml centrifuge tube. Then, the mixtures were shaken at 25 °C for 24 h to reach desorption equilibrium. The experimental variables for desorption studies include, equilibrium solution pH, concentrations of different desorbing cations, and the number of desorption cycles. After centrifugation, the supernatant was passed through a 0.45 μm filter, and the equilibrium concentration of CIP in the supernatant was determined via UV/VIS spectrophotometer. For experiments involving in the influence of desorption cycles, after centrifuging, analyzing, and decanting the supernatant, another portion of 20 mL desorbing solution was added, and the mixture was shaken for 24 h. A total of five desorption cycles were performed. For desorption kinetic study, the mixtures were shaken on a reciprocal shaker at 150 rpm for 0.25, 0.5, 1, 2, 4, 8, 16, 24 h. The amount of TC or CIP desorbed was calculated from the equilibrium TC or CIP concentrations at each desorption cycle.

Chemical and instrumental analysis

The equilibrium TC concentrations were analyzed by a UV/VIS Spectrophotometer (SmartSpec 3000, Bio-Rad Corp.) or with a Milton Roy Spectronic 601 spectrophotometer (Ivyland, PA) at a detection wavelength of 254 nm. The detection limit was 0.002 mM and the linear response range was from 0.004 to 0.1 mM with the coefficient of determination $r^2 = 0.9997$. The CIP concentration was analyzed by a TU-1900 UV/VIS spectrophotometer (Puxi Instrumental Inc., Beijing, China) or with a Milton Roy Spectronic 601 spectrophotometer (Ivyland, PA) at a detection wavelength of 275 nm. The calibration curve was established with 5 standards between 0-20 mg/L with the coefficient of determination r^2 higher than 0.999.

The Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations were analyzed using an HPLC system with a mobile phase of 3.5 mM cupric sulfate and a PRP-X200 cation chromatographic column. At a flow rate of 2.0 mL/min, the retention time was 1.4, 1.9, 2.9, and 3.9 min for Na^+, K^+, Mg^{2+} and Ca^{2+}, respectively. The detection limit was 0.05 mM for the cations, and the linear range was from 0.1 to 1 mM.

Powder XRD analyses were performed on an X'Pert PRO (Pananlytical, The Netherland) with Cu-Kα radiation at 40 kV and 30 mA or on a Rigaku D/Max-IIIa with Ni-filtered Cu-Kα radiation at 30 kV and 20 mA, or on a Bruker D8 Advance diffractometer with a Cu-Kα radiation at 40 kV and 40 mA and a SOL-XE energy-dispersive detector. Samples were scanned from 2° to 20° 2θ at 1°/min with a scanning step of 0.01°/step. A 1° divergent slit and scatter slit and 0.3 mm receiving slit were used.

Bacterial strains, reagents, MIC determination

Standard aseptic microbiology protocols were used for all experiments. Two *Escherichia coli* isogenic strains were used: XL1Blue (XLF'Tc) was the TC-resistant control strain and XL1Blue MR F'Kan (XLF'Kn), 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 (MIC) were determined for the XL1 lab strains using a variation of the microtiter plate method described previously [16]. 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.

Antimicrobial activity tests

The antimicrobial activities of TC were tested against the XLF'Tc, and XLF'Kn 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 XLF'Kn and 10 μg/mL for XLF'Tc. 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:

$$CFU/mL = \frac{Colonies\ \ present}{Volume\ \ spotted(ml)} \times Dilution\ \ \ factor \quad (1)$$

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 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 of"), and then each tube ("prior" and "day of" LB clay samples) was inoculated with 110 µL of log-phase *E. coli* XLF'Tc or XLF'Kn 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+ 4 concentrations of CIP was mixed with 1.8 mL LB in a sterile test tube. Each tube was then inoculated with 0.10 mL of bacteria in log-phase XLF'Kn 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 hour incubation period, these samples were plated to determine CFU/mL as previously described.

Results and Discussion

Antibiotic adsorption on different types of clay minerals

Adsorption of TC on different MMT at ambient pH is plotted in Fig. 3. The experimental data were fitted well by the Langmuir type isotherm:

$$C_S = \frac{K_L S_m C_L}{1 + K_L C_L} \qquad (2)$$

where C_S is the amount of TC adsorbed on solid at equilibrium (mg/g), C_L the equilibrium solute concentration (mg/L), S_m the apparent adsorption capacity or adsorption maximum (mg/g), and K_L the Langmuir coefficient (L/mg). TC adsorption maxima reached to a few hundred mg/g, indicating superior capacity of TC uptake by MMT. When the capacity values were converted into mmol/g, they corresponded to 0.74, 0.96, 0.36, and 0.75 mmol/g, or 0.87, 0.78, 0.51, and 1.13 CEC of the minerals for SWy-2, SAz-1, SYn-1, and SHCa-1, respectively. In contrast, TC adsorption on kaolinite was much lower, reaching a capacity of 0.009 mmol/g (Fig. 4). The difference in TC uptake by MMT and kaolinite could be attributed to their drastic difference in CEC values.

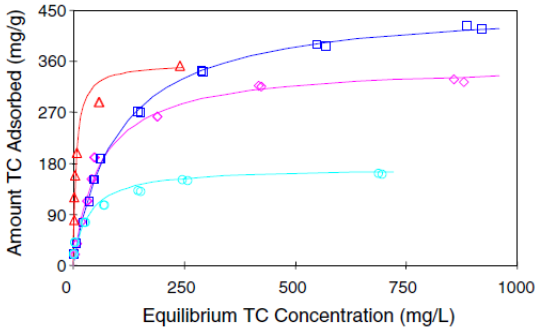

Fig. 3. TC adsorption on SWy-2 (◇), SAz-1 (□), Syn-1 (○), and SHCa-1 (Δ) at pH 4–5. Lines are Langmuir fit to the observed data.

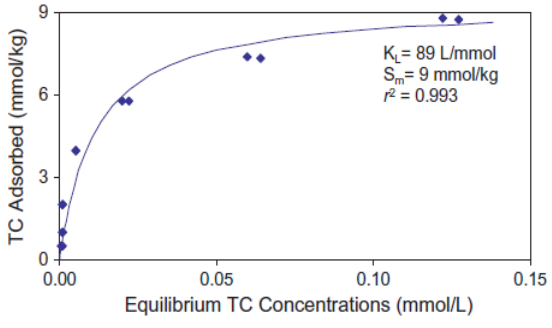

Fig. 4. TC adsorption on KGa-1b. The line is the Langmuir fit to the observed data.

Solution pH had less effect on TC adsorption onto MMT when less than 7.7, the pK_{a2} value of TC (Fig. 5). A similar observation was found for T adsorption on kaolinite (Fig. 6). When solution pH is less than the pK_{a2} value but greater than the pK_{a1} value of TC at 3.3, the TC will be in zwitterionic form TCH_2^0. The significant TC adsorption at this pH range confirmed that the ammonium groups still played an important role in TC uptake even in zwitterionic form.

In comparison to TC adsorption, CIP adsorption on MMT and kaolinite was similar (Figs. 7 and 8). Plot of desorption of exchangeable cations against CIP adsorption resulted in a slope of 1 (Fig. 8 inset), confirming that cation exchange was the dominating mechanism of CIP uptake on clay minerals. similarly, when solution pH was less than the pK_{a2} value of 8.7, CIP adsorption was less affected by changes in solution pH (Figs. 9 and 10), again confirming that protonation of the amine group in the piperazine moiety still played an important role in CIP uptake even in when ICP is in its zwitterionic form. As the solution pH was greater than the pK_{a2} CIP adsorption was greatly reduced.

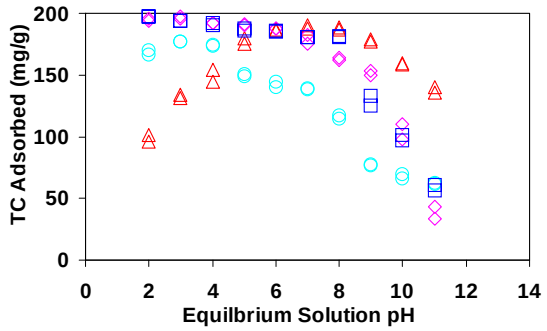

Fig. 5. TC adsorption on SWy-2 (◇), SAz-1 (□), Syn-1 (○), and SHCa-1 (Δ) as affected by equilibrium solution pH.

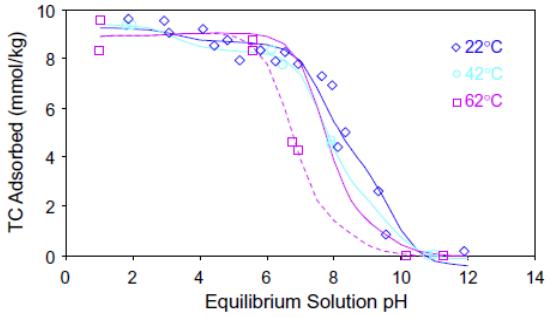

Fig. 6. TC adsorption on kaolinite as affected by solution pH. Lines are numerical fits to the observed data using pK_{a1}, pK_{a2}, and pK_{a3} values.

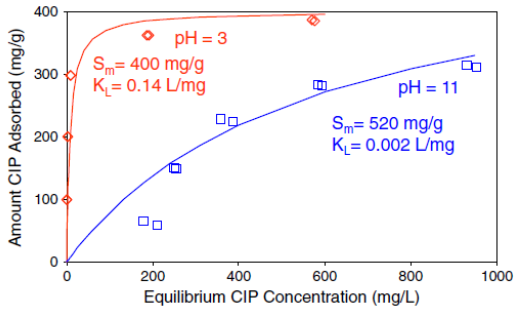

Fig. 7. CIP adsorption on MMT at pH 3 and 11. The lines are Langmuir fits to the observed data.

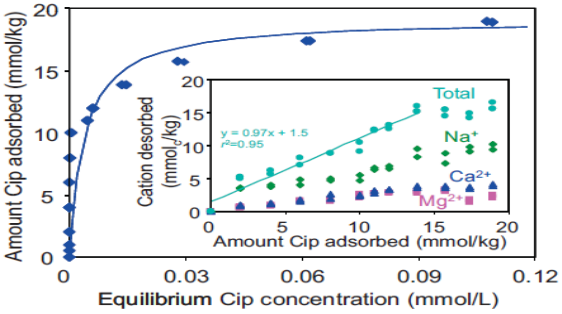

Fig. 8. CIP adsorption on kaolinite. The line is the Langmuir fit to the observed data. The inset shows desorption of exchangeable cations accompanying CIP adsorption.

XRD analyses after TC adsorption on MMT showed drastic difference in comparison to the mixtures of MMT and TC at the same ratio (Fig. 11). For the physical mixture both MMT and TC peaks were present. However, there was no expansion of d_{001} spacing of MMT in the mixture (Fig. 11a). In contrast a significant increase in d_{001}-spacing was found after TC uptake on MMT (Fig. 11b).

Similarly, with progressive increase in CIP adsorption on MMT, the d_{001}-spacing expanded from 12.5 to 20.7 Å (Fig. 12a). Under neutral condition, the solubility of CIP decreased, resulting in CIP precipitates in addition to CIP intercalation (Fig. 12b). Under alkaline conditions, a separate peat at 23.4 Å showed up at higher CIP adsorption levels (Fig. 12c).

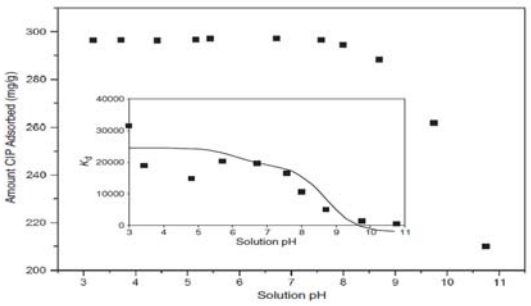

Fig. 9. CIP adsorption on MMT as affected by solution pH. The line of the inset shows the change of K_d as a function of pH.

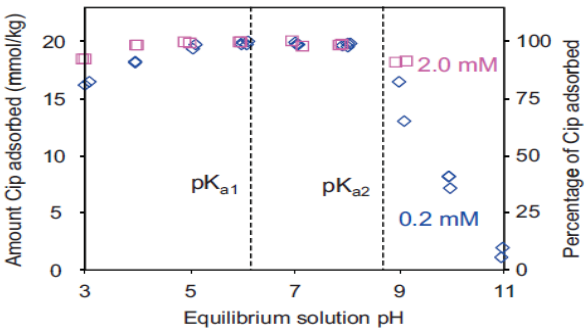

Fig. 10. Effect of solution pH on CIP adsorption on kaolinite at initial CIP concentrations of 0.2 and 2.0 mM.

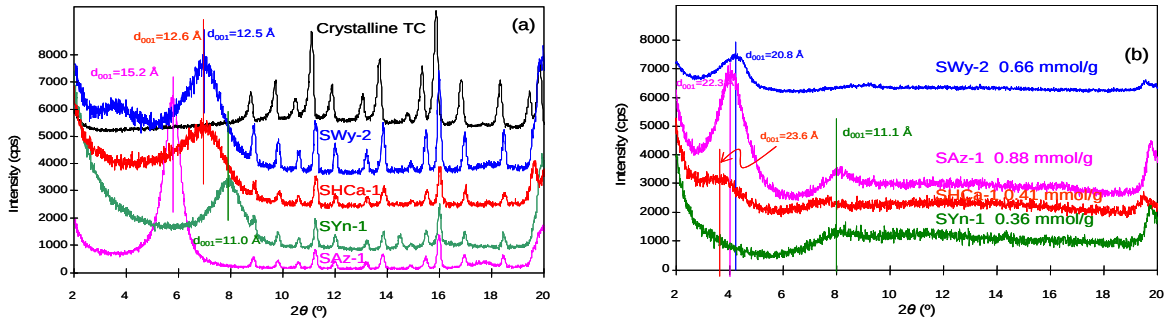

Fig. 11. X-Ray diffraction patterns of raw MMT mixed with TC (a) and MMT intercalated with TC (b).

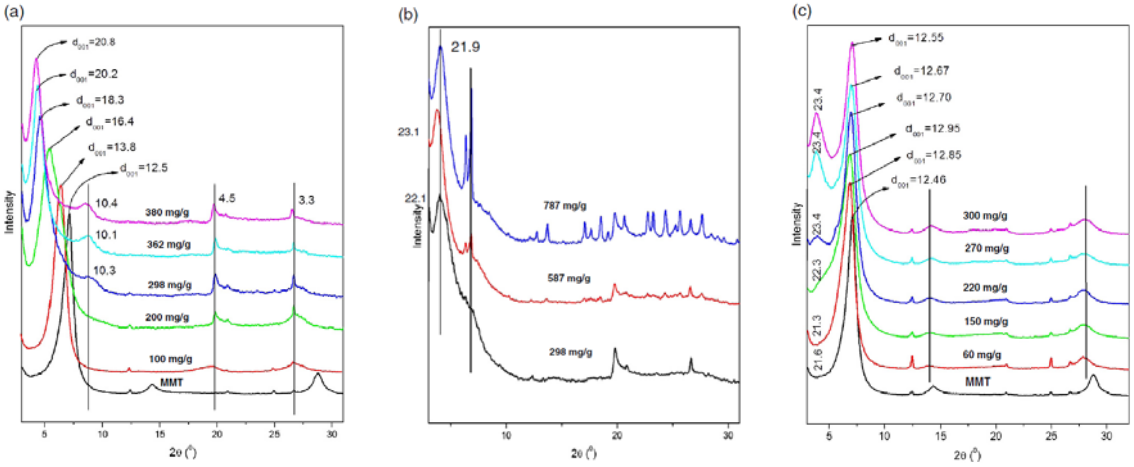

Fig. 12. XRD patterns of MMT intercalated with different amounts of CIP in acidic (a), neutral (b), and alkaline (c) conditions. The d-spacings are in Å.

Desorption of antibiotics from different types of clay minerals

Regardless of the initial TC adsorption levels, TC desorption by solutions of different cations from Ca-MMT reached equilibrium at 8 h or less Fig. 13a-c. In addition, as the amount of TC adsorption increased, desorption of TC increased (Fig. 13d). Moreover, as the solution pH increased, particularly as the solution pH was greater than the pK_{a2} value of TC, significant TC desorption was observed (Fig. 13e).

Desorption of CIP, on the other hand, from kaolinite and MMT showed hysteresis and was instantaneous (Fig. 14a-d). Again, desorption of CIP increased significantly as the solution pH was

greater than the pK_{a2}, when CIP was in its anionic form (Fig. 14e&f). Meanwhile, more CIP desorbed from kaolinite or MMT surfaces at a higher CIP loading level.

The XRD analysis of TC desorption by different cations under different time and different desorption cycles showed no change in d_{001}-spacing, suggesting that the removal of TC from MMT was from the external surfaces instead of from the interlayer (Fig. 15).

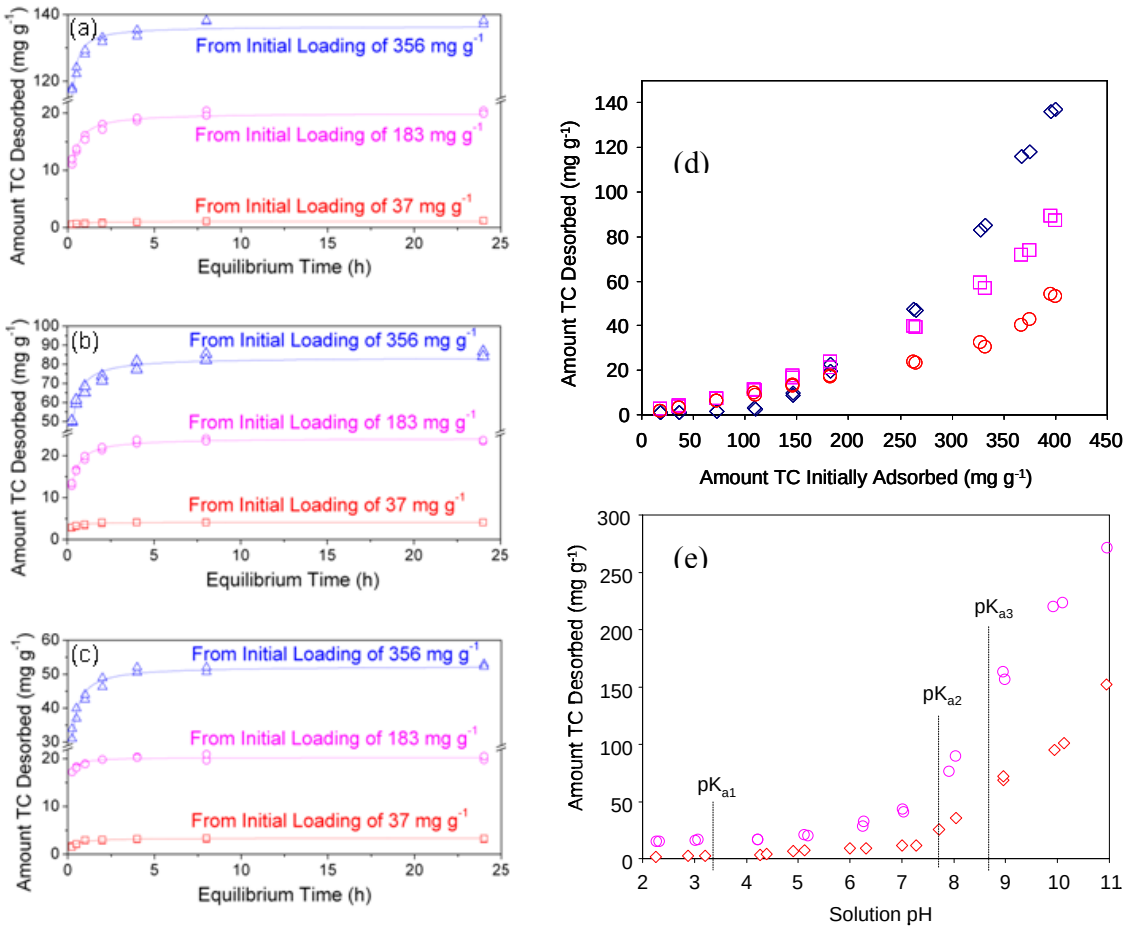

Fig. 13. Desorption kinetics of TC from SAz-2 by 0.05 M $AlCl_3$ (a), $CaCl_2$ (b), and NaCl (c). The solid lines are pseudo-second order kinetic fit to the observed data. TC desorption from SAz-2 at different initial loadings by $AlCl_3$ (◇), $CaCl_2$ (□), and NaCl (○) (d). TC desorption from SAz-2 as affected by solution pH at initial TC loadings of 187 mg/g (◇) and 400 mg/g (○) (e).

Antimicrobial activity of TC and CIP

The antibiotic minimum inhibitory concentrations were determined for each of the *E. coli* strains. XLF'Kn had a TC MIC of 1x 10^{-3} mg/mL and a CIP MIC of 2.5 x 10^{-4} mg/mL. The XLF'Tc, with an MIC of 1.25 x 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.

Antimicrobial effectiveness of TC and CIP adsorbed on MMT

The impact of MMT on antibiotic growth inhibition profile was examined by measuring the cell number after a three hour exposure to antibiotics in the presence or absence of the clay.

When neither clay nor antibiotic was present, XLF'Tc and XLF'Kn reached a similar cell density (Fig. 16). For the XL'Kn strain, cell growth was slowed in the presence of 0.05 mg/mL such that the resulting density after 3 hour incubation was only one third that of the antibiotic-free culture. At the highest concentration tested (0.5 mg/mL), no colonies resulted as bacteriostatic antibiotics could 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 from 5.0 x10^7 to 5.5 x10^3 CFU/mL. Thus, even the growth of the TC-resistant strain was inhibited greatly at this high level of TC.

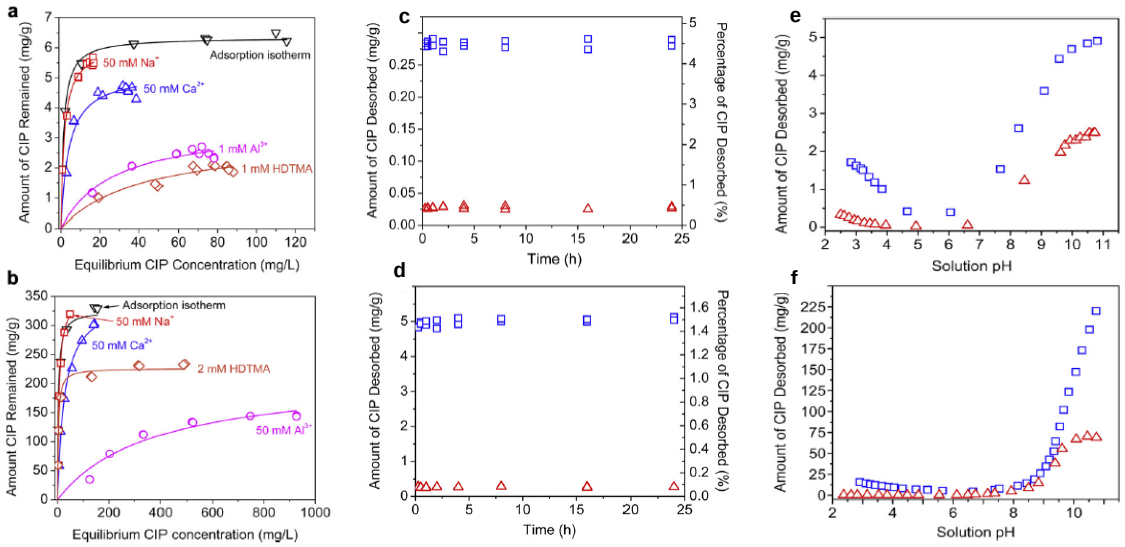

Fig. 14. Desorption isotherm of CIP from kaolinite (a) and MMT (b) under different Na^+, Ca^{2+}, Al^{3+}, and HDTMA concentrations. CIP desorption by deionized water as a function of desorption time and initial CIP loading of 50% (△) and 100% (□) from kaolinite (c) and MMT (d). Effect of solution pH on CIP desorption from kaolinite (e) and MMT (f) at initial loading of 50% (△) and 100% CEC values (□).

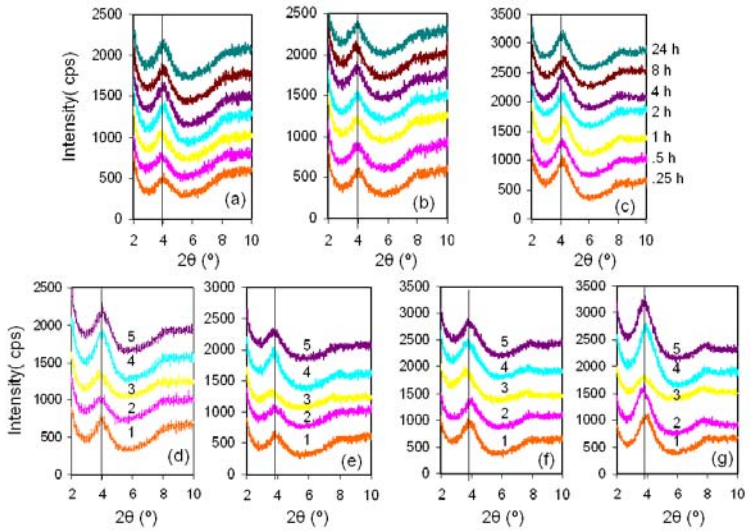

Fig. 15. XRD patterns of SAz-2 desorbed by 0.05 M $AlCl_3$ (a), $CaCl_2$ (b), and NaCl (c) at different time; desorbed by 0.05 M $AlCl_3$ (d), $CaCl_2$ (e), NaCl (f), and water (g) under different desorption cycles.

With MMT alone, both the XLF'Tc and XLF'Kn strains reached cell densities comparable to the no clay controls (Figs. 16&17). 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 CIP MIC for the XLF'Kn was 2.5 x 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 at a TC concentration of 0.5 mg/mL (Fig. 18). However, a complete inhibition was seen at 0.1 mg/mL, showing that the antibiotics was still active even in the presence of MMT. Part of the inhibition could be due to CIP that was not adsorbed on the clay but the major inhibition was associated with bound CIP (Fig. 19). The results from this study agree well with a previous observation that CIP remained biologically active over time and adsorption did not completely eliminate the effects of this compound [17].

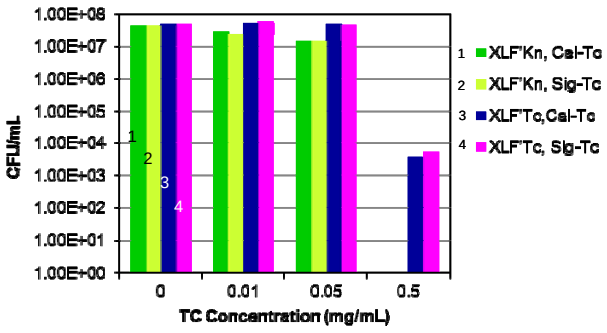

Fig. 16. Effect of TC alone on bacterial growth. The XLF'Kn strain is sensitive to TC. The XLF'Tc strain is resistant to TC due to the TC resistance gene on the F' plasmid. The efficacies of TC from different commercial sources were compared.

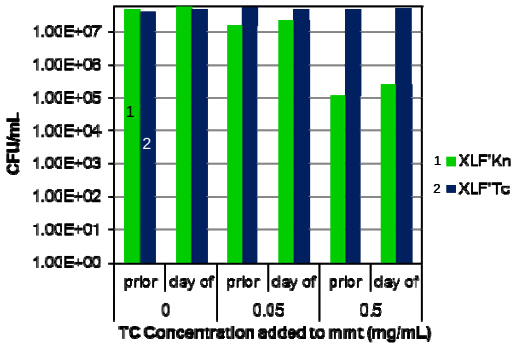

Fig. 17. Effects of MMT+TC on bacterial growth of XLF'Tc and XLF"Kn strains. For the "prior" samples, the MMT-TC was mixed with LB immediately after the antibiotic loading. In the "day of" samples, the medium addition was delayed until the day of the bacterial growth experiments.

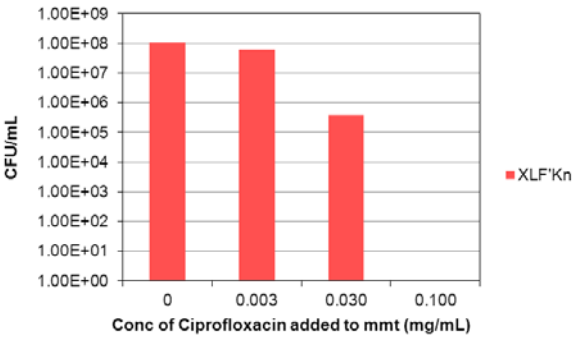

Fig. 18. Effect of CIP+MMT on bacterial growth of XLF'Kn.

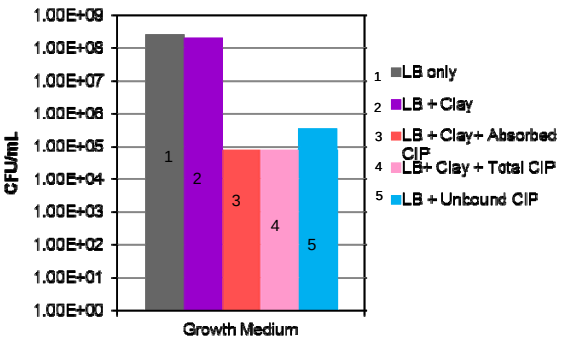

Fig. 19. Cell growth studies on the influence of present CIP on the growth of bacteria.

The large decrease in effectiveness of TC's antimicrobial activity in the presence of MMT and the ability of CIP to still function in the same situation could be attributed to different mechanisms of

antibiotic-microbial interactions. TC works by binding the 30S ribosomal subunit, and through an interaction with 16S rRNA, it can prevent the docking of amino-acylated tRNA. While for CIP, it functions by inhibiting DNA gyrase, a type II topoisomerase, and also topoisomerase IV, and kills bacteria by interfering with the enzymes that allow DNA to unwind during replication, thus stopping DNA and protein synthesis. In the current study, identifying exact mechanisms causing the difference in reduction of antimicrobial activity when adsorbed on MMT was difficult. Nevertheless, exposure to sub-MIC levels of certain antibiotics could selects for development of low-level resistance to other functionally- and structurally- unrelated antibiotics [18]. By comparison, 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 [19].

Conclusions and Recommendations

MMT had larger capacity for TC and CIP adsorption, which would limit their mobility in subsurface environment. Desorption of TC and CIP from MMT only occurred from the external surfaces. Solution pH had a strong influence on antibiotics sorption and desorption. The strong adsorption of TC or CIP on MMT resulted in a significant decrease in their antibiotic activities at input concentrations significantly higher than the MIC 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 the sub-MIC or EC_{50} levels may induce bacteria resistance to the antibiotics adsorbed, which needs to be further investigated.

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Appendix A:

Journal Publications:

Li, Z., Stockwell*, C., Niles*, J., Minegar*, S., Hong, H. (2013) Uptake and removal of sulfa drugs sulfadiazine using hydrophilic zeolite. *Appl. Environ. Soil Sci.*, accepted.

Lv, G., Stockwell*, C., Niles*, J., Minegar*, S., Li, Z., Jiang, W.-T. (2013) Uptake and retention of amitriptyline by kaolinite. *J. Colloid Interface Sci.* in press. http://dx.doi.org/10.1016/j.jcis.2013.08.026

Chang, P.-H., Li, Z., Jean, J.-S., Jiang, W.-T., Wu, Q., Lin, K.-H., Kraus*, J. (2013) Desorption of tetracycline from montmorillonite by aluminum, calcium and sodium: an indication of intercalation stability, *Int. J. Environ. Sci. Technol.*, in press. http://dx.doi.org/10.1007/s13762-013-0215-2

Lv, G., Pearce*, C.W., Gleason*, A., Liao, L., MacWilliams, M.P., Li, Z. (2013) Influence of montmorillonite on antimicrobial activity of tetracycline and ciprofloxacin, *J. Asian Earth Sci.*, in press. http://dx.doi.org/10.1016/j.jseaes.2013.04.025

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Wu, Q., Li, Z., Hong, H. (2013) Adsorption of the quinolone antibiotic nalidixic acid onto montmorillonite and kaolinite, *Appl. Clay Sci.*, **74**, 66-73. http://dx.doi.org/10.1016/j.clay.2012.09.026

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* Denotes undergraduate students from University of Wisconsin – Parkside.

Conference Presentation: (Only invited presentations are listed)

"Intercalation of pharmaceuticals to clays" Keynote to 2012 International Conference on Advanced Material and Manufacturing Science (ICAMMS 2012), Beijing, Dec. 20, 2012.

"Interactions between Pharmaceuticals and Clays in Aqueous Solution", to China University of Geosciences (Wuhan), Jan. 5, 2012.

"Interactions between Pharmaceuticals and Clays in Aqueous Solution", to Geosciences Department, University of Wisconsin - Milwaukee, Nov. 10, 2011.

"Interaction between Some Antibiotics and Swelling Clays in Aqueous Solution", American Chemical Society Annual Meeting, San Francisco, March 21–25, 2010.

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