# BIOAVAILABILITY OF TETRACYCLINE IN WATER AND SOIL TO *ESCHERICHIA COLI* FOR EXPRESSION OF ANTIBIOTIC RESISTANCE

By

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## ABSTRACT

### BIOAVAILABILITY OF TETRACYCLINE IN WATER AND SOIL TO *ESCHERICHIA COLI* FOR EXPRESSION OF ANTIBIOTIC RESISTANCE

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Tetracyclines are a class of antimicrobials extensively used as human and veterinary medicine, and in livestock production since they were discovered in the 1940s. A large portion of tetracyclines administered to humans and animals are excreted and subsequently released into the environment, where they pose potential risks to ecosystem and human health. There is a growing concern that the presence of antibiotics such as tetracycline at trace levels in the environment is related to the emergence and ever-increasing abundance of antibiotic resistance genes in natural and engineered microbial populations. However, basic knowledge at the molecular scale of bacterial access to tetracyclines present in environmental matrices and expression of antibiotic resistance genes remain nearly unknown. In this study, we used the *E. coli* MC4100/pTGM whole-cell bioreporter as an effective tool to investigate bioavailability of tetracycline in water and soil to bacteria for expression of antibiotic resistance genes. Our hypothesis was that the speciation of tetracycline dissolved in water and sorption by soil minerals controls the bioavailabilities for bacterial uptake and subsequent activation of antibiotic resistance genes. The results revealed that activation of antibiotic resistance in the *E. coli* bioreporter responded linearly to intracellular tetracycline concentration. The extent of tetracycline uptake by *E. coli* was modulated by tetracycline speciation. We have identified that zwitterionic tetracycline as the primary species favorable for bacterial

uptake. Geochemical factors such as pH, salt composition and concentration influenced the fractional distributions of tetracycline species in aqueous solution and hence altered uptake by *E. coli*. In addition, the presence of organic ligands could also alter tetracycline speciation by releasing tetracycline from its metal complexes in aqueous solution. For tetracycline associated with Mg-smectite, desorption of tetracycline from clay to solution was the major exposure pathway for bacterial uptake and subsequent activation of antibiotic resistance in the diluted clay suspensions. In clay film cultivation, clay-sorbed tetracycline was still bioaccessible to *E. coli* evoking strong expression of antibiotic resistance. Direct contact of the *E. coli* bioreporters with clay surfaces and further formation of biofilms plausibly facilitated tetracycline transfer to bacteria. Overall, this study greatly advances the fundamental understanding of bioavailability of tetracycline in the environment to bacteria for expression of antibiotic resistance genes.

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# CHAPTER I

# LITERATURE REVIEW, RESEARCH HYPOTHESES AND OBJECTIVES

## INTRODUCTION

Tetracycline antibiotics have been widely administered to humans and animals since they were discovered in the 1940s. This class of antibiotics, collectively referred to as tetracyclines, includes chlortetracycline, oxytetracycline, tetracycline, demethylchlortetracycline, rolitetracycline, methacycline, doxycycline, minocycline, and the most recent member tigecycline (Durckheimer, 1975; Meagher et al., 2005; Passarell et al., 2009). These compounds all share a similar core structure consisting of four linearly fused 6-carbon cyclic rings with attached amide, tricarbonyl methane, diketone, and dimethylammonium functional groups. Tetracyclines are broad-spectrum antimicrobials exhibiting activities against a wide range of gram-positive and gram-negative bacterial strains plus atypical organisms such as chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites (Chopra and Roberts, 2001).

The effective antimicrobial action and the absence of major adverse side effects have led to large-scale production and extensive use of tetracyclines in human and animal infection therapy, and in livestock feeding operations. Antibiotics administered to humans commonly end up in the influents to municipal wastewater treatment plants (WWTPs) (Ding et al., 2011; Gao et al., 2012a; Gobel et al., 2004; Karthikeyan and Meyer, 2006; Spongberg and Witter, 2008; Ternes, 1998). However, the current treatment processes operating in WWTPs are not specifically designed to effectively remove pharmaceuticals such as tetracyclines, and hence, certain fractions are discharged in effluents from WWTPs (commonly referred to as reclaimed water). Removal efficiencies of WWTPs for pharmaceuticals in general range from approximately 10 to 95% (Gao et al., 2012a; Karthikeyan and Meyer, 2006; Spongberg and Witter, 2008).

Irrigation of agricultural lands/crops using reclaimed water from WWTPs has been increasingly adopted in many arid and semi-arid regions to promote sustainable crop production and enhance profitability. One suspected consequence of global climatic change is an increase in the duration and frequency of drought periods in many regions of the world causing greater demands of fresh water for agricultural irrigation (Gleick, 2003). Moreover, the ever-increasing world population increases the requirements for all natural resources as exemplified by the escalating use of fresh water, which is arguably our most essential natural resource. On a global basis, ~70% of the freshwater currently consumed is used for crop irrigation, 20% for industrial purposes and the remaining 10% for domestic use (Zimmerman et al., 2008). As a partial solution to the problem of freshwater stress/shortage, secondary or tertiary treated effluents from WWTPs are being increasingly utilized for crop irrigation. Unfortunately, this practice undoubtedly introduces pharmaceuticals, including many tetracyclines, into agroecosystems (Chefetz et al., 2008; Kinney et al., 2006; Wang and Gardinali, 2012; Yao et al., 2012). The consequences to human and ecosystem health of chronic exposure to an undefined mixture of pharmaceutical chemicals designed to be bioactive at low concentrations are unknown (Boonsaner and Hawker, 2010; Boxall et al., 2006; Calderon-Preciado et al., 2011; Macherius et al., 2012; Wu et al., 2010), but potentially of enormous significance.

Tetracyclines and many other veterinary pharmaceuticals are commonly administered to livestock for disease control, and added into feeds at subtherapeutic levels to improve feeding efficiency, growth rate and animal health. The subtherapeutical doses applied as feed supplements enables highly intense livestock management systems as exemplified by concentrated animal feeding operations (CAFOs). This practice was

rapidly adopted once antibiotics were first shown to be effective in promoting livestock growth in the early 1950s (Bartley et al., 1950; Gustafson and Bowen, 1997; Stokstad and Jukes, 1950). According to the Food and Drug Administration, in 2011 more than 13 million kilograms of antimicrobial drugs were administered for livestock production in the United States (2011). Among the variety of pharmaceuticals used, tetracyclines rank as the most highly used group of antibiotics at 5.6 million kilograms per annum, equivalent to ~42% of the total antibiotics used annually for livestock production. Large fractions of tetracyclines used in animal feeding operations are excreted with manure and urine either as parent compounds or bioactive metabolites (Aga et al., 2005; Jacobsen et al., 2004). For instance, 40-80% of oxytetracycline administered in livestock feed is excreted unchanged (Halling-Sorensen et al., 2001; Kay et al., 2004).

After a period of storage (e.g. several months), manure waste (feces and urine) containing high levels of antibiotics (i.e. $\mu g\ kg^{-1}$ to $mg\ kg^{-1}$) are commonly land applied to agricultural fields as an inexpensive means of disposal, and for their ancillary fertilizer value. As a consequence, antibiotics are introduced into the soils and waters of agroecosystems (Hamscher et al., 2005; Larsbo et al., 2009; De Liguoro et al., 2003; Snow et al., 2008; Song et al., 2010). These antibiotics may be decomposed to a certain degree during manure storage, but degradation rates are typically slow (relative to holding time) under the anaerobic conditions prevalent in animal manure storage pits. Thus, significant amounts of antibiotics remain in the manure waste, and subsequent land application results in the dissemination of multiple classes of veterinary antibiotics and hormones in the receiving soil and hence to surface water, groundwater, and sediment (Christian et al., 2003a; Dolliver and Gupta, 2008; Jacobsen et al., 2004; Kolpin et al.,

2002). For example, tetracyclines have been found in liquid manure at concentrations up to 40 mg $L^{-1}$ (Kay et al., 2004), and up to 200 μg $kg^{-1}$ tetracycline in soils fertilized with liquid manure (Hamscher et al., 2002). In a two-year liquid manure application (to soil) study, the rate of tetracycline accumulation exceeded the rate of degradation, suggesting that tetracyclines can accumulate over time, hence acting as "persistent" compounds in soils (Hamscher et al., 2002).

Under the current practice of CAFOs, subtherapeutic use of antibiotics contributes to lowering the costs of meat and related food products, and to maintaining high profits for farmers. Unfortunately, the widespread dissemination of antibiotics in the environment emanating from feeding operations, potentially promotes the development and proliferation of antibiotic resistant bacterial strains. The occurrence of (multi)drug-resistant human pathogens is a direct and ever-increasing threat to human health, and has been referred to as a "ticking time bomb" in the popular press. Banning the use of antibiotics for growth promotion in CAFOs has often been suggested (especially by the medical community), and implemented in the European Union. However, the economic benefits for livestock producers and the concomitant increase in food price are major concerns, so this practice continues in the United States. It has been estimated that the phaseout of subtherapeutic use of antibiotics would cost hundreds of millions to billions of dollars annually for swine production alone in the U.S. (Brorsen et al., 2002; Wade and Barkley, 1992), so the economic pressures for the continued use of antibiotics in livestock feed are powerful.

In addition to dissemination of tetracyclines from land application of manure waste and biosolids, and direct discharges from WWTPs, tetracyclines are also sprayed

into aquaculture ponds and onto fruit trees for disease control (Cabello, 2006; McManus et al., 2002; Schnabel and Jones, 1999; Seyfried et al., 2010). Currently, CAFOs, aquaculture facilities, and municipal WWTPs are the primary sources for the dissemination of antibiotics to surface water, groundwater, soils and sediments (Boxall et al., 2003; Calamari et al., 2003; Halling-Sorensen et al., 2002; Jacobsen et al., 2004; Kolpin et al., 2002; Lalumera et al., 2004; De Liguoro et al., 2003; McArdell et al., 2003; Miao et al., 2004; Sacher et al., 2001). Among these sources, the amount of tetracyclines used in livestock production is much greater than that used for human disease control, and the public perceives CAFOs as the primary concern. The first comprehensive national reconnaissance on chemicals of emerging concern in streams of the U.S. showed that during 1999-2000 approximately 50% of surface waters from 139 streams and rivers across 30 states were contaminated by antibiotics. Tetracyclines were found in water at concentrations of 0.11 $\mu g\ L^{-1}$ for tetracycline, 0.34 $\mu g\ L^{-1}$ for oxytetracycline, and 0.69 $\mu g\ L^{-1}$ for chlortetracycline (Kolpin et al., 2002).

Although the levels of tetracyclines found in the environment are far below the thresholds required to exhibit medicinal inhibitory effects on bacterial populations, they exert selective pressure resulting in enrichment of antibiotic resistance genes and increase of antibiotic resistant bacterial populations in the environment (Alexander et al., 2009; Chapin et al., 2005; Chee-Sanford et al., 2001; Pruden et al., 2006; Reinthaler et al., 2003; Rhodes et al., 2000; Whittle et al., 2003; Witte, 1998). Development of antibiotic resistance in microorganisms, and antibiotic resistance gene transfer between organisms have become ever-increasing global health threats originating partially from to the widespread distribution of antibiotics in the environment due to unsatisfactory waste

management at animal production facilities (Campagnolo et al., 2002; Chee-Sanford et al., 2009; Cotta et al., 2003; Davies, 1994; Gilchrist et al., 2007a; Pruden et al., 2006; Reinthaler et al., 2003; Rhodes et al., 2000; Whittle et al., 2003; Witte, 1998). In natural environments, many microorganisms can carry multiple and mobile antibiotic resistance genes which can be horizontally transferred between bacterial species, and even into those residing in animals and humans (Davies, 1994; Gilliver et al., 1999; Rhodes et al., 2000; Schnabel and Jones, 1999; Smith et al., 1999). Acquisition of multi-antibiotic resistance may render pathogenic bacteria particularly virulent by preventing effective treatment by standard antibiotics. The therapeutic use of antibiotics to treat livestock disease is generally less likely to promote the emergence and propagation of antimicrobial resistant bacteria because of the relatively short-term and infrequent administration of antibiotics to individual animals (Levy, 1998). In contrast, subtherapeutic use of antibiotics as feed supplements supplies a continuous and low-dosed source of antibiotics. In addition, this practice has been widely used for large numbers of animals, e.g. CAFOs. The low-dosed, prolonged course of antibiotic exposure provides a long-term constant selective pressure on bacteria to outgrow other bacteria and hence propagate antibiotic resistant strains.

## TETRACYCLINE CHEMISTRY IN THE ENVIRONMENT

Tetracycline molecules contain three ionizable functional groups, *viz.* tricarbonylmethane, diketone and dimethylammonium, attached to the fused 6-carbon ring structure (Figure 1.1A). These functional groups dissociate in aqueous solution to form cations (+ 0 0), zwitterions (+ - 0), and anions (+ - - , 0 - -) with the fractional distributions depending on solution pH (Figure 1.1B). In addition, the ionic species can

complex with naturally occurring inorganic cations (e.g. $Ca^{2+}$ and $Mg^{2+}$) in aqueous solution in a variety of steric configurations (Gulbis et al., 1976; Othersen et al., 2003, 2006; Wessels et al., 1998). All these species can potentially interact with geosorbents in a variety of ways such as replacement of exchangeable cations on clays, and complexation with multivalent cations on soil organic matter. For example, the dimethylammonium moiety on C4 in species I, II, and III can exchange inorganic cations associated with cation-exchange sites in soils (Figueroa et al., 2004; Kulshrestha et al., 2004; Sassman and Lee, 2005). The phenolic diketone on C10, C11 and C12, and the C3 hydroxy group can deprotonate and produce anions, which can complex with divalent and multivalent cations (Figueroa and Mackay, 2005; Porubcan et al., 1978; Sithole and Guy, 1987a, 1987b). Experimental results show that at typical soil pH values sorption of tetracycline occurs primarily at soil domains containing negatively charged sites, most importantly in soil organic matter and smectite clays (Chang et al., 2009; Figueroa et al., 2004; Kong et al., 2012; Kulshrestha et al., 2004). Sorption of tetracyclines to smectite clay minerals ranged from 250 to 600 $\mu mol\ g^{-1}$ (Ding et al., 2011; Figueroa et al., 2004), approximately 8 to 150 times the sorption to Fe-oxides (Figueroa and Mackay, 2005; Gu and Karthikeyan, 2005). Sorption of tetracycline to soil organic matter, e.g. humic acid, was comparable to sorption by smectite clays (Ding et al., 2012; Gu et al., 2007; MacKay and Canterbury, 2005). Sorption by Ca-saturated clay minerals or humic acids demonstrated a greater affinity than that by the corresponding Na-exchanged sorbents, indicating formation of complexes between $Ca^{2+}$ and tetracycline (Figueroa et al., 2004; MacKay and Canterbury, 2005; Sithole and Guy, 1987a). It is apparent from studies

described above that the electrostatic interactions (cation exchange and complexation) between tetracycline and soil components are the predominant sorption mechanisms. Partitioning into soil organic matter is predictably a comparatively minor sorption process due to their high compatibility with water and low octanol-water partitioning coefficients (i.e. $\log K_{OW} < 1.4$) (Chiou, 2002; Chiou et al., 1983; Herbert and Dorsey, 1995; Moffat et al., 2004; Varanda et al., 2006).

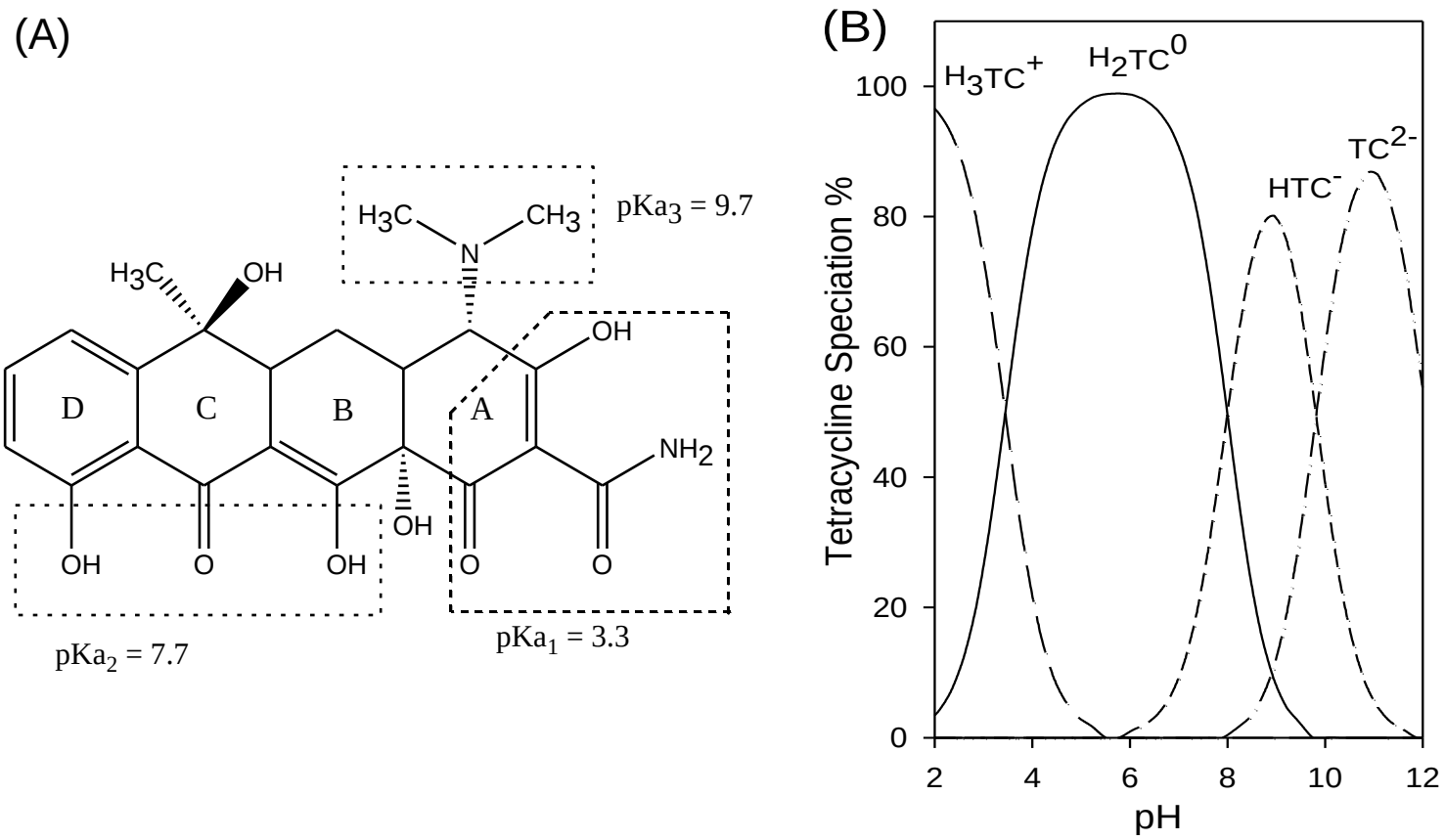


Figure 1.1. (A) Chemical structure and pKa values of tetracycline, and (B) Fractional distribution of tetracycline species as a function of pH values in aqueous solution. $H_3TC^+$, $H_2TC^0$, $HTC^-$, and $TC^{2-}$ represent the cation, zwitterion, anion, and divalent anion of tetracycline species.

## BIOAVAILABILITY

Sorption by soil/sediment plays a dominant role in controlling the distribution, transport, and bioavailability of tetracyclines in the environment. It is postulated that the role of tetracyclines released into the environment in the development and preservation of antibiotic resistance gene is strongly linked to their speciation in aqueous solution and to their sorptive interactions with natural geosorbents. Bioavailability is a concept to describe the accessibility of contaminants in various environmental matrices to target organisms, and is a critical link in understanding the relationship between environmental contamination and impacts on ecosystem and human health. In order for tetracyclines to manifest physiological effects on microbes, they must be available for active transport into the cells of susceptible bacteria and exert bacteriostatic effects by inhibiting protein biosynthesis, and hence preventing the multiplication of cells (Figure 1.2). Bioavailability describes the uptake of contaminants from bioaccessible matrices (water and possibly soil phases) to target organisms resulting in the corresponding biological responses. It is generally assumed that contaminants in aqueous phase are bioavailable. Sorption/desorption processes regulate the contaminant concentration in water, which renders it bioaccessible to directly contact the cell membrane and bioavailable as it moves into the cell (National Research Council, 2003; Pignatello, 2009). However, the bioavailability of soil- or sediment-bound contaminants for microbial uptake and use remains controversial and poorly understood (Feng et al., 2000; Guerin and Boyd, 1992). The bioavailability of soil-sorbed contaminants is especially important for strongly soil-sorbed chemicals such as tetracyclines. As described above, tetracyclines present in the environment are predominantly bound to soils/sediments. Thus the vast majority of

exposure risk of antibiotics stems from the soil-sorbed tetracyclines. If sorption substantially lowers bioaccessibility and bioavailability to bacteria, this would significantly reduce the risk that tetracyclines in the environment contribute to the development of resistant strains of infectious bacteria. However, several studies reported that soil-sorbed tetracyclines were still biologically active and possibly influenced the growth of bacteria in which tetracycline desorption from soils to solution was assumed to be the prerequisite step (Chander et al., 2005; Goetsch et al., 2012). One approach to lower risks posed by tetracycline was to encourage greater sorption to solid phases, perhaps through in-situ sorbent amendment i.e. biochars to soils (Ippolito et al., 2012; Laird et al., 2010). A recent study shows that soil-sorbed tetracycline did not apparently exert selective pressure on bacteria (Subbiah et al., 2011), suggesting the reduced risk of sorbed tetracycline. However, the bioavailability of soil-sorbed tetracycline remains unknown, in large part due to lack of detailed desorption data, and development of appropriate molecular methods to measure bioavailability (National Research Council, 2003).

Current dogma thus simply assumes that only contaminants in aqueous phase are bioavailable. Accordingly, desorption processes regulate the amount of bioavailable contaminants in water which directly contact the cell membrane; soil- or sediment-bound contaminants that do not desorb into water are considered not available for microbial uptake. However, some previous studies have used macroscopic measurements of contaminant degradation or mineralization, and provided evidence that bacteria can access pools of soil-sorbed organic chemicals without the requirement of desorption into bulk solution (Calvillo and Alexander, 1996; Feng et al., 2000; Guerin and Boyd, 1992,

1997; Ortega-Calvo and Saiz-Jimenez, 1998; Park et al., 2003; Tang et al., 1998; Xia et al., 2010). Although these studies provide strong evidence that bacteria were somehow experiencing and accessing the pool of soil-sorbed chemicals, they were unable to provide molecular-scale mechanistic insights into the physical, chemical, or microbiological determinants of bioavailability.

Bioavailability links the accessible antibiotics in various environmental matrices to the uptake by target microorganisms, which is a prerequisite for the manifestation of the associated biological responses. Tetracycline chemistry in various environmental settings could alter bioavailability to microbes and hence the ensuing activation of antibiotic resistance genes. Tetracycline demonstrates multiple species in aqueous phase with the fractional distribution depending on solution pH, ionic strength and composition. These species determine the specific interactions with clay minerals and soil organic matter. However, little is known about how tetracycline speciation in aqueous solution and sorption by soils influence their bioavailability to bacteria. An important research question is whether, and to what extent, tetracyclines in the environmental matrices are bioavailable to microbes and become available for uptake.

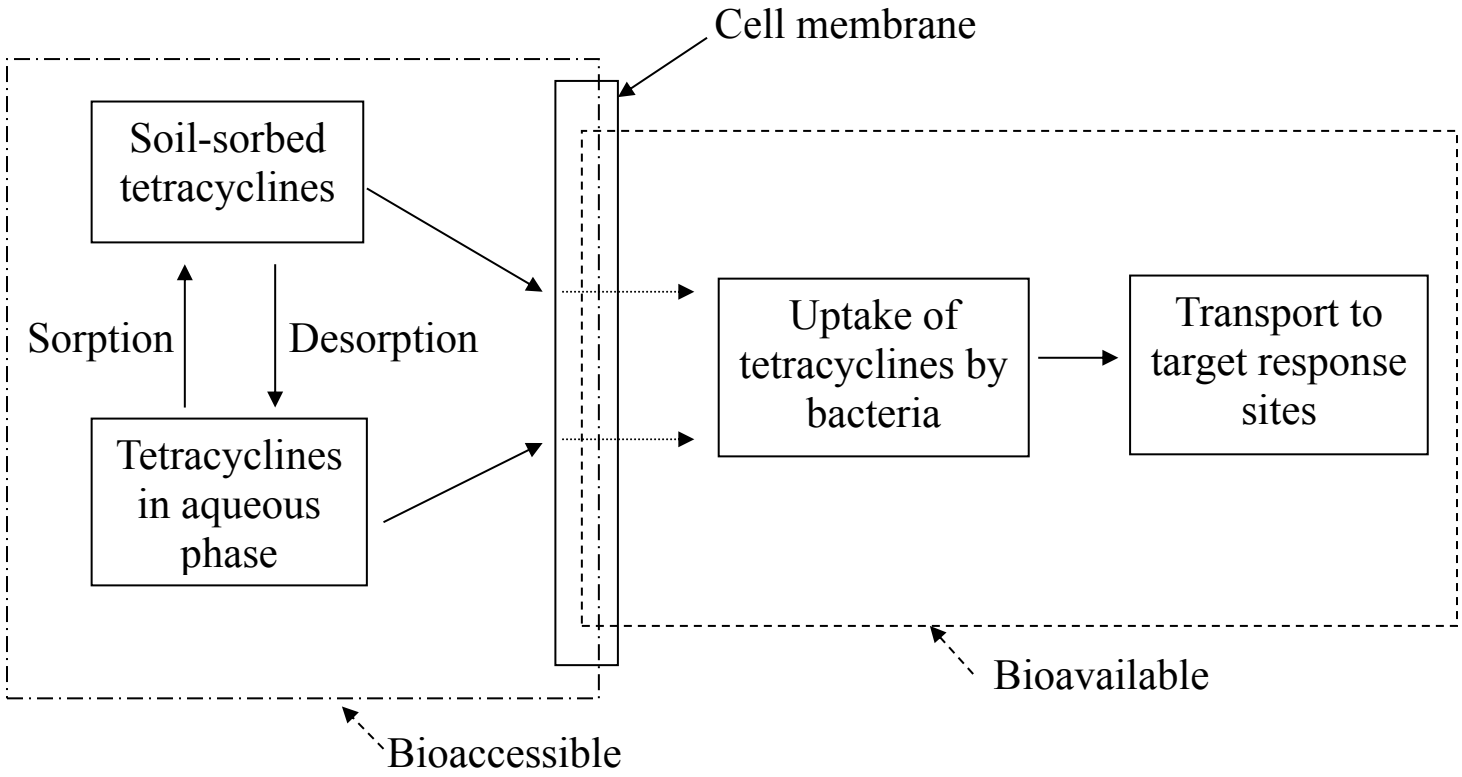


Figure 1.2. Bioaccessible vs. bioavailable fraction of tetracycline in soil and water

## TETRACYCLINES RESISTANCE IN BACTERIA

It is well established that tetracycline inhibit protein biosynthesis by preventing the attachment of aminoacyl-tRNA to ribosomal acceptor sites (Chopra and Roberts, 2001). Tetracyclines could cross the outer membrane of gram-negative enteric bacteria through OmpF and OmpC porin channels as positively charged metal-tetracycline coordination complexes (Chopra and Roberts, 2001). The metal-tetracyline complexes then dissociate in the periplasm, and the slightly lipophilic tetracycline penetrate the cytoplasmic membrane (Chopra and Roberts, 2001). Once in the cytoplasm, tetracyclines bind reversibly to the 30S ribosomal subunit, which prevents aminoacyl-tRNA binding. During these steps the elongation step of protein synthesis is halted in the presence of tetracyclines hence inhibiting bacterial growth (Sapunaric et al., 2005). To avoid growth inhibition, bacteria could develop, maintain and transfer tetracycline resistance genes over the course of evolution. The major mechanisms of conferring resistance to bacteria are activation of efflux pumps and ribosome protection. The genes associated with efflux pump involves *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(Z), *tet*(30), *tet*(31), *tet*(K), *tet*(L), *otr*(B), *tcr*3,*tetA*(P), *tet*(V), *tet*(Y), which are coded for membrane-associated proteins in order to export tetracycline from bacterial cells in an energy-dependent pathway (Chopra and Roberts, 2001). The efflux proteins generally reside in the membrane, and exchange a proton for cation-tetracycline complex against a concentration gradient (Roberts, 1996). The expression of active efflux is tightly regulated and only induced upon exposure to tetracyclines, which is accomplished by the TetR transcriptional repressor protein. Upon binding tetracyclines, a conformational change in TetR is induced, releasing it from the tetracycline promoter allowing

transcription of the active efflux pump gene (Ramos et al., 2005). This type of control is conserved in all tetracycline-resistance encoding efflux pumps in gram-negative bacteria (Roberts, 1996). Ribosome protection proteins, coded by *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q), *tet*(T),*otr*(A), and *tetB*(P) genes, are cytoplasmic proteins that protect the ribosomes from interactions with tetracyclines (Chopra and Roberts, 2001). Ribosome protection proteins may be derived from the elongation factors EF-G and EF-Tu, and lost original function and were adapted to the functions in tetracycline resistance. For the instance of Tet(M) and Tet(O) which have been well-studied, the ribosome protection proteins could reduce the tetracycline binding to ribosomes in the presence of GTP (Connell et al., 2003).

In the environment, tetracycline antibiotic resistance genes *tet*(O), *tet*(Q), *tet*(W), *tet*(M), *tetB*(P), *tet*(S), *tet*(T), and *otr*(A) were frequently found in bacteria from animal lagoons, and detected in downstream surface water as well as in ground water in proximity of CAFOs (Chee-Sanford et al., 2001; Gao et al., 2012a; Pruden et al., 2006, 2012; Smith et al., 2004). Tetracycline resistance genes were more frequently detected at sites where tetracyclines were present due to land application of animal manure, as compared to non-manured sites (Heuer et al., 2011). In pristine areas where tetracyclines were not detected, the abundance of tetracycline resistance genes was orders of magnitude less than that at the sites contaminated with tetracyclines (Pei et al., 2006). There is a paucity of data on the long-term effects of environmental pollution by antibiotics on antibiotic resistant bacteria. A recent study by Knapp et al (Knapp et al., 2010), showed significant increases of antibiotic resistance genes in agricultural soils over the past six decades. In archived soil samples from five Dutch arable field sites, the

abundance of genes coding for resistance to tetracyclines increased exponentially in copy numbers relative to the 16S rRNA gene, which coincided with the dramatic increase in production and use of tetracyclines since 1950. Tetracyclines are the most frequently found chemicals of emerging concern in agricultural soils and surface waters, and tetracycline resistance genes are also commonly found in agricultural ecosystems. However, little is known about the extent to which tetracyclines bound to geosorbents or dissolved in water are bioavailable to bacterial strains, which is a prerequisite for exerting selective pressure on the bacteria leading to the maintenance or further development of antibiotic resistance in response to the selective pressure.

**RESEARCH OBJECTIVES**

The overall objective in this study was to evaluate bioavailability of aqueous-phase and soil-sorbed tetracyclines to bacteria (*Escherichia coli*) for uptake which evokes expression of antibiotic resistance gene *tet*(M). This study provided fundamental science-based knowledge regarding the impacts of tetracycline present in soil and water on the expression of antibiotic resistance. Tetracycline antibiotics are a class of chemicals of emerging concern most commonly utilized in concentrated livestock feeding operations, and in disease control for humans and animals. Since their introduction in the 1950s, tetracyclines have become ubiquitous in the environment, and coincidently the abundance of antibiotic resistance genes has increased exponentially in soils. However, basic knowledge at the molecular scale of bacterial access to tetracyclines present in environmental matrices remains nearly non-existent. Furthermore, when tetracyclines are dissolved in the aqueous phase, they can form complexes with the cations commonly found in natural waters such as $Ca^{2+}$ and $Mg^{2+}$. In addition, organic ligands in natural

water can potentially form complexes with metal cations, and hence alter the solution chemistry of tetracycline. Little is known about the impacts of such coordination complexes on the tetracycline uptake by bacterial cells, and the subsequent expression of antibiotic resistance. For the tetracyclines sorbed by soils, bacteria might have the high potential to take up these sorbed fractions in a more convenient manner when cells are in close contact to soil surfaces where tetracyclines are held. In this study, we investigated the links between environmental chemistry of aqueous phase and soil-sorbed tetracyclines and the uptake of tetracyclines by bacterial cells, i.e. *E. coli* bioreporter, where tetracycline evoked antibiotic resistance gene which was quantified by the emission of *gfp*. Our hypothesis was that the speciation of tetracyclines dissolved in water and sorption by soil minerals controls their bioavailabilities for uptake by bacteria and subsequent activation of antibiotic resistance genes. Transport of tetracyclines into bacterial cells, i.e. the bioavailability of tetracyclines, whether as complexes in aqueous solution or sorbed by soils, is prerequisite for these compounds to exert selective pressure on bacteria that ultimately leads to enrichment of antibiotic resistance genes. As such, tetracycline bioavailability is a question of fundamental importance that directly impacts risks associated with antibiotic resistance.

**SIGNIFICANCE AND IMPACTS**

The results from this study could be utilized in predictive conceptual models to evaluate the impacts of tetracyclines in the environment on the enrichment and preservation of antibiotic resistance genes in the agroecosystems. This study could greatly advance the fundamental understanding of the relationship between development and preservation of antibiotic resistance and the presence of tetracycline in water and soil.

This information is critically needed to assess the risks of tetracycline, a chemical of emerging concern, in the environment, and guides efforts to achieve sustainable production of agricultural ecosystems, for example, establishing guideline for the appropriate use of reclaimed water in agricultural irrigation, and reasonable land application of animal waste while maintaining soil quality and health, as well as the diversity of soil microbial communities. Most previous exposure and risk models for organic contaminants and pesticides simply used the total concentrations to assess their toxic effects. However, many recently defined chemicals of emerging concern develop multiple species in the environment, each with potentially markedly different impacts on microbial communities. The knowledge of the environmental chemistry of antibiotics, particularly in the form of a speciation model, should be incorporated into the overall exposure and risk assessment framework for evaluating the potential risks associated with antibiotics in soils. The information obtained from this study could improve the risk assessment of chemicals of emerging concern, and facilitate the development of scientifically informed management schemes to minimize the potential of developing antibiotic resistance, hence sustaining agroecosystems for agricultural food production.

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# CHAPTER II

# TETRACYCLINE SPECIATION IN AQUEOUS SOLUTION CONTROLS BACTERIAL UPTAKE AND SELECTIVE PRESSURE ON ANTIBIOTIC RESISTANCE

**ABSTRACT**

Tetracyclines are a class of antimicrobials extensively used in human and veterinary medicine, and in livestock production. A large portion of tetracyclines administered to humans and animals are excreted and subsequently released into the environment, where they pose potential risks to ecosystem and human health. Tetracycline contains ionizable functional groups that manifest several species of differing charges, with the fractional distributions of each species depending on pH-pKa relationship in the aqueous phase. These species could interact with naturally abundant cations (e.g., $Ca^{2+}$ and $Mg^{2+}$) to form metal-tetracycline complexes in water. In this study, we used the *E. coli* MC4100/pTGM whole-cell bioreporter as an effective tool to investigate tetracycline uptake from the solution phase under varying conditions (pH, salt composition and concentration) as indicated by activation of antibiotic resistance. The results revealed that activation of antibiotic resistance in the *E. coli* bioreporter responded linearly to intracellular tetracycline concentration which was measured by liquid chromatography coupled to tandem mass spectrometer. At higher solution pH (e.g., pH = 8.0) comparatively less tetracycline entered *E. coli* cells than that at lower solution pH of 6.0 and 7.0 indicating reduced antibiotic effectiveness as aqueous phase pH increases. Inorganic cations such as $Mg^{2+}$ and $Ca^{2+}$ in solution form metal-tetracycline complexes, which manifests reduced uptake of tetracycline by *E. coli*. Increasing $Mg^{2+}$ and $Ca^{2+}$ concentrations enhanced the formation of such complexes hence further decreasing the uptake and effectiveness of tetracycline as indicated by a diminished bioresponse. Among the tetracycline species present in solution including both metal-complexed and free

(non-complexed) species, zwitterionic tetracycline was identified as the predominant species that most readily passes through the cell membrane activating the antibiotic resistance gene in the *E. coli* whole-cell bioreporter. Understanding the relationship between tetracycline speciation and uptake by bacteria is fundamental knowledge needed to assess tetracycline exposure of bacteria in the environment. Geochemical factors such as pH and metal cations modulate the selective pressure exerted by tetracycline for the development of antibiotic resistant bacteria.

## INTRODUCTION

Tetracycline antibiotics have been widely administered to humans and animals since they were discovered in the 1940s. Their effective antimicrobial actions and the lack of major adverse side effects have led to the extensive use in livestock production for disease control, and as feed supplement at subtherapeutic levels to improve feeding efficiency, growth rate and general health. According to United States Food and Drug Administration (FDA), in 2011 more than 13.5 million kilograms of antimicrobials were sold and distributed for use in domestic food-producing animals (FDA, 2011). Among the variety of pharmaceuticals available, tetracyclines ranked as the most highly used group of antibiotics at 5.6 million kilograms per annum, equivalent to ~42% of the total antibiotics used annually for livestock production in the United States. Large fractions of tetracyclines used in animal feeding operations or disease treatment are excreted with feces and urine (manure waste) either as parent compounds or bioactive metabolites (Aga et al., 2005; Jacobsen et al., 2004). After a period of storage, manure wastes containing certain levels of tetracyclines (e.g., $\mu g\ kg^{-1}$ to $mg\ kg^{-1}$) are usually land applied to agricultural fields as an inexpensive disposal management approach, and also for their ancillary value as a fertilizer. This practice introduces these antibiotics into soils and waters of agroecosystems, which are subsequently transported to other environmental compartments. As a result, tetracyclines have been frequently detected in soils (Hu et al., 2010; Jacobsen et al., 2004), surface waters (Batt and Aga, 2005; Christian et al., 2003b; Kolpin et al., 2002; Wei et al., 2011) and even in groundwater (Chee-Sanford et al., 2001; Gottschall et al., 2012; Hu et al., 2010).

Although the levels of tetracyclines found in the most environmental media are below the thresholds required to exhibit medical inhibitory effects on bacterial populations, they plausibly exert selective pressure on bacteria resulting in the development of antibiotic resistance genes and the enrichment of antibiotic resistant bacterial populations in the environment (Chee-Sanford et al., 2001; Gibbs et al., 2004; Gilchrist et al., 2007b; Hong et al., 2013; Looft et al., 2012). There is a growing concern that the presence of antibiotics such as tetracycline at trace levels in the environment is related to the emergence and ever-increasing abundance of antibiotic resistance genes (ARGs) in natural microbial populations. Previous studies reported that tetracycline ARGs, such as tet(O), tet(Q), tet(W), tet(M), tetB(P), tet(S), tet(T), and otr(A), were frequently found in bacteria from animal lagoons, and detected in downstream surface water as well as in ground water in proximity of animal production facilities (Chee-Sanford et al., 2001; Gao et al., 2012b; Smith et al., 2004). Tetracycline resistance genes were more frequently detected at the sites where tetracyclines were present due to land application of animal manures, as compared to non-manured sites. In pristine areas where tetracyclines were not detected, the abundance of tetracycline resistance genes was orders of magnitude less than that at the sites contaminated with tetracyclines (Pei et al., 2006; Storteboom et al., 2010). A recent study (Knapp et al., 2010) showed significant increases of antibiotic resistance genes in agricultural soils over the past six decades. In archived soil samples from five Dutch arable field sites, the abundance of genes coding for resistance to tetracyclines increased exponentially in copy numbers relative to the 16S rRNA gene, which coincided with the dramatic increase in production and use of tetracyclines since 1950s.

The concurrence of tetracycline in the environment and the increasing abundance of ARGs suggest that the exposure of microbial communities to tetracycline might pose selective pressure for the development and proliferation of bacteria containing tetracycline resistance genes. In order for tetracyclines to manifest physiological effects on microorganisms, they must be available for transport into the cells and exert bacteriostatic effects on susceptible bacteria. Little is known about how tetracycline speciation in the environment affects bacterial uptake, which modulates the degree of selective pressure and hence the abundance of genes for antibiotic resistance.

The tetracycline molecule contains three ionizable functional groups i.e. tricarbonylmethane, diketone and dimethylammonium, which are substituted at various positions on a fused 6-carbon ring structure (Figure 1.1A). These ionizable functional groups form cations, zwitterions and anions in aqueous solution with the fractional distributions depending on solution pH (Figure 1.1B). Additionally, the anionic species could form complexes with naturally occurring inorganic cations (e.g., $Ca^{2+}$ and $Mg^{2+}$) in aqueous solution (Carlotti et al., 2012; Othersen et al., 2003, 2006; Wessels et al., 1998). It has been observed that the presence of inorganic cations in solution increases the minimum inhibitory concentration (MIC) of tetracycline to bacteria (Avery et al., 2004; Lunestad and Goksøyr, 1990; Nanavaty et al., 1998), indicating the cation-tetracycline complexation could reduce uptake and hence effectiveness of tetracyclines to inhibit bacterial growth. However, these studies were conducted at relatively high tetracycline concentrations (e.g., > 1 mg $L^{-1}$) with the purpose of quantifying the MICs of tetracycline to bacteria.

In this study, the primary objective was to evaluate the effects of tetracycline speciation in the aqueous solution on bacterial uptake and the associate selective pressure on antibiotic resistance. We hypothesize that tetracycline species of varying net charge (cations vs. zwitterions vs. anions) manifest differential uptake by bacteria as indicated by their differing capabilities to activate antibiotic resistance in the *E. coli* bioreporter. Quantifying the effects of speciation on tetracycline uptake is of fundamental importance because this modulates the degree of uptake and hence selective pressure for the development of antibiotic resistance. To test our hypothesis, we altered solution pH, and type and concentration of salts, which are primary determinants of tetracycline speciation, and measured tetracycline uptake and the corresponding expressions of antibiotic resistant responses of the *E. coli* bioreporter. The bioreporter is *E. coli* strain MC4100 containing the plasmid pTGM with transcriptional fusion between a tetracycline inducible promoter ($P_{tet}$) and a fluorescence-assisted cell sorting optimized *gfp* gene (Hansen and Sørensen, 2000). Tetracycline uptake by the bioreporter was quantified using a high performance liquid chromatography integrated with tandem mass spectrometry (LC-MS/MS). The degree of promoted antibiotic resistance was quantified by measuring bacterial growth rate and the fluorescence emitted from the *E. coli* bioreporter. The effects of tetracycline speciation in solution on bacterial uptake and antibiotic responses in the bioreporter were examined to elucidate the predominant tetracycline species that optimizes cellular concentration of the antibiotic and the activation of antibiotic resistance genes.

## MATERIALS AND METHODS

### *Chemicals*

Tetracycline hydrochloride (purity ≥ 95%), ampicillin sodium salt (purity ≥ 95%), methanol (HPLC grade), and 3-(N-morpholino)propanesulfonic acid (MOPS, buffer range 6.5-7.9) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride, potassium chloride, calcium chloride, magnesium chloride, ethylenediaminetetraacetic acid (EDTA), formic acid, sodium phosphate dibasic, and potassium phosphate monobasic were purchased from J.T. Baker (Philipsburg, NJ). Bacto tryptone and Bacto yeast extracts were purchased from Becton, Dickinson and Company (Sparks, MD). Acetonitrile (HPLC grade) and hydrochloric acid (37%) were purchased from EMD Chemicals (Gibbstown, NJ). 2-(N-morpholino)ethanesulfonic acid (MES, buffer range 5.5-6.7) was purchased from Fisher Biotech (Fair Lawn, NJ).

### *Bacterial Strain*

The *E. coli* strain MC4100/pTGM used as the whole-cell bacterial bioreporter in this study was generously provided by Dr. Søren Johannes Sørensen at the University of Copenhagen. This strain was constructed by inserting *tet*(M) gene (encoding tetracycline resistance by ribosomal protection) into plasmid pTGM (Bahl et al., 2005), which contains a transcriptional fusion between a *tetR*-regulated $P_{tet}$ promoter and flow cytometry-optimized *gfp* gene (*gfp*mut3) encoding green fluorescence protein (GFP). As tetracycline enters the *E. coli* bioreporter cell, it deactivates the TetR repressor protein in the $P_{tet}$ promoter and activates *gfp* gene transcription. The pTGM construct contains a tetracycline resistance gene *tet*(M) that inhibits tetracyclines from killing the cells, and maintains the intracellular concentration of tetracycline. Meanwhile, the GFP translated

from the expression of *gfp* gene emits the fluorescence signal with the intensity proportional to the $P_{tet}$ activity that drives antibiotic resistance gene expression in natural settings. The quantification limit of this bioreporter for tetracycline concentration in aqueous solution is ~5 μg $L^{-1}$ (Bahl et al., 2005; Hansen et al., 2001).

*Cultivation of Bacteria*

The *E. coli* bioreporter was cultured in a low-salt Luria-Bertani (LB) media which contained 10.0 g of tryptone, 5.0 g of yeast extracts and 0.5 g of NaCl in 1.0 L of 0.22 μm-filtered MilliQ-water. The pH of the media was adjusted to 6.0, 7.0, and 8.0 using 100 mM of MES buffer or 50 mM of MOPS buffer. The LB media were autoclaved at 121 ℃ for 30 min. The *E. coli* bioreporter was inoculated and cultivated in 25.0 mL of LB media amended with 100 mg $L^{-1}$ ampicillin, and incubated on a horizontally-moving shaker at 150 rpm and 30 ℃. When the bacterial culture grew to mid-log phase as indicated by optical density at 600 nm ($OD_{600}$) of the culture approaching ~0.7, 0.5 mL of the culture was diluted 100 fold into 50.0 mL of freshly prepared LB media containing 100 mg $L^{-1}$ampicillin. The LB media was prepared so that it contained final tetracycline concentrations of 0, 25, 50, 75, 100 and 125 μg $L^{-1}$ in the presence of KCl, $CaCl_2$, or $MgCl_2$ at concentrations of 0, 1.0and 5.0 mM. All culture samples were prepared in triplicate. For each treatment 1.0 mL of culture sample was collected every 30 min, and analyzed for the emitted fluorescence (*gfp*mut3 excitation wavelength = 488 nm; emission wavelength = 511 nm) using a SpectraMax M2 spectrofluorometer (Molecular Devices, Sunnyvale, CA).

*Relationship between Bacterial Biomass and Optical Density*

The *E. coli* bioreporter was cultivated in 25 mL of LB media at pH 7.0. As growth continued, the $OD_{600}$ values increased from 0.2 to 1.5. At the point in time when these $OD_{600}$ values were obtained, the cultures were collected, and centrifuged at 15000 g and 4 ℃ for 30 min to obtain bacterial pellets. The supernatants were decanted, and the pellets were re-suspended in pre-weighed glass centrifuge tubes with 25 mL of MilliQ-water followed by another centrifugation step at 1900 g and at 4 ℃ for 30 min. This water-washing step was repeated one more time, and the pellets freeze dried for 5 days to obtain the dried bacterial biomass. The dried bacterial cells were weighed, and used to establish the relationship between bacterial biomass and the measured $OD_{600}$.

*Estimation of Promoter Activity*

The measured fluorescence emitted from the bioreporter, GFP maturation and bacterial growth rate are factors used to calculate promoter activity in the *E. coli* bioreporter at steady state. Assuming that GFP does not undergo proteolytic degradation during moderate time interval (up to 4.0 h), the model of Leveau and Lindow (2001) can be used to quantify promoter activity according to **equation 1**:

$$P = f_{ss} \times \mu \times (1 + {}^{\mu}/_{m}) \qquad \text{eq. 1}$$

where $P$ is promoter activity (relative unit of immature GFP per OD unit per hour, RU $OD^{-1}$ $h^{-1}$), $f_{ss}$ represents the fluorescene at steady state during bacterial growth (relative unit of fluorescent mature GFP per OD unit, RU $OD^{-1}$). $\mu$ ($h^{-1}$) is growth rate, and $m$ ($h^{-1}$) is the maturation constant for GFP maturing to fluorescent GFP ($m$ = 1.54 $h^{-1}$ for

*gfp*mut3) (Leveau and Lindow, 2001). The term $f_{ss}$ is obtained from a plot of fluorescence vs. $OD_{600}$. The term $\mu$ is obtained from a plot of natural logarithm of optical dentistry vs. time using **equation 2**:

$$OD_{600} = OD_{600,0} \exp(\mu \times t) \qquad \text{eq. 2}$$

where $t$ is culture time (h), and $OD_{600}$ and $OD_{600,0}$ are the measured optical densities (600 nm) at time t and t = 0, respectively. This approach can circumvent the effects of dilution of GFPcontents and GFP maturation during the growth of bacteria, which enables comparison of antibiotic resistance among experimental settings.

*Analysis of Tetracycline Concentrations in Solution and in Bacteria*

Hydrophilic–lipophilic balanced (HLB) cartridge (Waters Corporation, Milford, MA) was used in solid phase extraction to extract tetracycline from aqueous phase. Cartridges were preconditioned prior to use by sequential rinses of methanol (3 mL), 0.1 M HCl (3 mL) and water (6 mL). When the $OD_{600}$ value of the *E. coli* bioreporter cultures approached ~0.5, the bacteria were separated from the culture media by centrifugation at 15000 g and 4 ℃ for 30 min. The supernatants (5.0 mL) were collected and passed through pre-conditioned HLB cartridges at a flow rate of 2.0 mL $min^{-1}$ to extract tetracycline. The cartridges were then washed with a 1:9 (v/v) methanol/water solution (5 mL). Tetracycline retained on the HLB cartridges was eluted with 1:1 (v/v) methanol/water solution (5.0 mL) containing 150 mg $L^{-1}$ of EDTA, then with additional 5.0 mL of methanol containing 1% (v/v) formic acid. The eluted solutions were combined and analyzed for tetracycline concentration using LC-MS/MS. The extraction recovery for tetracycline from the LB media was 86% with a standard deviation of 10%.

After centrifugation, the bacterial cell pellets were rinsed twice with 25 mL of phosphate-buffered saline solution (PBS solution: 80.0 g of NaCl, 2.0 g of KCl, 14.4 g of $Na_2HPO_4$ and 2.4 g of $KH_2PO_4$ dissolved in 1 L of water, pH 7.4) that had been diluted 10 times; centrifugation followed each rinse. Then 10 mL of McIlvain buffer (12.9 g of citric acid monohydrate, 10.9 g of $Na_2HPO_4$ and 37.2 g of EDTA dissolved in 1 L of water) was used to suspend the cell pellets and remove tetracycline from the cells. The mixture was votexed (1 min), sonicated (10 min), and then centrifuged (15000 g for 15 min). The extraction step was repeated and the supernatants were combined prior to solid phase extraction. The cell extract containing tetracycline (20 mL) was passed through a preconditioned HLB cartridge and eluted as described above. The extraction recovery of tetracycline was measured at 108% with a standard deviation of 14%.

Tetracycline concentration was quantified using LC-MS/MS. The system consisted of a high-performance liquid chromatography (Shimadzu, Columbia, MD) fully integrated with a triple quadrupole mass spectrometer (Applied Biosystems Sciex 3200, Foster City, CA). A C18 column (Gemini, 5μm, 50×2.0 mm, Phenomenex Inc., Torrance, CA) was used with a flow rate at 0.15 mL $min^{-1}$. The mobile phase consisted of phase A (95% MilliQ-water and 5% acetonitrile containing 0.1% formic acid and 1 mM ammonium acetate) and phase B (100% acetonitrile containing 0.5% formic acid and 1 mM ammonium acetate). The mobile phase gradient was programmed as: 0 to 1 min phase A linearly decreased from 95% to 5% and phase B increased to 95%, 1 to 6 min the mobile phase held at a ratio of 5% phase A and 95% phase B, 6 to 6.5 min phase A linearly increased to 95% and phase B decreased to 5%, and then held at this rates until 7.5 min. The tetracycline concentration was quantified in multiple reaction monitoring

mode with precursor/product ion pair m/z 445.4/410.0; two other pairs of precursor/product transitions (m/z 445.4/428.2 and 445.4/339.3) were used to further confirm tetracycline fingerprints.

*Tetracycline Speciation in Aqueous Phase*

Fractional distributions of tetracycline speciation in aqueous solution in the presence of chloride salts at pH of 6.0, 7.0 and 8.0 were calculated using MINEQL+ software version 4.5 (Environmental Research Software, Hallowell, ME). The input parameters were set to mimic the actual experimental conditions. The tetracycline concentration was input in units of mM, and the solution pH was set at 6.0, 7.0 and 8.0. The cation concentrations were input as a sum of those added (i.e., 0, 1.0, or 5.0 mM KCl, $MgCl_2$ or $CaCl_2$) and pre-existing in the LB media (0.13 mM $Ca^{2+}$ and 0.26 mM $Mg^{2+}$). The equilibrium constants (*K*) for the associations of $H^+$, $Ca^{2+}$and $Mg^{2+}$ with tetracycline anionic species used in the calculations are summarized in Table 2.1. It was assumed that the presence of relatively low levels of electrolytes used in the experiments did not significantly alter values of the equilibrium constants.

Table 2.1: Equilibrium constants for association of $H^+$, $Ca^{2+}$ and $Mg^{2+}$ with tetracycline (TC) in aqueous solution.

| Reactions | Constants | log *K* | References |
|---|---|---|---|
| $H^+ + TC^{2-} \leftrightarrows HTC^-$ | $K_{HTC^-}$ | 9.7 | Gu and Karthikeyan, 2005 |
| $H^+ + HTC^- \leftrightarrows H_2TC^0$ | $K_{H_2TC^0}$ | 7.7 | |
| $H^+ + H_2TC^- \leftrightarrows H_3TC^+$ | $K_{H_3TC^+}$ | 3.3 | |
| $Ca^{2+} + HTC^- \leftrightarrows CaHTC^+$ | $K_{CaHTC^+}$ | 3.4 | Werner et al., 2006 |
| $Ca^{2+} + TC^{2-} \leftrightarrows CaTC^0$ | $K_{CaTC^0}$ | 5.8 | |
| $Mg^{2+} + HTC^- \leftrightarrows MgHTC^+$ | $K_{MgHTC^+}$ | 3.9 | |
| $Mg^{2+} + TC^{2-} \leftrightarrows MgTC^0$ | $K_{MgTC^0}$ | 4.1 | |

## RESULTS AND DISCUSSION

### *Relationship between Bacterial Biomass and Optical Density*

The *E. coli* bioreporter was cultivated in 25 mL of LB media at pH of 7.0, and the samples were collected at different growth stages ranging between $OD_{600}$ of 0.2 to 1.5. The weights of bacterial biomass in culture suspensions obtained by freeze drying were plotted against the corresponding $OD_{600}$ values and a linear relationship was observed (Figure 2.1). This relationship was utilized to estimate the bacterial biomass present in culture suspension based on $OD_{600}$ values, which was used to normalize intracellular tetracycline concentrations to dry bacterial biomass.

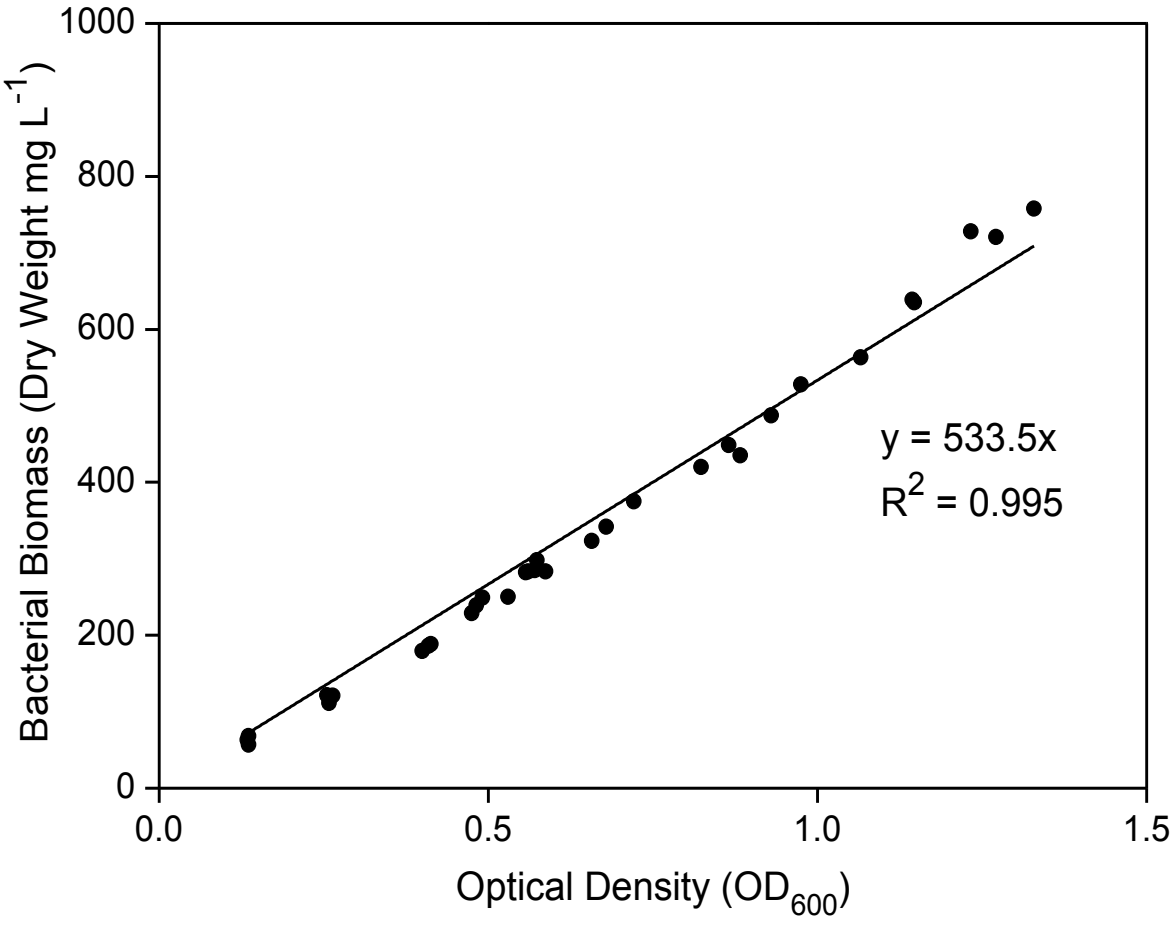


Figure 2.1. Correlation between bacterial biomass in culture suspension and optical density measured at 600 nm.

*Bacterial Uptake of Tetracycline and Expression of Antibiotic Resistance*

When the *E. coli* bioreporter is exposed to tetracycline in aqueous solution, tetracycline enters the cells and activates the antibiotic resistance genes, which causes the GFP to emit fluorescence. Tetracycline uptake by the *E. coli* whole-cell bioreporter and the corresponding antibiotic resistant response (promoter activity) at varying levels of tetracycline in LB media at pH of 6.0, 7.0 and 8.0 is shown in Figure 2.2. Bacterial uptake (represented as intracellular tetracycline concentration) and antibiotic resistant response (promoter activity) increased with increasing tetracycline concentration present in ambient aqueous solution. For example, at pH 6.0 intracellular tetracycline concentrations of 9.0 ± 2.2, 17.6 ± 1.9, 32.2 ± 2.2, 41.8 ± 3.6, and 52.9 ±3.5 $\mu g\ g^{-1}$ resulted from tetracycline aqueous concentrations of 25, 50, 75, 100, and 125 $\mu g\ L^{-1}$, respectively (Figure 2.2A). Correspondingly, the promoter activity increased proportionally, yielding values of 215 ± 17, 414 ± 28, 753 ± 2, 1165 ± 67 and 1712 ± 50 RU $OD^{-1}\ h^{-1}$, respectively (Figure 2.2B). These results indicate that the increasing intracellular tetracycline concentration resulted in enhanced bacterial bioresponse to the antibiotic tetracycline.

Interestingly when the *E. coli* bioreporter was exposed to the same tetracycline concentration, but at different pHs, uptake of tetracycline and the concomitant expression of antibiotic resistance changed. At higher pH (e.g., 8.0) in the LB media, less tetracycline was taken up by the *E. coli* cells than at a lower pH (e.g., 6.0 and 7.0); the corresponding promoter activity was also diminished at pH of 8.0. Specifically, at the tetracycline concentration of 100 $\mu g\ L^{-1}$ in the solution, the intracellular concentrations

were 41.8 ± 3.6, 47.9 ± 6.6, and 18.6 ± 1.0 μg $g^{-1}$ at pH of 6.0, 7.0 and 8.0, respectively, and the corresponding promoter activities were 1165 ± 67, 1405 ± 2, and 505 ± 18 RU $OD^{-1}$ $h^{-1}$, respectively. Diminished bacterial uptake and promoter activity at pH 8.0 relative to pH 6.0 and 7.0 suggests that the speciation of tetracycline in the aqueous phase could influence bacterial uptake and hence modulate selective pressure for the expression of antibiotic resistance.

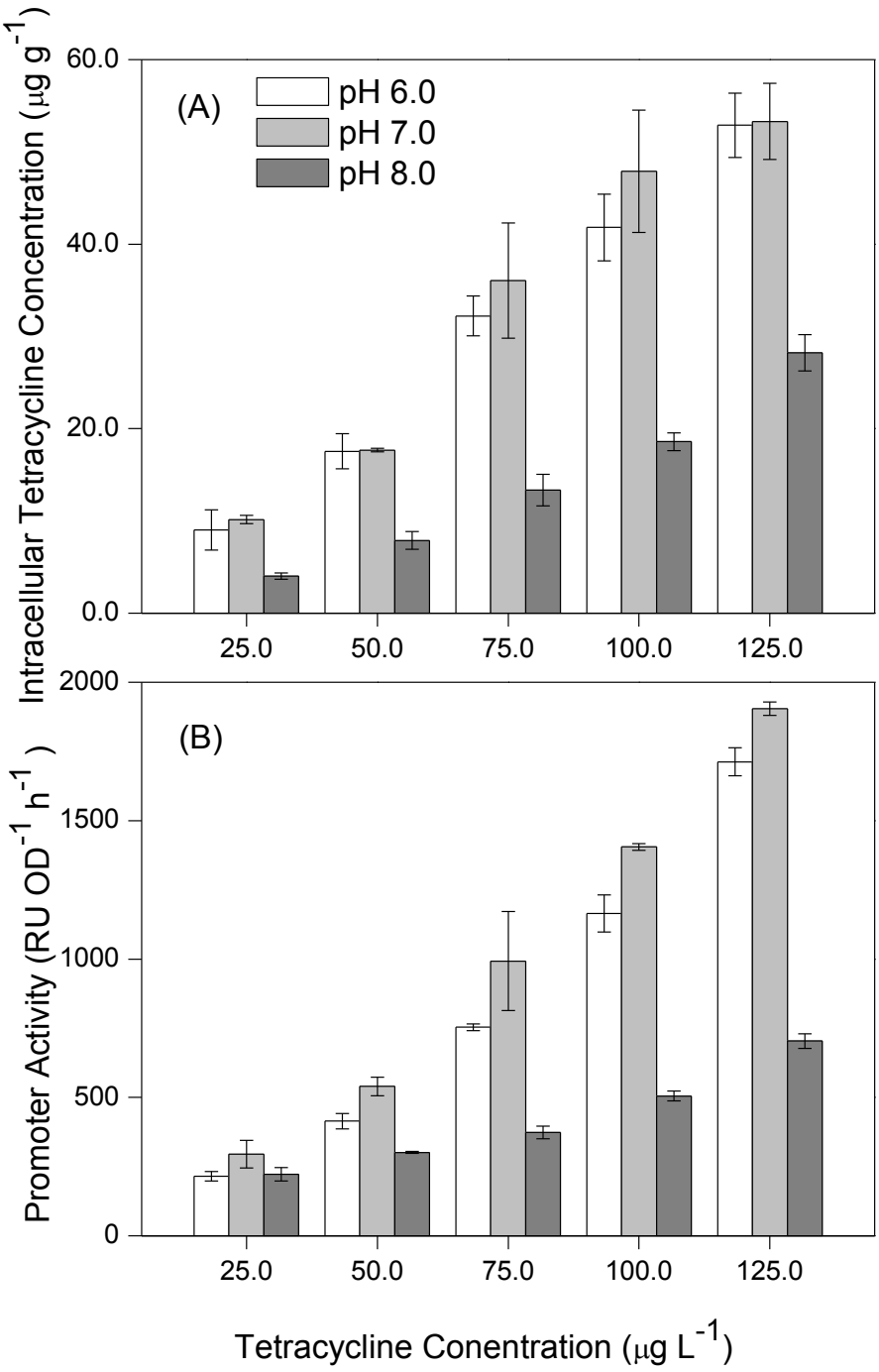


Figure 2.2. (A) Tetracycline uptake by the *E. coli* whole-cell bioreporter and (B) calculated promoter activity in LB media containing several levels of tetracycline at pH 6.0, 7.0 and 8.0.

*Effects of Metal Cation-Tetracycline Complexation on Bacterial Uptake and Promoter Activity*

To further investigate the effects of tetracycline speciation on bacterial uptake and as indicated by the expression of antibiotic resistance in the bioreporter, $CaCl_2$, $MgCl_2$ and KCl were added to LB media at concentrations of 1.0 and 5.0 mM. Tetracycline present in aqueous solution can complex with divalent metal cations, whereas monovalent cation (e.g. $K^+$) manifests minimal complexation with tetracycline (Hosny et al., 1999; Jin et al., 2007; Palm et al., 2008; Werner et al., 2006). Tetracycline complexation is expected *a priori* to shift the fractional distribution of tetracycline species in the ambient solution plausibly manifesting alterations in bacterial uptake. When exposed to the same concentration of tetracycline (e.g. 100 $\mu g\ L^{-1}$), the presence of $Mg^{2+}$ and $Ca^{2+}$ in aqueous solution apparently diminished tetracycline uptake by the *E. coli* bioreporter as well as diminished promoter activity (Figures 2.3A, 2.3B, 2.3C and 2.3D). The presence of $K^+$ caused little or no inhibition of uptake; at pH 6.0 and 8.0 no change occurred, and at pH 7.0 intracellular tetracycline concentrations increased only 9.7% and 15.0% in the presence of 1.0 and 5.0 mM KCl compared to controls without added KCl (Figures 2.3E and 2.3F). In comparison, 1.0 and 5.0 mM $Mg^{2+}$ reduced tetracycline uptake by 11.6% and 58.7% at pH 6.0, 69.0% and 87.1% at pH 7.0, and 73.0% and 84.8% at pH 8.0, with concomitant decreases in promoter activity of 34.5% and 69.5% at pH 6.0, 73.8% and 84.2% at pH 7.0, and 54.0% and 58.4% at pH 8.0, respectively. Similar effects were also elicited by added $CaCl_2$. Specifically, tetracycline

uptake was reduced 28.2% and 55.1% at pH 6.0, 70.4% and 78.4% at pH 7.0, and 61.9% and 76.3% at pH 8.0 in the presence of 1.0 and 5.0 mM $CaCl_2$, with concomitant decreases in promoter activity of 36.3% and 65.4% at pH 6.0, 57.5% and 81.0% at pH 7.0, and 40.1% and 54.6% at pH 8.0, respectively. These results clearly demonstrate that formation of $Mg^{2+}$- and $Ca^{2+}$-tetracycline complexes in the ambient solution abates tetracycline uptake by the *E. coli* bioreporter and hence the selective pressure on expression of antibiotic resistance genes. As such, this geochemical factor, as well as increasing pH, could plausibly reduce the selective pressure of tetracycline of the development of antibiotic resistant bacteria.

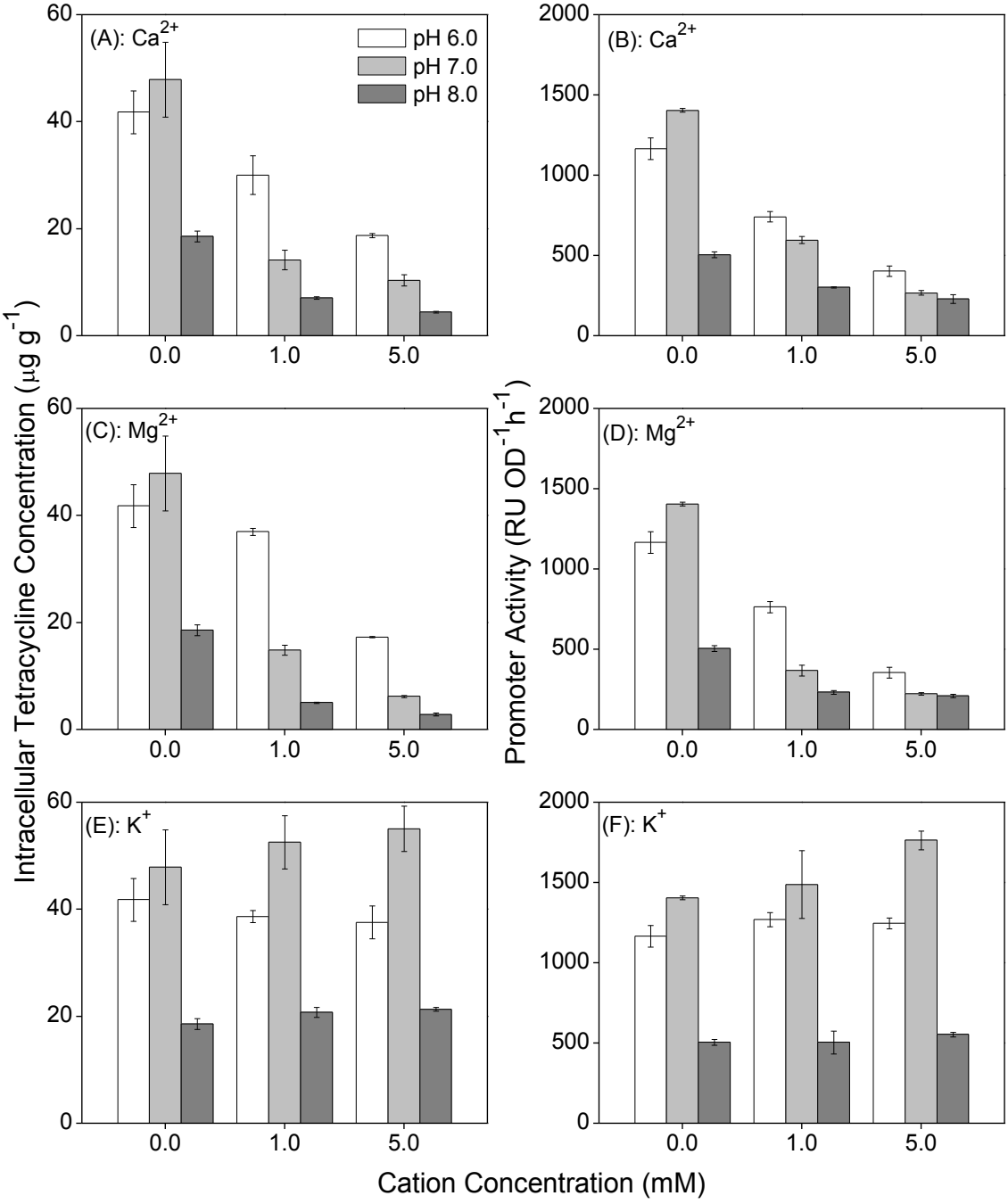


Figure 2.3. Tetracycline uptake by *E. coli* whole-cell bioreporter in the presence of (A) $Ca^{2+}$, (C) $Mg^{2+}$ and (E) $K^{+}$, and the expressed intensities of promoter activity (B, D and F). Tetracycline concentration was 100 μg $L^{-1}$ in LB media.

*Relationship between Intracellular Tetracycline Concentration and Promoter Activity*

As described above, tetracycline can enter the *E. coli* bioreporter and evoke the expression of antibiotic resistance genes (as evidenced by the emitted fluorescence), which allows self-protection of the bacterial cells. However, the same concentrations of tetracycline resulted in varying magnitudes of cellular uptake and antibiotic promoter activity depending on the geochemical conditions of ambient solution pH (Figure 2.2) and the presence and type of metal cations (Figures 2.3). Geochemical conditions are of fundamental importance because they modulate the degree of tetracycline uptake by *E. coli*. This is shown clearly when the promoter activities expressed in the *E. coli* bioreporter are plotted against the corresponding intracellular tetracycline concentration across the range of pH values as well as type and concentration of metal cations (Figure 2.4). The plot includes all data collected from the experiments at different tetracycline concentrations in the solution, three pH values, and the presence and absence of $MgCl_2$, $CaCl_2$ and KCl. The results show a highly linear relationship between promoter activity and intracellular concentration ($R^2$ = 0.85). This clearly indicates that only tetracycline molecules that enter the *E. coli* cells can evoke antibiotic resistance, and that external solution conditions do not directly affect this activity. In a series of experiments in which all performed at the same total initial aqueous phase concentration of tetracycline, the *E. coli* bioreporter responded differently as reflected by differences in the measured promoter activity. For instance, at a constant 100 μg $L^{-1}$ of tetracycline, but different external solution conditions, the promoter activity expressed ranged from 201 to 2013 RU $OD^{-1}$ $h^{-1}$ (filled dots in Figure 2.4). Increase of solution pH above 7.0 or the presence

of divalent metal cations ($Ca^{2+}$ and $Mg^{2+}$) generally lessened tetracycline uptake by the *E. coli* and diminished the expression of antibiotic response. This observation is also apparent for the bacteria exposed to other tetracycline concentrations (Figure 2.4). Overall, it is clear that solution pH, type and composition of salts influenced expression of antibiotic resistance via their influence on bacterial uptake of tetracycline. This is shown convincingly by the highly linear relationship between intracellular tetracycline concentration and promoter activity. Solution chemistry influences tetracycline speciation which is the primary determinant of tetracycline uptake by bacteria.

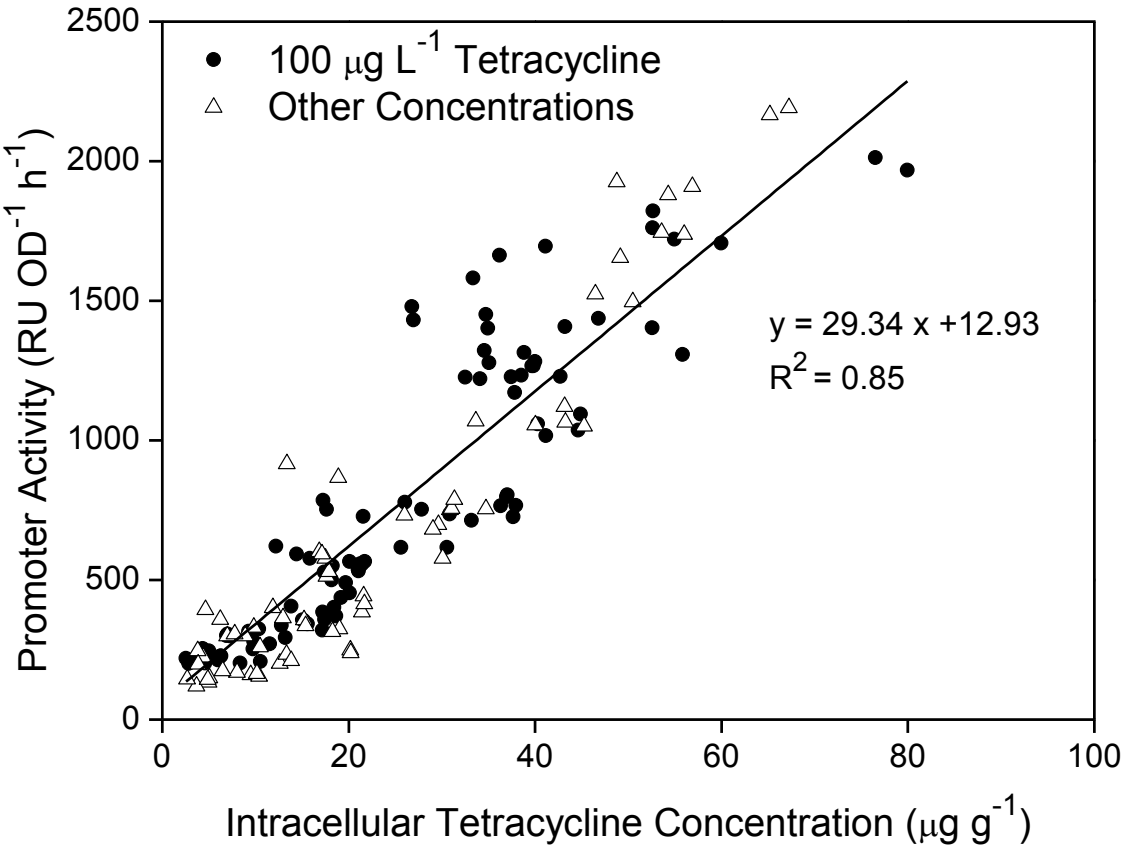


Figure 2.4. Relationship between intracellular tetracycline concentration and the expressed promoter activity of the *E. coli* bioreporter. The plot includes all data collected at tetracycline concentration of 100 μg $L^{-1}$ and at other concentrations (25.0, 50.0, 75.0 and 125.0 μg $L^{-1}$) at different pHs, in the presence and absence of $Mg^{2+}$, $Ca^{2+}$, and $K^{+}$.

*Effective Aqueous Tetracycline Speciation for Bacterial Uptake*

Aqueous tetracycline speciation is postulated to control tetracycline uptake by bacteria. However, it was unknown which species most effectively penetrated the bacterial membrane and passed into the cells. To identify the preferred species for bacterial uptake, the fractional distribution of tetracycline species in aqueous solution was estimated using solution conditions similar to those used in our experiments (Table 2.2). The three ionizable functional groups of tetracycline result in the formation of four tetracycline species (based on charge) in aqueous solution, viz. cation, zwitterion, anion and divalent anion. The added metal cations react with tetracycline anions, forming 1:1 metal-tetracycline complexes, whose relative fractional concentrations depends on their complexation constants (Martin, 1979; Schmitt and Schneider, 2000; Werner et al., 2006).

Table 2.2: Tetracycline speciation in aqueous phase with different pHs and metal cation concentrations.

| Metal Cation | Metal Cation Concentration (mM) | pH | Tetracycline Species (%) | | | |
|---|---|---|---|---|---|---|
| | | | $H_2TC^0$ | $HTC^-$ | $MHTC^+$ | $MTC^0$ |
| -- | 0.0 | 6.0 | 96.4 | 1.0 | 2.6 | 0.0 |
| | | 7.0 | 73.8 | 7.4 | 17.9 | 0.9 |
| | | 8.0 | 17.7 | 17.7 | 42.5 | 22.1 |
| $Ca^{2+}$ | 1.0 | 6.0 | 94.2 | 0.9 | 4.7 | 0.1 |
| | | 7.0 | 58.7 | 5.9 | 29.1 | 6.3 |
| | | 8.0 | 5.6 | 5.6 | 27.5 | 61.2 |
| | 5.0 | 6.0 | 85.8 | 0.9 | 12.9 | 0.4 |
| | | 7.0 | 32.4 | 3.2 | 48.4 | 15.9 |
| | | 8.0 | 1.5 | 1.5 | 22.7 | 74.3 |
| $Mg^{2+}$ | 1.0 | 6.0 | 89.8 | 0.9 | 9.4 | 0.0 |
| | | 7.0 | 46.7 | 4.7 | 48.0 | 0.7 |
| | | 8.0 | 7.2 | 7.2 | 75.0 | 10.5 |
| | 5.0 | 6.0 | 69.8 | 0.7 | 29.5 | 0.0 |
| | | 7.0 | 18.8 | 1.9 | 78.9 | 0.4 |
| | | 8.0 | 2.2 | 2.2 | 90.9 | 4.8 |

At pH of 6.0, 7.0 and 8.0, tetracycline exists primarily as zwitterion ($H_2TC^0$), anion ($HTC^-$), and divalent metal-anion complexes ($MHTC^+$ and $MTC^0$); these four species comprise > 99% of the total tetracycline present in solution. The tetracycline cation ($H_3TC^+$) and divalent anion ($TC^{2-}$) are estimated as < 1% of the total tetracycline in the solution, and therefore not reported in Table 2.2. The experimental conditions studied provided a wide range of tetracycline species to evaluate the effects of tetracycline speciation on bacterial uptake. The zwitterion ranged from 1.5 to 96.4 %, the anion from 0.7 to 17.7 %, and the combined metal-tetracycline complexes contributed to 2.6 to 97.0 % of tetracycline in the solution. The LB media contained 0.13 mM $Ca^{2+}$ and 0.26 mM $Mg^{2+}$, which were included in the calculations of tetracycline speciation. As a result, a portion of tetracycline was present as metal complexes in our experiments without the added salts, and this fraction became more significant at higher pH, e.g. 2.6% at pH 6.0 and 64.6% at pH 8.0 (Table 2.2).

To further explore the dominant tetracycline speciation contributing to the uptake of tetracycline by the *E. coli* bioreporter, we combined all collected data on bacterial uptake of tetracycline from LB media containing varying levels of tetracycline, at different pHs and in the presence and absence of $CaCl_2$, $MgCl_2$ and KCl, and estimated the fractional distributions of tetracycline species in the media. A correlation analysis was conducted to assess the relationship between intracellular tetracycline concentrations and the concentrations of the four major tetracycline species in the solution ($H_2TC^0$, $HTC^-$, $MHTC^+$ and $MTC^0$). Intracellular tetracycline concentrations ranged from 2.6 to 44.6 μg

$g^{-1}$. The corresponding tetracycline species concentrations were estimated as 1.5 to 96.4 μg $L^{-1}$ for $H_2TC^0$, 0.7 to 17.7 μg $L^{-1}$ for $HTC^-$, 0.2 to 41.7 μg $L^{-1}$ for $CaHTC^+$, 1.8 to 90.2 μg $L^{-1}$ for $MgHTC^+$, 0.0 to 74.2 μg $L^{-1}$ for $CaTC^0$, and 0.0 to 2.2 μg $L^{-1}$ for $MgTC^0$. Such a wide range of tetracycline concentrations in the bacteria and in the ambient media allowed a reliable data analysis to identify the most favorable species for bacterial uptake. Correlation analysis between intracellular tetracycline concentration and fractional contents of $H_2TC^0$, $HTC^-$, $MHTC^+$, and $MTC^0$ yielded correlation coefficients (*r*) of 0.859, -0.083, -0.735, and -0.457, respectively. The significant positive correlation between intracellular tetracycline concentration and $H_2TC^0$ indicates that zwitterionic tetracycline is the most favorable species for uptake by the *E. coli* bioreporter. In contrast, the negative correlations between intracellular tetracycline concentrations and metal-tetracycline complexes indicate that these species inhibit tetracycline uptake by bacteria.

The preferential uptake of zwitterionic tetracycline can be further elucidated by correlating tetracycline uptake with zwitterionic tetracycline concentration in LB media (Figure 2.5). It is apparent that as the concentration of zwitterionic species increased, intracellular tetracycline concentration also increased, even though the overall tetracycline concentration varied in the media (Figure 2.5). For instance at 100 μg $L^{-1}$ of tetracycline present in the media, the presence or absence of $Mg^{2+}$ and $Ca^{2+}$ at different pHs (6.0, 7.0 and 8.0) shifted the fractional distributions of tetracycline species resulting in the zwitterionic tetracycline concentration ranging from 1.5 to 96.4 μg $L^{-1}$ (Table 2.2).

Correspondingly, the bacterial uptake increased from 4.4 to 42.0 μg $g^{-1}$ (Figure 2.5). Clearly, it is the tetracycline zwitterionic species in ambient solution, rather than the total tetracycline concentration, that is the primary determinant of bacterial uptake and hence activation of antibiotic resistance. Controlling the concentration of zwitterions provides a means to modulate tetracycline uptake by bacterial cells hence determining the degree of selective pressure of the development and proliferation of antibiotic resistance.

Several previous studies on the minimum inhibitory concentration (MIC) of tetracycline required to hinder bacterial growth indicate that the formation of metal-tetracycline complexes reduce tetracycline effectiveness thereby increasing the MIC levels (Avery et al., 2004; Lunestad and Goksøyr, 1990; Nanavaty et al., 1998). The tetracycline concentrations used in the MIC studies were 5 to 100 times greater than the concentrations used in this study. In general, $Mg^{2+}$-complexed tetracyclines are believed to quickly pass through outer membrane porin channels (e.g. OmpF) of *E. coli* following the Donnan potential, and accumulate in the periplasm (Thanassi et al., 1995). The formed complex is likely to dissociate in the periplasm of the cell due to lower pH, and yield the neutral species of tetracycline that could diffuse through the inner lipid bilayer membrane (Nikaido and Thanassi, 1993). The neutral tetracycline species could also diffuse into *E. coli* cells but at a much slower rate (50 to 100 times) less than that of $Mg^{2+}$-tetracycline complex (Nikaido and Thanassi, 1993). The results obtained from this study are generally consistent with these previous studies though tetracycline concentrations were 1-2 orders of magnitude less than those used in the previous studies. However, we have demonstrated that zwitterionic tetracycline is the dominant species

entering *E. coli* cells, and that the formation of metal-tetracycline complexes is not prerequisite for the diffusion process. At pH 6.0, and without the added salts, only 2.6 % of the total tetracycline is present as metal-tetracycline complexes. Under these conditions, zwitterions are the dominant species present (96.4%) (Table 2.2), and uptake by the *E. coli* bioreporter was much greater than that in the presence of $MgCl_2$ (Figures 2.2 and 2.3). Fluorescence emitted from the bioreporter was measured as a function of exposure time to tetracycline (0 to 4 h at intervals of 30 min). The fluorescence intensities of treatments without the added salts were greater than those samples with added $MgCl_2$ and $CaCl_2$ (data not shown), again indicating the formation of metal-tetracycline complexes does not facilitate passage of tetracycline into bacterial cells compared to tetracycline itself. Neutral tetracycline could slowly diffuse through the outer membrane of *E. coli*, which is without porin channels, and enters the periplasm (Thanassi et al., 1995). However, this route of tetracycline accumulation in *E. coli* is less significant compared to uptake by the bacteria with porin channels (Thanassi et al., 1995). The results from this study indicate that zwitterionic tetracycline favorably passes through the membrane resulting in maximal uptake by the bacterial bioreporter. The formation of metal cation-tetracycline complexes clearly was not a necessary step or responsible for the substantial uptake of tetracycline by the *E. coli* bioreporter observed herein.

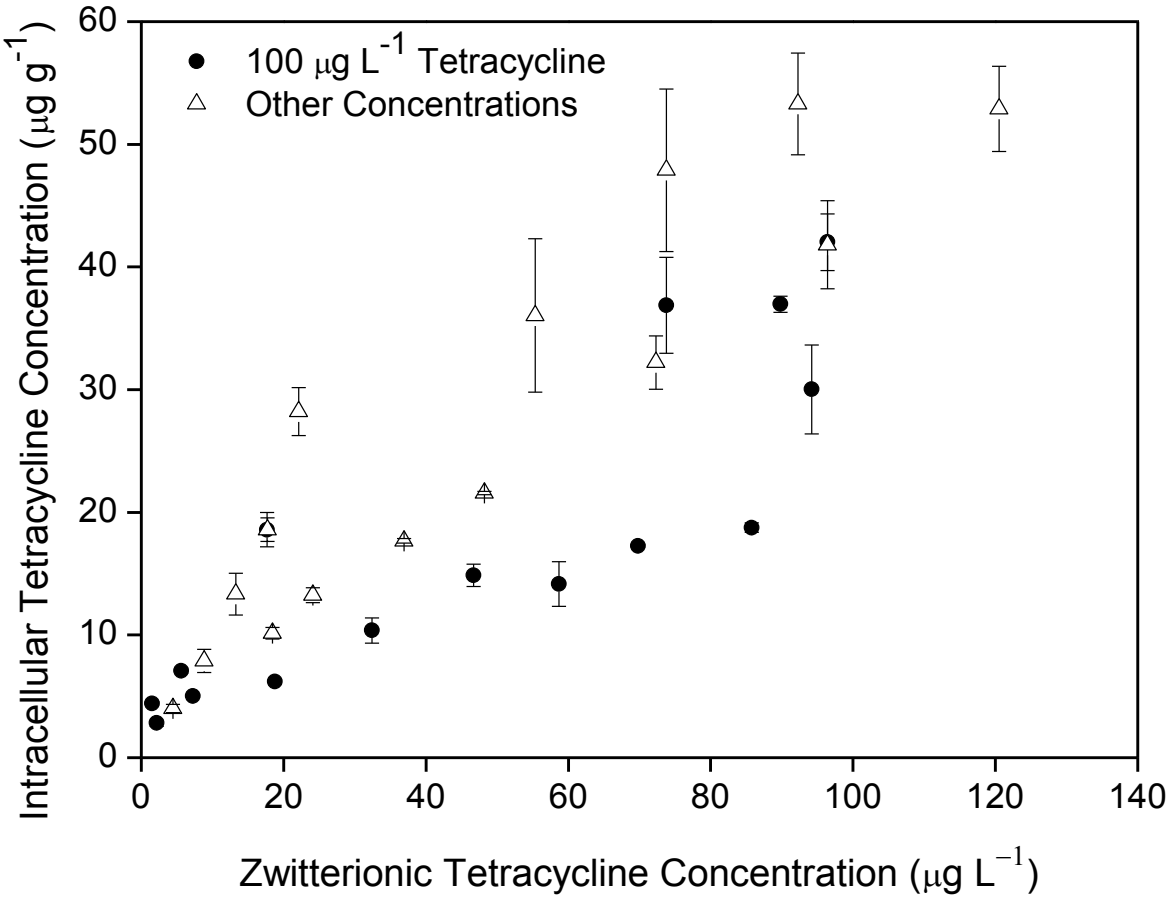


Figure 2.5. Relation of tetracycline zwitterion concentration in the ambient media and intracellular tetracycline concentration. Other tetracycline concentrations referred to as 25.0, 50.0, 75.0 and 125 μg $L^{-1}$ in LB media.

## ENVIRONMENTAL IMPLICATIONS

Tetracycline antibiotics are frequently found in the environment hence exerting potential selective pressure on the development and proliferation of antibiotic resistance in microbial communities. This study demonstrates the passage of tetracycline present in aqueous solution into bacterial cells (in this instance *E. coli*), and activate bacterial antibiotic resistance genes. The extent of uptake is modulated by tetracycline speciation. Geochemical factors such as pH, salt composition and concentration influences the fractional distributions of tetracycline species in aqueous solution and hence alters uptake by *E. coli*. We have identified that zwitterionic tetracycline as the primary species favorable for bacterial uptake. These results suggest that bacteria exposed at the same total external concentration of tetracycline will have varying intracellular tetracycline depending on aqueous geochemistry which affects the fractional distribution of each tetracycline species based on net charge. Geochemical conditions that favor formation of zwitterions will promote microbial uptake and maximize selective pressure for the development of antibiotic resistance genes. For instance, relatively high tetracycline concentrations are commonly found in animal manure which is frequently applied to soils; however, the comparatively high content of salts present in manure collection pits could plausibly form complexes with tetracyclines hence mitigating their potential uptake by bacteria， and antimicrobial impacts to the associated microbial communities. The subsequent land application of these manure wastes (feces plus urine) could, to some extent, dilute the salt content leading to the dissociation of metal-tetracycline complexes and formation of more zwitterionic species which would enhance tetracycline uptake and maximize selective pressure. Importantly, fundamental knowledge of the effects of

tetracycline speciation on bacterial uptake suggests approaches to minimize intracellular concentrations thereby modulating the extent of selective pressure posed by such antibiotics. Furthermore, results from this study can be utilized in predictive models to assess the impacts of aqueous phase tetracyclines in the environment on the enrichment and preservation of antibiotic resistance in bacteria. Most previous exposure and risk models for organic contaminants and pesticides simply use total antibiotic concentrations to assess their potential adverse effects. However, many pharmaceuticals can develop multiple species of differing charge in the environment with each possessing markedly different propensities for microbial uptake. We suggest that the speciation chemistry of tetracycline should be incorporated into the overall exposure and risk assessment framework for evaluating the potential risks associated with this and similar antibiotics in the environment. The fundamental knowledge gained from this study could also facilitate the development of scientifically informed management schemes to mitigate the potential enrichment of tetracycline antibiotic resistance in microbial populations associated with animal feeding operations and in soil receiving animal manure from such facilities for its ancillary fertilizer value.

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# CHAPTER III

## ORGANIC ACIDS ENHANCE TETRACYCLINE UPTAKE BY *E. COLI* AND ACTIVATION OF ANTIBIOTIC RESISTANCE GENES

**ABSTRACT**

Tetracyclines are a class of antimicrobials used extensively in concentrated livestock feeding operations. A large portion of tetracyclines administered to animals are excreted in manure and subsequently released into the environment though land application, where they pose potential risks to ecosystem and human health. Although it is known that the formation of metal-tetracycline complexes can reduce bioavailability of aqueous-phase tetracycline to *E. coli*, many naturally-occurring organic ligands also form complexes with metal cations, which could further alter the bioavailability of tetracycline. In this study, we investigated the influence of acetic acid, succinic acid, malonic acid, oxalic acid and citric acid on tetracycline uptake from water containing $Mg^{2+}$ and $Ca^{2+}$ by the *E. coli* bioreporter. The results revealed that the presence of organic acid ligands altered tetracycline speciation in a manner that enhanced its uptake by *E. coli*. The increase in bacterial uptake and antibiotic resistant response is positively related to the degree of complexation of metal cations in the order of citric acid > oxalic acid > malonic acid > succinic acid > acetic acid. The magnitude of this effect increased with increasing organic acid concentration in aqueous solution. A significant positive correlation between the concentration of intracellular tetracycline and zwitterionic tetracycline in aqueous solution indicated that (net) neutral tetracycline is the species that most readily enters the *E. coli* bioreporter cells. Understanding the influence of naturally-occurring organic acid ligands on tetracycline speciation and uptake by *E. coli* allows improved assessment of the potential risks for developing antibiotic resistant bacteria in the natural aqueous environment.

## INTRODUCTION

Tetracyclines are the most commonly used antibiotics in concentrated livestock feeding operations and in disease control for humans and animals. In 2011, approximately 5.6 million kilograms of tetracyclines were used for livestock production and disease control in the United States, equivalent to ~42% of the total antibiotics used in food-producing animals (2011). Large fractions of tetracyclines used in animal feeding operations are excreted with manure and urine either as the parent compounds or bioactive metabolites (Aga et al., 2005; Jacobsen et al., 2004). Land application of animal wastes containing high levels of tetracyclines (i.e. $\mu g\ kg^{-1}$ to $mg\ kg^{-1}$) is commonplace, which introduces these tetracycline antibiotics into the soils and waters of agroecosystems. As a result, tetracyclines have been frequently detected in soils (Hu et al., 2010; Jacobsen et al., 2004), surface waters (Batt and Aga, 2005; Christian et al., 2003b; Kolpin et al., 2002; Wei et al., 2011) and even in groundwater (Chee-Sanford et al., 2001; Gottschall et al., 2012; Hu et al., 2010). Tetracyclines introduced into the environment in this fashion plausibly exert selective pressure on indigenous bacteria resulting in the development of antibiotic resistance genes and the enrichment of antibiotic resistant bacterial populations (Chee-Sanford et al., 2001; Gibbs et al., 2004; Gilchrist et al., 2007b; Hong et al., 2013; Looft et al., 2012).

In order for tetracyclines to manifest maximal selective pressure and other biological effects on microorganism, they must pass though cytoplasmic membrane and interact with specific receptors. Due to the presence of ionizable functional groups, tetracyclines form multiple species of varying net charge in aqueous solution; the fractional distribution of these species depends on solution pH, ionic strength and

composition. The presence of one species vs. another can plausibly alter the bioavailability to microbes and hence the activation of antibiotic resistance genes. For example, inorganic cations (e.g. $Mg^{2+}$ and $Ca^{2+}$) in solution reduce the bioavailability of aqueous tetracycline to *E. coli* by forming metal-tetracycline complexes (Chapter II). In natural environments there are many naturally-occurring organic acids which could function as ligands and compete with tetracycline to form complexes with common inorganic cations such as $Ca^{2+}$ and $Mg^{2+}$ (Aiken et al., 2011; Mantoura et al., 1978). Changes in the complexation of tetracycline by inorganic cations in solution could potentially influence the uptake of tetracycline by bacteria hence altering the selective pressure exerted on native bacterial communities.

The objective of this study was to inspect whether, and to what extent, organic acid ligands shift the impact of metal cation i.e. $Mg^{2+}$ on tetracycline bioavailability in aqueous solution. We hypothesize that small naturally-occurring organic acids could compete with tetracycline in the formation of complexes with metal cations in aqueous solution thereby altering the speciation of tetracycline and its bioavailability to *E. coli*. To test this hypothesis, we selected several organic acid ligands commonly found in the aqueous environment, which display varying binding affinities for $Ca^{2+}$ and $Mg^{2+}$**.** It is expected *a priori* that the presence of these organic ligands in water will alter the complexation of tetracycline with $Ca^{2+}$ and/or $Mg^{2+}$**,** and in doing so change the degree of tetracycline uptake by bacteria and the antibiotic resistant responses evoked in the *E. coli* bioreporter. The bioreporter used was *E. coli* strain MC4100 containing the plasmid pTGM with transcriptional fusion between a tetracycline inducible promoter ($P_{tet}$) and a

fluorescence-assisted cell sorting optimized *gfp* gene (Hansen and Sørensen, 2000). Tetracycline uptake by the bioreporter (i.e. intracellular concentration) was quantified using high performance liquid chromatography integrated with tandem mass spectrometry (LC-MS/MS). Shifts in the fractional distributions of tetracycline species induced by the organic acids were estimated in an attempt to relate bacterial uptake of tetracycline (bioavailability) with the presence of competing organic acid ligands that from complexes with $Ca^{2+}$ and $Mg^{2+}$.

## MATERIALS AND METHODS

### *Chemicals*

Tetracycline hydrochloride (≥ 95%), ampicillin sodium salt (≥ 95%), methanol (HPLC grade), and MOPS buffer (pH range 6.5-7.9) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride, calcium chloride, magnesium chloride, oxalic acid, citric acid, acetic acid, ethylenediaminetetraacetic acid (EDTA), formic acid, sodium phosphate dibasic, and potassium phosphate monobasic were purchased from J.T. Baker (Philipsburg, NJ). Bacto tryptone and Bacto yeast extracts were purchased from Becton, Dickinson and Company (Sparks, MD). Malonic acid and succinic acid were purchased from MP Biomedicals (Solon, OH). Acetonitrile (HPLC grade) and hydrochloric acid (37%) were purchased from EMD Chemicals (Gibbstown, NJ). MES buffer (pH range 5.5-6.7) was purchased from Fisher Biotech (Fair Lawn, NJ).

### *Tetracycline Exposure to E. coli*

The *E. coli* strain MC4100/pTGM used as the whole-cell bacterial bioreporter in this study was constructed by inserting *tet*(M) gene (encoding tetracycline resistance by ribosomal protection) into plasmid pTGM, which contains a transcriptional fusion

between a *tetR*-regulated $P_{tet}$ promoter and flow cytometry-optimized *gfp* gene (*gfp*mut3) encoding green fluorescence protein (GFP) (Bahl et al. 2005). The *E. coli* bioreporter was cultured in a low-salt Luria-Bertani (LB) media (10.0 g of tryptone, 5.0 g of yeast extracts and 0.5 g of NaCl in 1.0 L of 0.22 μm-filtered MilliQ-water). The medium pH was adjusted to 7.0 using 50 mM of MOPS buffer followed by an autoclave at 121 ℃ for 30 min. Then, the *E. coli* bioreporter was inoculated in the LB medium amended with 100 mg $L^{-1}$ of ampicillin, and incubated on a horizontally-moving shaker at 150 rpm in a 30 ℃ incubation room. When the bacterial culture grew to mid-log phase as indicated by optical density at 600 nm ($OD_{600}$) of the culture approaching approximate 0.7, the culture was diluted 100 fold into 50.0 mL of freshly prepared LB media amended with 100 mg $L^{-1}$ of ampicillin. The LB media also contained 100 μg $L^{-1}$ of tetracycline and 5.0 mM of $MgCl_2$. In addition, selected common organic acid ligands were added to the culture individually. These ligands included citric acid (2.0, 4.0, 6.0, 8.0, 10.0 and 20.0 mM), oxalic acid (20.0 mM), malonic acid (20.0 mM), succinic acid (20.0 mM), and acetic acid (20.0 mM). All culture samples were prepared in triplicate. For each treatment 1.0 mL of culture sample was collected every 30 min to measure the emitted fluorescence from *gfp*mut3 with excitation wavelength of 488 nm and emission wavelength of 511 nm using a SpectraMax M2 spectrofluorometer (Molecular Devices, Sunnyvale, CA).

*Estimation of Promoter Activity*

Promoter activity (*P*) in the *E. coli* bioreporter at steady state was quantified according to the model developed by Leveau and Lindow (2001): $P = f_{ss} \times \mu \times (1 + {}^{\mu}/_{m})$, in which $f_{ss}$ (relative unit of fluorescent mature GFP per OD unit, RU $OD^{-1}$) is the

measured fluorescence emitted from the bioreporter at stead state, $m$ (1.54 $h^{-1}$ for *gfp*mut3) is the maturation constant for GFP maturing to fluorescent GFP, and $\mu$ ($h^{-1}$) is the bacterial growth rate. The $f_{ss}$ value was obtained as the slope of linear plot of measured fluorescence intensity against $OD_{600}$ values. The $\mu$ value was determined from the slope of natural logarithm of $OD_{600}$ of bacterial culture against time (h). This approach can circumvent the effects of dilution of GFP contents and GFP maturation during the growth of bacteria, which enables the comparison of the expression of antibiotic resistance (promoter activity) among varying experimental settings.

*Analysis of Tetracycline Concentrations in Solution and in Bacteria*

When $OD_{600}$ value of the *E. coli* cultures approached to approximate 0.5, the cultural suspensions were centrifuged at 15000 g and at 4 ℃ for 30 min. The supernatants (5.0 mL) were collected, and passed through pre-conditioned hydrophilic–lipophilic balance (HLB) cartridges (Waters Corporation, Milford, MA) to sorb tetracycline from solution. The pre-conditioning procedure of HLB cartridge consisted of a sequential rinse with methanol (3 mL), 0.1 M HCl (3 mL) and water (6 mL). The tetracycline-loaded cartridges were then washed with 1:9 (v/v) methanol/water solution (5 mL). Tetracycline retained on HLB cartridges was eluted with 1:1 (v/v) methanol/water solution (5.0 mL) containing 150 mg $L^{-1}$ of EDTA, and additional 5.0 mL of methanol containing 1% of formic acid. The eluted solutions were combined and analyzed for tetracycline concentration using a Shimadzu high-performance liquid chromatography fully integrated with an Applied Biosystems Sciex 3200 triple quadrupole mass spectrometer (LC-

MS/MS). A Gemini C18 column (5 µm, 50×2.0 mm, Phenomenex Inc., Torrance, CA) was used with a flow rate at 0.15 mL $min^{-1}$. The mobile phase consisted of phase A (95% MilliQ-water, 5% acetonitrile, 0.1% formic acid and 1 mM ammonium acetate) and phase B (100% acetonitrile, 0.5% formic acid and 1 mM ammonium acetate). Tetracycline was quantified in multiple reaction monitoring mode with precursor/product ion pair m/z 445.4/410.0. The extraction recovery for tetracycline from the LB media was 86% with a standard deviation of 10%.

After the centrifugation, the bacterial cell pellets were rinsed twice with 25 mL of 10-fold diluted phosphate-buffered saline solution (PBS solution: 80.0 g of NaCl, 2.0 g of KCl, 14.4 g of $Na_2HPO_4$ and 2.4 g of $KH_2PO_4$ dissolved in 1.0 L of water, pH 7.4). Then 10.0 mL of McIlvain buffer (12.9 g of citric acid monohydrate, 10.9 g of $Na_2HPO_4$ and 37.2 g of EDTA dissolved in 1.0 L of water) was used to suspend the cell pellets and dissolve tetracycline from the cells. This mixture was vortexed for 1 min, sonicated for 10 min followed by centrifugation at 15000 g for 15 min. This extraction step was repeated one more time. The supernatants were combined, subject to solid-phase extraction using HLB cartridge, and analyzed for tetracycline by LC-MS/MS as described above. The extraction recovery of tetracycline was measured at 108% with a standard deviation of 14%.

*Estimation of Tetracycline Speciation in Aqueous Phase*

Fractional distributions of tetracycline species in aqueous solution in the presence of 5.0 mM $MgCl_2$ and several selected organic acid ligands at pH 7.0 were calculated using MINEQL+ software version 4.5 (Environmental Research Software, Hallowell,

ME). The present organic ligands competed with tetracycline to complex with divalent cation $Mg^{2+}$, hence altering the distributions of tetracycline species. The parameter inputs in the MINEQL+ database were set to mimic the conducted experimental conditions. Tetracycline initial concentration was input as $2.25 \times 10^{-4}$ mM (100 μg $L^{-1}$) in aqueous phase, and solution pH was set at 7.0. The concentration of added organic acid ligands (citric acid, oxalic acid, malonic acid, succinic acid and acetic acid) was set as described above. The metal cation concentrations included 5.0 mM $Mg^{2+}$ from the added $MgCl_2$, 0.13 mM for $Ca^{2+}$ and 0.26 mM for $Mg^{2+}$ from the LB media. The acidic dissociation constants of tetracycline, and equilibrium constants (log*K*) for the association of $Ca^{2+}$and $Mg^{2+}$ with tetracycline anionic species used in the calculations are summarized in Table 3.1. The complexation constant for the association of $Ca^{2+}$and $Mg^{2+}$ with the selected organic acid ligands are also listed in Table 3.1. We assumed that the presence of the relatively low ionic strength in aqueous solution does not significantly alter the values of these equilibrium constants.

Table 3.1: Dissociation equilibrium constants of tetracycline and selected organic acids in aqueous solution and their complexation constants with $Ca^{2+}$ and $Mg^{2+}$.

| Compounds | Reactions | pKa | log *K* | |
|---|---|---|---|---|
| | | | $Ca^{2+}$ | $Mg^{2+}$ |
| Tetracycline | $H_3TC^+ \leftrightarrows H^+ + H_2TC^-$ | 3.3[a] | -- | -- |
| | $H_2TC^0 \leftrightarrows H^+ + HTC^-$ | 7.7[a] | -- | -- |
| | $HTC^- \leftrightarrows H^+ + TC^{2-}$ | 9.7[a] | -- | -- |
| | $M^{2+} + HTC^- \leftrightarrows MHTC^+$ | -- | 3.4[b] | 3.9[b] |
| | $M^{2+} + TC^{2-} \leftrightarrows MTC^0$ | -- | 5.8[b] | 4.1[b] |
| Acetic acid | $CH_3COOH \leftrightarrows H^+ + CH_3COO^-$ | 4.76[d] | -- | -- |
| | $M^{2+} + CH_3COO^- \leftrightarrows MCH_3COO^+$ | -- | 1.18[c] | 1.27[c] |
| Succinic acid | $(CH_2)_2(COOH)_2 \leftrightarrows H^+ + HOOC(CH_2)_2COO^-$ | 4.21[d] | -- | -- |
| | $HOOC(CH_2)_2COO^- \leftrightarrows H^+ + (CH_2)_2(COO^-)_2$ | 5.64[d] | -- | -- |
| | $M^{2+} + CH_2(COO^-)_2 \leftrightarrows MCH_2(COO)_2$ | -- | 2.00[d] | 2.00[d] |
| Malonic acid | $CH_2(COOH)_2 \leftrightarrows H^+ + HOOCCH_2COO^-$ | 2.85[d] | -- | -- |
| | $HOOCCH_2COO^- \leftrightarrows H^+ + CH_2(COO^-)_2$ | 5.70[d] | -- | -- |
| | $M^{2+} + CH_2(COO^-)_2 \leftrightarrows MCH_2(COO^-)_2$ | -- | 2.35[d] | 2.85[d] |
| Oxalic acid | $(COOH)_2 \leftrightarrows H^+ + HOOCCOO^-$ | 1.25[d] | -- | -- |
| | $HOOCCOO^- \leftrightarrows H^+ + (COO^-)_2$ | 4.27[c] | -- | -- |
| | $M^{2+} + (COO^-)_2 \leftrightarrows M(COO)_2$ | -- | 3.00[c] | 3.43[c] |

$M^{2+}$: metal cation $Ca^{2+}$ or $Mg^{2+}$; [a] data from Gu and Karthikeyan, 2005; [b] data from Werner et al., 2006; [c] data from MINEQL+ database (Version 4.5, 2002); [d] data from Martell, 1997

Table 3.1 (cont'd)

| Compounds | Reactions | pKa | log *K* | |
|---|---|---|---|---|
| | | | $Ca^{2+}$ | $Mg^{2+}$ |
| Citric acid | $HOOCCOH(CH_2)_2(COOH)_2 \leftrightarrows H^+ + HOOCCOH(CH_2)_2COOHCOO^-$ | 3.13[d] | -- | -- |
| | $HOOCCOH(CH_2)_2COOHCOO^- \leftrightarrows H^+ + HOOCCOH(CH_2)_2(COO^-)_2$ | 4.76[d] | -- | -- |
| | $HOOCCOH(CH_2)_2(COO^-)_2 \leftrightarrows H^+ + COH(CH_2)_2(COO^-)_3$ | 6.40[d] | -- | -- |
| | $M^{2+} + HOOCCOH(CH_2)_2COOHCOO^- \leftrightarrows M^+HOOCCOH(CH_2)_2COOHCOO$ | -- | 4.87[c] | 4.87[c] |
| | $M^{2+} + HOOCCOH(CH_2)_2(COO^-)_2 \leftrightarrows MHOOCCOH(CH_2)_2(COO)_2$ | -- | 2.86[c] | 2.86[c] |
| | $M^{2+} + COH(CH_2)_2(COO^-)_3 \leftrightarrows MCOH(CH_2)_2(COO)_3^-$ | -- | 1.10[c] | 1.10[c] |

## RESULTS AND DISCUSSION

*Effects of Organic Acid Ligands on Bacterial Uptake of Tetracycline-Metal Complexes*

Tetracycline in aqueous solution enters the *E. coli* bioreporter cells and activates the antibiotic resistance genes which cause the emission of fluorescence by the GFP which is proportional to tetracycline concentrations in the cells (Chapter II). The presence of $MgCl_2$ in aqueous solution reduced the uptake of tetracycline by the *E. coli* bioreporter as indicated by a corresponding decrease in the antibiotic resistant response (expressed as promoter activity). Reduced tetracycline uptake is due primarily to the complexation between tetracycline and $Mg^{2+}$ which inhibits the diffusion into bacterial cells (Chapter II). Amendment of the LB media containing 100 $\mu g\ L^{-1}$ of tetracycline with 5.0 mM $MgCl_2$ reduced bacterial uptake of tetracycline by 92.7%; intracellular tetracycline concentration was reduced from 44.5 ± 0.2 to 3.3 ± 0.1 $\mu g\ g^{-1}$, and the corresponding promoter activity decreased from 1477 ± 90 to 184 ± 4.5 RU $OD^{-1}\ h^{-1}$ (Figure 3.1). This inhibitory effect of $Mg^{2+}$ was mitigated by the presence of organic acid ligands (20 mM) in the media. The organic acids were observed to promote tetracycline uptake in the presence of $Mg^{2+}$ as evidenced by the activation and expression of antibiotic resistant genes (Figure 3.1). Apparently the organic acid ligands competed with tetracycline forming metal cation complexes with $Mg^{2+}$ (or $Ca^{2+}$) in a manner that enhanced uptake; the intracellular tetracycline concentration increased from 3.3 ± 0.1 $\mu g\ g^{-1}$ to 5.4 ± 0.5, 6.4 ± 0.3, 13.3 ± 1.8, 44.3 ± 2.2, 70.3 ± 4.0 $\mu g\ g^{-1}$ for the amendments of acetic acid,

succinic acid, malonic acid, oxalic acid and citric acid, respectively (Figure 3.1A), and the corresponding estimated promoter activities increased proportionally from 184 ± 4.5 RU $OD^{-1}$ $h^{-1}$ to 186 ± 6, 221 ± 9, 428 ± 26, 1582 ± 141 and 2533 ± 36 RU $OD^{-1}$ $h^{-1}$, respectively (Figure 3.1B).

The observed increase in bacterial uptake and expression of the antibiotic resistant response is positively related to the capability of the organic acid ligands to complex with $Mg^{2+}$. Figure 3.1 shows that the *E. coli* bioreporter exposed to a constant concentration of tetracycline (100 μg $L^{-1}$) and $Mg^{2+}$ (5.0 mM) manifested varying extents of tetracycline uptake and as expressed by the magnitude of the antibiotic resistance response in the presence of a constant concentration of organic acid ligands (20.0 mM). Among the tested organic acid ligands, citric acid is the strongest (chelating) ligand for $Mg^{2+}$ (Table 3.1). The comparatively higher effectiveness of citric acid in complexing with $Mg^{2+}$ is demonstrated by the release of more tetracycline for bacterial uptake. Compared with oxalic acid, citric acid was 59% more effective in inducing an increase in bacterial uptake of tetracycline, or 60% more effective based on the bioresponse. When comparing the other relatively weaker ligands (to citric acid), acetic acid, succinic acid and malonic acid were only 7.7%, 9.1% and 18.9% as effective as citric acid for enhancing tetracycline uptake, and the corresponding increases of promoter activities were 7.3%, 8.7% and 16.7% of that amended with citric acid. These results suggest that the presence of naturally-occurring organic acid ligands could alter the predominant tetracycline species present in solution and in this fashion enhance its uptake by *E. coli*. Clearly, the stronger metal-binding ligand leads to greater tetracycline bioavailability, i.e.

greater uptake by the *E. coli* bioreporter cells with concomitant increase in bioresponse to the antibiotic.

It is noted that in the LB media amended with citric acid, tetracycline uptake by the *E. coli* bioreporter was 57.8% greater than that treatment with only 100 μg $L^{-1}$ of tetracycline, and the corresponding promoter activity increased 71.5% (Figure 3.1). This indicates that more tetracycline was bioavailable in the treatment amended with citric acid. In addition to competition between citric acid and tetracycline for metal cations, the enhanced tetracycline bioavailability could be also due to the increased disassociation of tetracycline from constituents present in the LB media matrices due to the presence of citric acid, which will be further elucidated below.

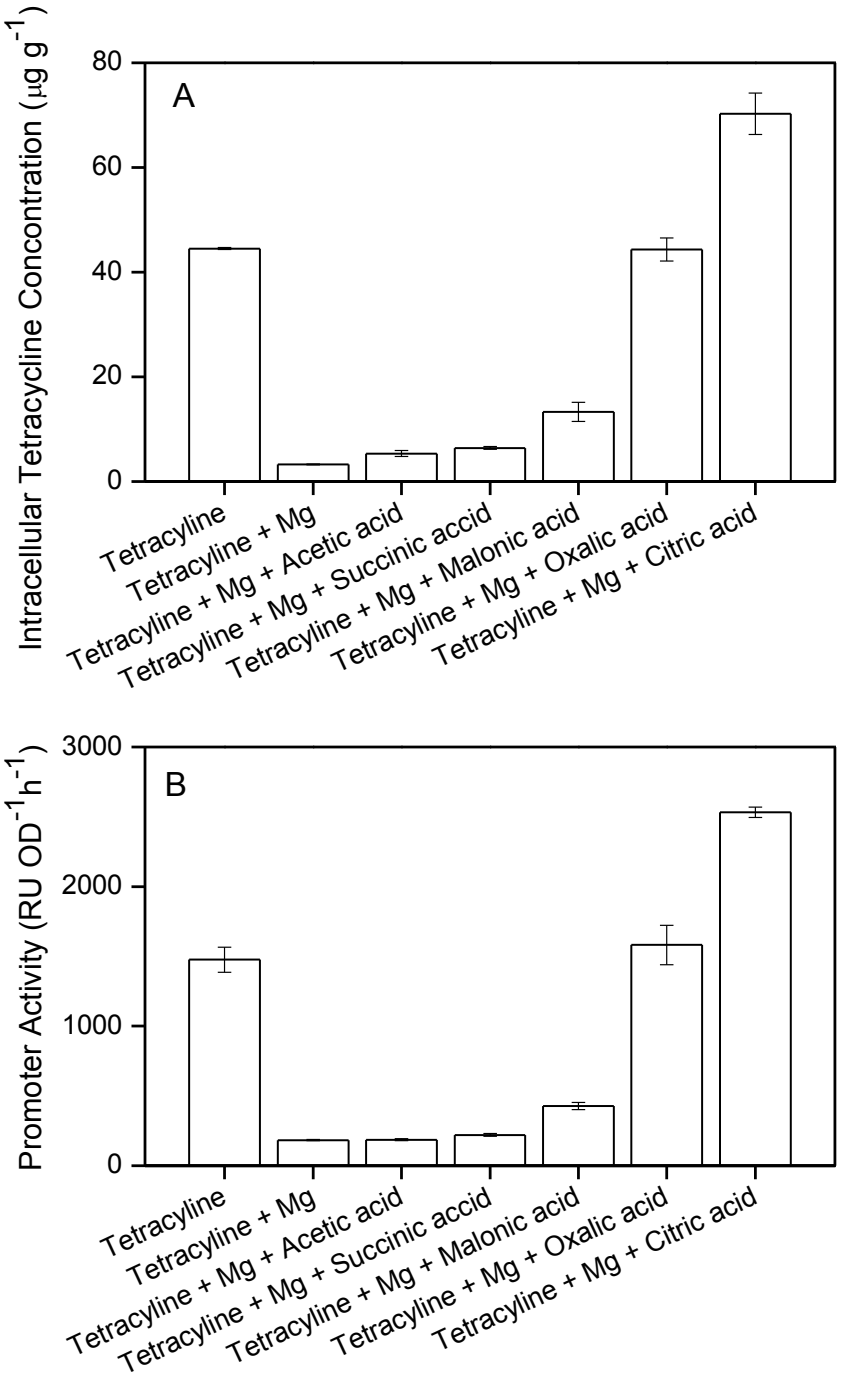


Figure 3.1. (A) Uptake of tetracycline by *E. coli* whole-cell bioreporter and (B) calculated promoter activity of the bioreporter exposed to 100 μg $L^{-1}$ of tetracycline in the LB media (pH 7.0) in the presence of 5.0 mM $Mg^{2+}$ and 20.0 mM of organic acid ligands.

The LB media containing 100 μg $L^{-1}$ of tetracycline and 5.0 mM of $MgCl_2$ was amended with a series of increasing citric acid concentrations (0 to 20 mM). It is apparent that bacterial uptake of tetracycline and antibiotic resistant response increased with increasing citric acid concentration (Figure 3.2). The intracellular tetracycline concentration was measured (by LC-MS/MS) as 3.4 ± 0.0 μg $g^{-1}$ for the citric acid-free treatment, and increased to 14.4 ± 0.2, 30.8 ± 0.3, 47.3 ± 0.9, 58.5 ± 1.6, 67.6 ± 2.9, and 70.3 ± 4.0 μg $g^{-1}$ at citric acid concentrations of 2.0, 4.0, 6.0, 8.0, 10.0 and 20.0 mM (Figure 3.2A), respectively. The estimated promoter activities increased from 184 ± 4 RU $OD^{-1}$ $h^{-1}$ for the treatment devoid of citric acid to 373 ± 9, 933 ± 18, 1683 ± 12, 2020 ± 52, 2230 ± 24, and 2533 ± 36 RU $OD^{-1}$ $h^{-1}$ in the same series of samples (Figure 3.2B). The added citric acid complexed $Mg^{2+}$ in aqueous solution, facilitating the release of the tetracycline species favoring bacterial uptake. The presence of naturally-occurring organic acid ligands could result in enhanced bacterial uptake of tetracycline hence increase the selective pressure for development of antibiotic resistance in microbial communities.

Amendment with relatively high concentration of citric acid e.g. > 6.0 mM could result in tetracycline uptake by the *E. coli* bioreporter and promoter activity greater than that in the control free of citric acid or $MgCl_2$. The tetracycline intracellular concentrations were 6.3%, 31.4%, 51.9% and 58.0%, and the corresponding promoter activities were 17.9%, 41.6%, 56.3% and 77.5% greater than the control at citric acid concentrations of 6.0, 8.0, 10.0and 20.0 mM, respectively (Figure 3.2). Again, these

results indicate that more tetracycline became bioavailable for bacterial uptake when a relatively large amount of citric acid was present in the ambient solution, i.e. more citric acid than needed to overcome of negative effect of 5.0 mM $Mg^{2+}$ The additional citric acid plausibly disrupted tetracycline interactions with constituents of the LB media hence releasing tetracycline into solution for bacterial uptake.

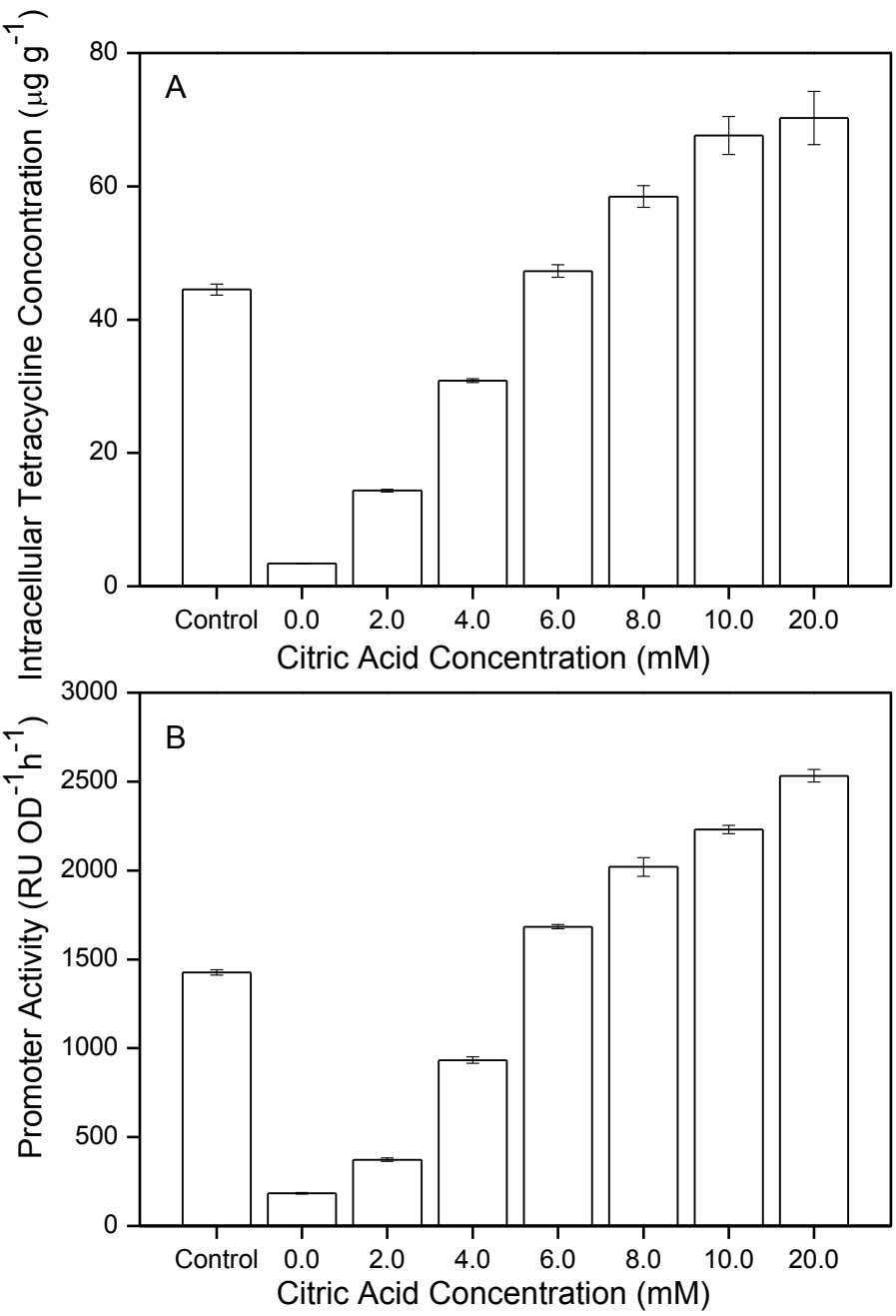


Figure 3.2. (A) Uptake of tetracycline by the *E. coli* bioreporter, and (B) the corresponding biological responses (promoter activity) in the presence of 5 mM $Mg^{2+}$ and citric acid at different concentrations. Tetracycline initial concentration: 100 μg $L^{-1}$, and pH: 7.0.

The enhanced bacterial uptake of tetracycline and the associated activation of the bioreporter could be due to the substitution of citric acid for tetracycline in $Mg^{2+}$-complexes thereby releasing effective tetracycline species for bacterial uptake, and/or an increase of tetracycline dissociation from constituents of the LB media. In LB media itself contains two divalent cations, i.e. $Mg^{2+}$ and $Ca^{2+}$, at concentrations of 0.13 mM and 0.26 mM, respectively, which could complex with ca. 18.8 % of the tetracycline (100 $\mu g\ L^{-1}$) present. The citric acid added to LB media alone competed with tetracycline for the two divalent cations, resulting in the dissociation of tetracycline from its metal complexes in a form available for bacterial uptake. Data in Figure 3.3 shows that when citric acid was added in increasing amounts (from 0 to 10.0 mM) the promoter activity increased from 1298 RU $OD^{-1}\ h^{-1}$ (citric acid-free control) to approximately 2000 RU $OD^{-1}\ h^{-1}$ at 2.0 mM of citric acid, then remained essentially constant. Simultaneously, the estimated zwitterionic fraction of tetracycline rapidly increased from 73.8% to 90.7% as the citric acid concentration was greater than 2.0 mM (Figure 3.3). The pre-existing metal cations e.g. $Ca^{2+}$ and $Mg^{2+}$ could complex with a portion of tetracycline (18.8%) in the LB media. The added citric acid complexed with these metal cations, hence freeing the metal-complexed tetracycline for bacterial uptake. However, in the presence of citric acid (> 2.0 mM), the promoter activity increased approximately 54.1%, which was much greater than the maximum release of tetracycline (16.9%) from metal-tetracycline complexes (Figure 3.3). This suggests that besides the release of tetracycline from its metal complexes, citric acid might also facilitates dissociation of tetracycline from the

LB matrices in which tetracycline could affiliate with some constituents in the LB media such as proteins/peptides. Tetracycline could bind to proteins (Kunin et al., 1973; Rolinson and Sutherland, 1965); the major constituents in the LB media are peptides/proteins with which tetracycline could be associated. The added citric acid plausibly also compete with tetracycline for these interaction sites in LB media, which drives the further release of tetracycline for bacterial uptake.

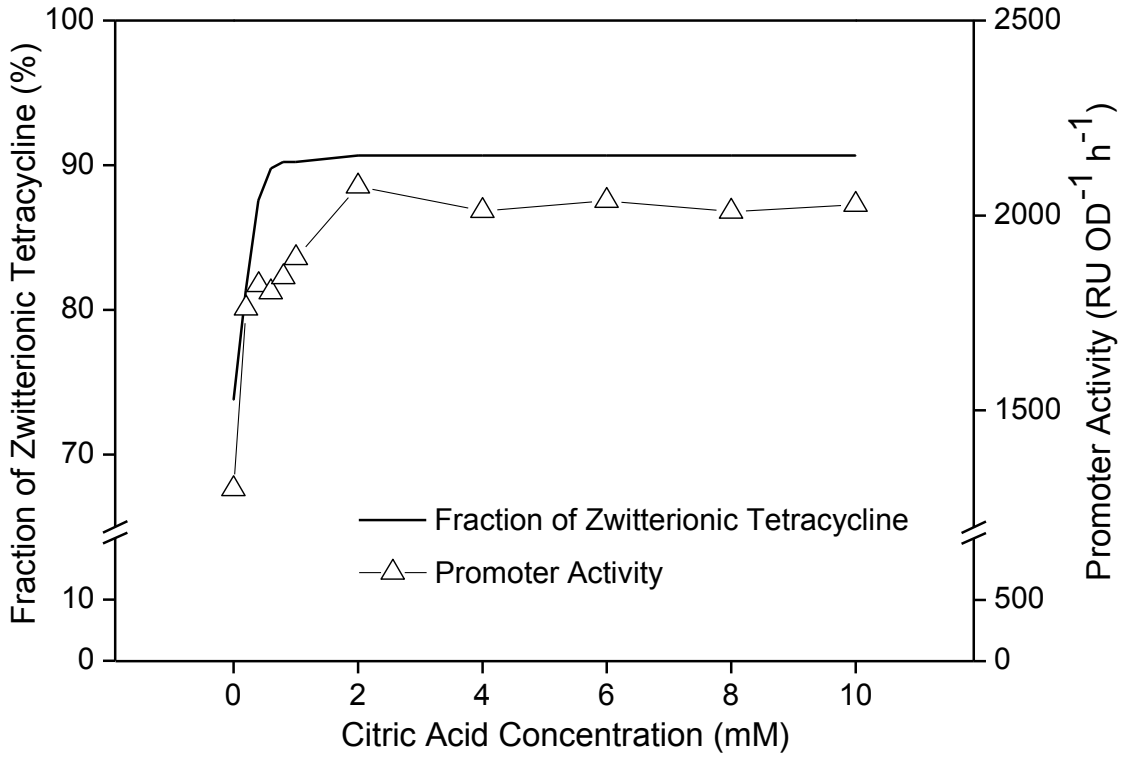


Figure 3.3. Fractional zwitterion tetracycline in LB media and promoter activity of the *E. coli* bioreporter as a function of amended citric acid concentration. Tetracycline initial concentration: 100 μg $L^{-1}$, pH: 7.0, neither $MgCl_2$ nor $CaCl_2$ was added in the LB media.

*Tetracycline Species Optimal for Bacterial Uptake*

The fractional distribution of tetracycline species in aqueous solution was estimated with the input condition similar to that used in the experiment (Table 3.2). The three ionizable functional groups of tetracycline result in four distinct tetracycline species in aqueous solution viz. cation, zwitterion, anion and divalent anion (Table 3.2). The added or LB media-derived metal cations complexed with tetracycline anions forming 1:1 metal-tetracycline complexes; the exact magnitude of the fraction depends on the complexation constants (Martin, 1979; Schmitt and Schneider, 2000; Werner et al., 2006). The amended organic acid ligands compete with tetracycline to form complexes with divalent metal cations present hence shifting the fractional distributions of tetracycline species.

At pH of 7.0, tetracycline existed primarily as zwitterion ($H_2TC^0$), anion ($HTC^-$), and metal-anion complexes ($MHTC^+$ and $MTC^0$), which account for more than 99% of the total tetracycline in aqueous solution (Table 3.2). The sum of tetracycline cation ($H_3TC^+$) and divalent anion ($TC^{2-}$) were estimated < 1% of the total tetracycline in solution and hence were not reported (Table 3.2). In the experiments conducted utilizing different organic acids and metal cations at differing concentrations, the zwitterion of tetracycline ranged from 18.8 to 90.7 %, the anion ranged from 1.9 to 8.9 %, and the combined metal-tetracycline complexes contributed to 0.4 to 79.2% of the total tetracycline in the solution. The LB media contained 0.13 mM $Ca^{2+}$ and 0.26 mM $Mg^{2+}$ which interacted with tetracycline forming metal-tetracycline complexes which accounts

~18.8 % of the total tetracycline (Table 3.2). Thus, a portion of tetracycline was present as metal complexes even in experiments without the added $MgCl_2$.

Table 3.2: Fractional distribution of tetracycline speciation in media at pH 7.0.

| Organic Ligands | Concentration of Organic Ligands (mM) | Metal Cation Concentration[a] (mM) | | Tetracycline Species (%) | | | |
|---|---|---|---|---|---|---|---|
| | | $Ca^{2+}$ | $Mg^{2+}$ | $H_2TC^0$ | $HTC^-$ | $MHTC^+$ | $MTC^0$ |
| -- | 0 | 0.13 | 0.26 | 73.8 | 7.4 | 17.9 | 0.9 |
| -- | 0 | 0.13 | 5.26 | 18.8 | 1.9 | 78.9 | 0.4 |
| Acetic acid | 20.0 | 0.13 | 5.26 | 23.6 | 2.2 | 73.9 | 0.4 |
| Succinic acid | 20.0 | | | 38.0 | 3.8 | 57.9 | 0.4 |
| Malonic acid | 20.0 | | | 67.6 | 6.6 | 25.7 | 0.3 |
| Oxalic acid | 20.0 | | | 83.1 | 8.1 | 8.7 | 0.1 |
| Citric acid | 2.0 | 0.13 | 5.26 | 26.5 | 2.7 | 70.3 | 0.4 |
| | 4.0 | | | 44.9 | 4.5 | 50.2 | 0.3 |
| | 6.0 | | | 84.0 | 8.3 | 7.6 | 0 |
| | 8.0 | | | 88.9 | 8.8 | 2.3 | 0 |
| | 10.0 | | | 89.8 | 8.9 | 1.3 | 0 |
| | 20.0 | | | 90.7 | 8.9 | 0.4 | 0 |

[a] including metal cations in the LB media, 0.13 mM for $Ca^{2+}$ and 0.26 mM for $Mg^{2+}$.

To evaluate which tetracycline species preferentially enters the bacterial cells (bioreporter), correlation analysis was conducted to identify any significant relations between the intracellular tetracycline concentration and the predominant tetracycline species present in the LB media using the data collected from this study, as well as data describing the bacterial uptake of tetracycline at different pH values and in the presence and absence of $CaCl_2$, $MgCl_2$ and KCl reported previously (Chapter II). Using the entire data set, correlation coefficients relating tetracycline intracellular concentration to fractional distribution of $H_2TC^0$, $HTC^-$, $MHTC^+$ and $MTC^0$ were 0.77, 0.49, -0.75 and -0.29, respectively. The significant positive correlation between intracellular tetracycline concentration and $H_2TC^0$ in the ambient solution indicated that zwitterionic tetracycline is the major species favored for uptake by the *E. coli* bioreporter. The negative relationship between intracellular tetracycline concentration and metal-tetracycline complexes implicates that these complex species could inhibit tetracycline uptake by bacteria. Organic acid ligands present in the media inhibited the formation of metal-tetracycline complexes, hence releasing tetracycline for bacterial uptake.

In the LB media containing the same initial concentration of tetracycline (100 μg $L^{-1}$), the presence of organic acid ligands shifted the fractional distributions of tetracycline species such that the zwitterionic tetracycline concentration ranged from 18.8 μg $L^{-1}$ for the control amended with 5.0 mM of $MgCl_2$ to 90.7 μg $L^{-1}$ in media amended with 20.0 mM of citric acid. The corresponding measured bacterial uptake increased from 3.3 to 89.7 μg $g^{-1}$ (Figure 3.4) with more uptake occurring in media amended with

organic ligands that formed stronger complexes with $Mg^{2+}$, such as citric acid and oxalic acid. A similar positive relation between bacterial uptake and amount of tetracycline zwitterions was previously observed for the data collected without the amendment of organic ligands (Chapter II); these data are included in Figure 3.4. Taken together, the data strongly support zwitterionic tetracycline as the favored species for bacterial uptake. This conclusion is supported by similar observations reported in previous studies on the determination of minimum inhibitory concentrations to bacteria (Nikaido and Thanassi, 1993; Thanassi et al., 1995; Yamaguchi et al., 1991). In Figure 3.4 when the zwitterionic tetracycline concentration was 90.7 $\mu g\ L^{-1}$ in the LB media amended with citric acid, bacterial uptake reached $70.3 \pm 4.0\ \mu g\ g^{-1}$ which was greater than the uptake from the LB media in the absence of organic ligands. We attribute this to the displacement of various bound or complexed forms of tetracycline by citric acid. Such competitive effects could plausibly result in the release of more tetracycline into aqueous solution in a form that readily enters bacterial cells and exerts its maximal selective pressure for the development of antibiotic resistance.

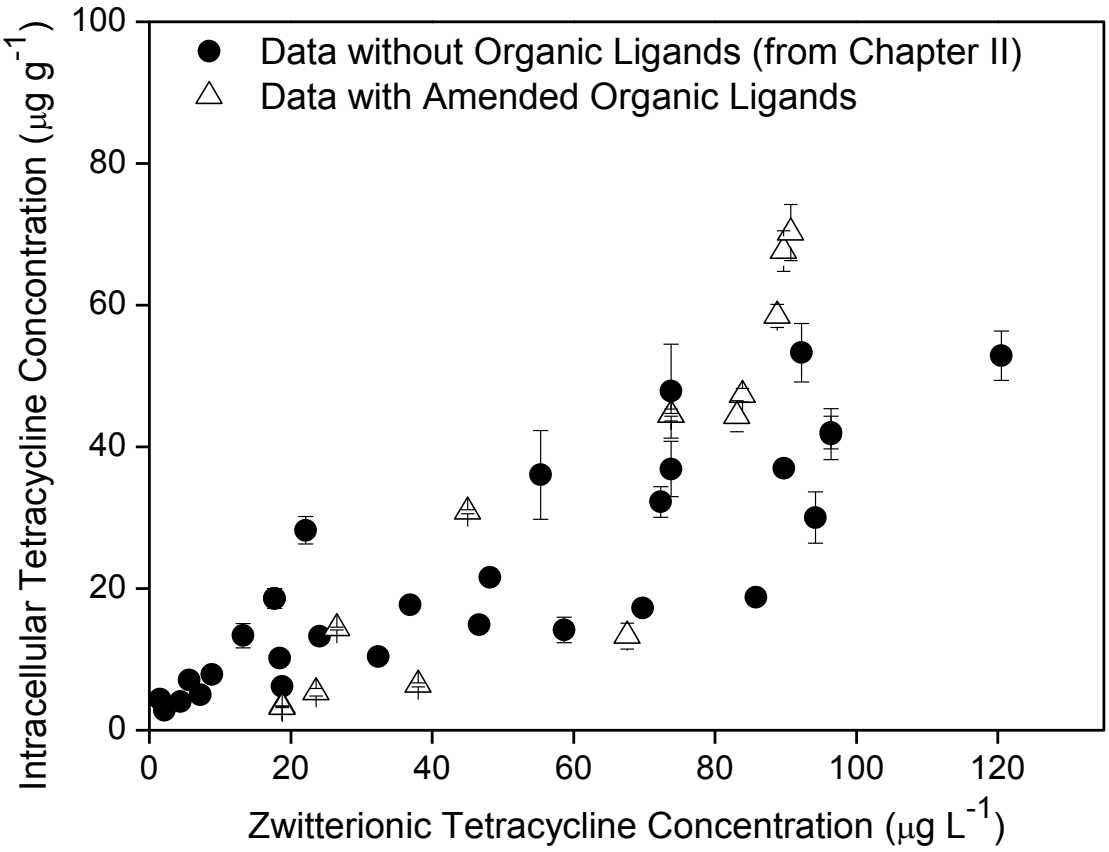


Figure 3.4. Relationship of tetracycline zwitterion concentration in the ambient media and intracellular tetracycline concentration.

*Relating Measured Intracellular Tetracycline Concentration to Promoter Activity*

Despite the fact that the *E. coli* bioreporter was consistently exposed to 100 μg $L^{-1}$ of tetracycline in all experiments, the expressed promoter activity ranged from 179 RU $OD^{-1}$ $h^{-1}$ in LB media amended with 5.0 mM $MgCl_2$, to 2572 RU $OD^{-1}$ $h^{-1}$ in LB media containing 20.0 mM citric acid (Figure 3.5). The presence of organic acid ligands in the LB media significantly enhanced the uptake of tetracycline and the magnitude of antibiotic resistance expression in the *E. coli* bioreporter. The maximum observed intracellular tetracycline concentration was 74.0 μg $g^{-1}$, which occurred in the presence of 20.0 mM citric acid, and was accompanied by the maximum expression of antibiotic response measured by promoter activity (Figure 3.5). Considering the entire data set described herein, a good linear relation between promoter activity and intracellular tetracycline concentration has been demonstrated for the *E. coli* bioreporter culture in the LB media, including that amended with naturally-occurring organic acids (Figure 3.5). The relations between bacterial uptake and promoter activity appear equally valued for experimental systems in which *E. coli* was exposed to varying levels of tetracycline at different pH values, and in the presence and absence of $CaCl_2$, $MgCl_2$ and KCl (reported in Chapter II). Analysis of the combined datasets demonstrated a highly significant linear relationship between promoter activity and intracellular tetracycline concentration with a correlation coefficient ($R^2$) of 0.98. This result strongly suggests that only intracellular tetracycline present in the *E. coli* bioreporter can evoke antibiotic resistance which can be quantified by the measurement of emitted fluorescent light as described herein.

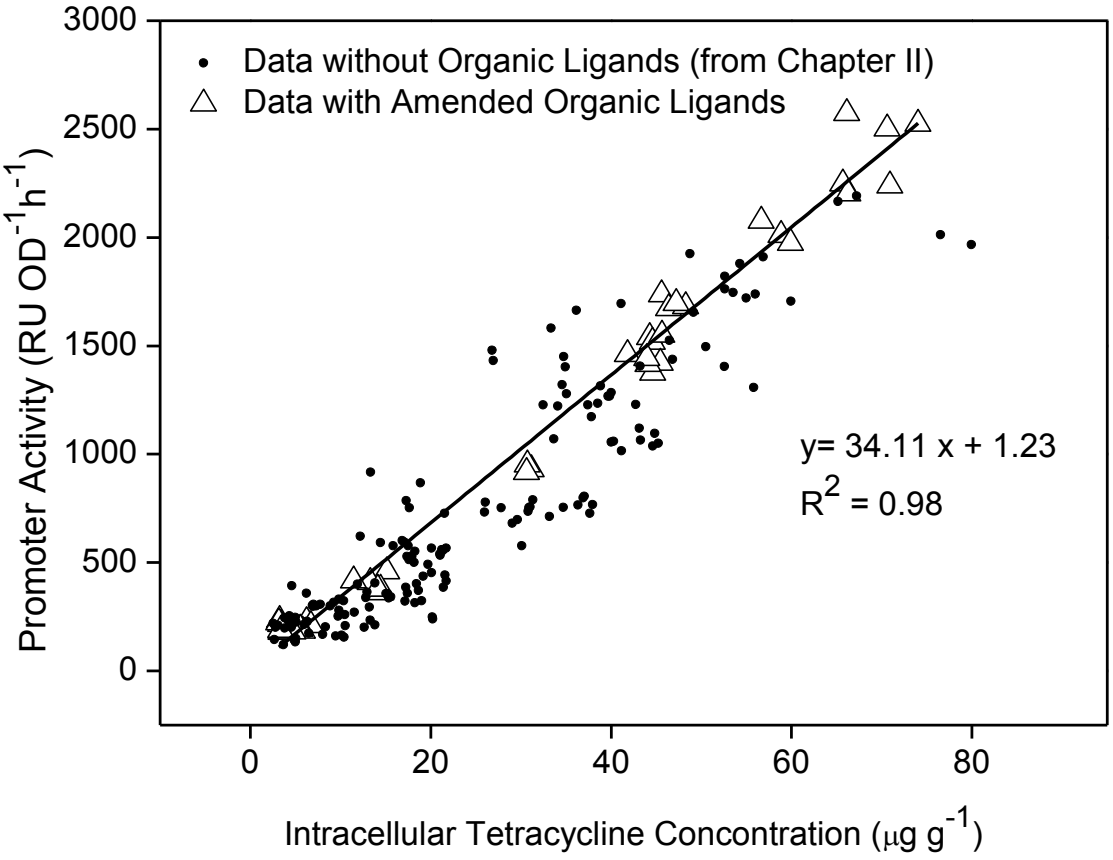


Figure 3.5. Relationship between intracellular tetracycline concentration and the expressed promoter activity of the *E. coli* bioreporter. Data included those collected with tetracycline initial concentration of 100 μg $L^{-1}$ with 5.0 mM of $Mg^{2+}$ at pH of 7.0 in the presence and absence of organic ligands, as well as the data collected in the study reported in Chapter II, that is, the *E. coli* was exposed to varying tetracycline concentrations at different pHs, in the presence and absence of metal cations.

## CONCLUSION

The presence of organic acid ligands was found to alter the fractional distribution of tetracycline species in aqueous solution. Such changes favored tetracycline uptake by *E. coli* which evoked the increased expression of antibiotic resistance. The enhanced bacterial uptake and antibiotic resistant response are positively related to the strength of divalent metal cation complexation in the order: citric acid > oxalic acid > malonic acid > succinic acid > acetic acid. Bacterial uptake of tetracycline and the associated antibiotic resistant response increased with increasing organic ligand concentration in the ambient aqueous solution. A significant positive correlation between intracellular tetracycline concentration and the aqueous phase zwitterionic tetracycline concentration was observed indicating that zwitterionic tetracycline is the major species which favors uptake by the *E. coli* bioreporter. The presence of naturally-occurring organic acid ligands in aqueous solution enhances uptake of tetracycline by bacteria, thereby maximizing the selective pressure for development and enrichment of antibiotic resistance in exposed microbial communities.

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# CHAPTER IV

# BIOAVAILABILITY OF CLAY-SORBED TETRACYCLINE TO *E. COLI* FOR ACTIVATION OF ANTIBIOTIC RESISTANCE GENES

**ABSTRACT**

The objective of this study is to determine bioavailability of clay-sorbed tetracycline to bacteria for expression of antibiotic resistance. Tetracyclines are a class of antimicrobials used extensively in human and veterinary medicine, and in livestock production. A large portion of tetracycline administered to humans and animals is excreted and subsequently released into the environment and could be strongly associated with soil minerals. It is not clear that whether tetracycline bound to soils is bioavailable to bacterial strains and evokes antibiotic resistance genes. In this study, we used *E. coli* MC4100/pTGM whole-cell bioreporter as an effective tool to investigate the bioavailability of smectite clay-sorbed tetracycline under varying conditions. In clay suspension, tetracycline sorbed by Mg-smectite was desorbed to aqueous phase, and became bioavailable to the whole-cell bioreporter. Desorption of tetracycline from clay to solution is the major exposure pathway for bacterial uptake and subsequent activation of antibiotic resistance in the diluted clay suspensions. Direct contact of the *E. coli* bioreporter with clay surfaces plausibly facilitated the transfer of clay-sorbed tetracycline to bacteria, but to a much lower extent. For tetracycline-loaded clays after multiple cycles of desorption, reducing volume of cultured solution was observed to evoke higher expression of antibiotic resistance from the bioreporter. Strong fluorescence emitted from bioreporter in the culture with clay films clearly indicated that clay-sorbed tetracycline is still bioaccessible to *E. coli*, and formation of biofilms facilitates bacterial uptake of tetracycline. Close contact of bacteria with soil surfaces, which are the most common in the environment, could significantly enhance bioavailability of soil-sorbed tetracycline, hence exposure and risks to the surrounding microbial communities.

## INTRODUCTION

Tetracycline antibiotics have been primarily used in livestock production for disease control, and as a feed supplement at subtherapeutic levels to improve feeding efficiency, growth rate and animal health. Tetracyclines ranked as the most highly used group of antibiotics in the United States in 2011; 5.6 million kilograms were administrated, which is equivalent to ~42% of the total antibiotics used for livestock production (2011). Of this, approximately 50 to 80% is excreted with manure and urine in unmetabolized forms (Halling-Sorensen et al., 2002). Even after a period of on-farm storage, these excreted wastes contain significant amounts of tetracyclines (Hamscher et al., 2002; Kay et al., 2004), and subsequent land application results in the dissemination of tetracyclines in the receiving soils (Hu et al., 2010; Jacobsen et al., 2004), adjacent surface waters (Batt and Aga, 2005; Christian et al., 2003b; Kolpin et al., 2002; Wei et al., 2011) and their sediments (Gibs et al., 2013; Luo et al., 2011; Zhou et al., 2011) as well as groundwater (Chee-Sanford et al., 2001; Gottschall et al., 2012; Hu et al., 2010). The presence of tetracyclines in these environmental matrices plausibly exerts selective pressure on indigenous bacterial populations leading to the development and proliferation of antibiotic resistant bacterial strains (Chee-Sanford et al., 2001; Gibbs et al., 2004; Gilchrist et al., 2007b; Hong et al., 2013; Looft et al., 2012; Seyfried et al., 2010).

Sorption of tetracyclines by soils and sediments plays a dominant role in controlling the soil/sediment-water distribution, transport, and bioavailability of these antibiotics in natural environments. The role of tetracyclines released into the soil environment in the development and preservation of antibiotic resistance genes in bacteria is plausibly linked to the sorptive interactions of these antibiotics with natural

geosorbents. Tetracyclines are known to bind extensively to geosorbents (e.g. clay) in soils with a sorption capacity ranging from 250 to 600 μmol $g^{-1}$ (Ding et al., 2011; Figueroa et al., 2004). However, the effect of tetracycline sorption on its availability for microbial uptake (i.e. its "bioavailability") remains poorly understood and controversial. A few studies reported that soil-sorbed tetracyclines were still biologically active and possibly influenced the growth of bacteria, but tetracycline desorption from soils to solution was assumed to be the prerequisite step (Chander et al., 2005; Goetsch et al., 2012). A recent study provided evidence that soil-sorbed tetracycline did not apparently exert selective pressure on bacteria, suggesting the reduced risk of sorbed tetracycline (Subbiah et al., 2011). The objective of this study is to determine whether, and to what extent, soil-sorbed tetracycline is bioavailability to bacteria.

Until very recently, the prevailing dogma simply assumed that only contaminants present in bulk aqueous solution are bioavailable. Accordingly, desorption was a prerequisite for the degradation of contaminants by bacteria, and only contaminants dissolved in bulk water could directly contact the cell membrane. Soil- or sediment-bound contaminants that do not desorb into bulk water remain unavailable for microbial uptake. However, more recent studies have utilized measurements of contaminant degradation and mineralization to provide compelling evidence that bacteria can access pools of soil-sorbed organic chemicals without the requirement of desorption into bulk solution (Calvillo and Alexander, 1996; Guerin and Boyd, 1992, 1997; Ortega-Calvo and Saiz-Jimenez, 1998; Park et al., 2003; Tang et al., 1998; Xia et al., 2010). Although these studies provide strong evidence that bacteria were somehow experiencing and accessing the pool of soil-sorbed chemicals, they were based on macroscopic measurements of

contaminants mineralization (to $^{14}CO_2$). These findings would be strengthened by molecular-scale evidence of bioavailability of soil-sorbed organic contaminants. Furthermore, insights into the physical, chemical, or microbiological determinants of bioavailability are lacking, and molecular-scale methods offer a unique opportunity to advance the state of knowledge regarding these fundamentally important coupled processes.

In this study the bioavailability of clay-sorbed tetracycline is examined utilizing a recently developed molecular method, namely the activation of antibiotic resistance genes in an *E. coli* bioreporter (Chapter II). It is hypothesized that desorption of clay-sorbed tetracycline is required for bacterial uptake and subsequent activation of antibiotic resistance gene. Alternatively, close contact between *E. coli* attached to mineral surfaces harboring sorbed tetracycline could facilitate bacterial uptake of the clay-sorbed antibiotic without the need for desorption into bulk water. To examine these questions, batch sorption/desorption experiments were conducted to achieve a range of clay-sorbed tetracycline concentrations. The *E. coli* bioreporter was cultivated with varying levels of clay-sorbed tetracycline using differing solid:solution ratios and water contents. The expression of the antibiotic resistance, measured as emitted fluorescence intensity using a flow cytometry, was used as a molecular probe of bioavailability. Tetracycline in the aqueous phase, and uptake by bacteria, was quantified using high performance liquid chromatography integrated with tandem mass spectrometry (LC-MS/MS). Scanning electron microscopy was also used to scrutinize the contact/attachment of the bioreporter bacteria with clay-mineral surfaces.

## MATERIALS AND METHODS

*Smectite Clay and Chemicals*

Reference Na-montmorillonite (SWy-2, Crook County, Wyoming, USA) was obtained from the Source Clays Repository of the Clay Minerals Society (Purdue University, Indiana). This clay has a cation exchange capacity of 850 $mmol_c\ kg^{-1}$ and a theoretical surface area of 800 $m^2\ g^{-1}$ (Borden, 2001). This smectite clay (20 g) was suspended with 1.0 L of 0.5 M NaCl aqueous solution for 24 h, and the clay fraction (< 2 μm) was collected using a wet sedimentation method. The cation exchange sites on smectite surfaces were saturated with $Mg^{2+}$ by washing the clay with 0.5 M $MgCl_2$ three times. The clay suspension was subsequently washed using Milli-Q deionized water five times to remove the excess $MgCl_2$ salt as indicated by a negative chloride test using $AgNO_3$. The clay suspension was then quickly frozen in a dry ice-acetone bath, and freeze dried to receive dried Mg-smectite powders. The clay was sterilized in the oven at 80 ℃ for 24 h prior to use in the bacterial cultural experiments described below.

Tetracycline hydrochloride (≥ 95%), ampicillin sodium salt (≥ 95%), methanol (HPLC grade), histodenz (iohexol), sodium pyrophosphate decahydrate, and 3-(N-morpholino)propanesulfonic acid (MOPS, buffer range 6.5-7.9) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride, ethylenediaminetetraacetic acid (EDTA), formic acid, sodium phosphate dibasic, and potassium phosphate monobasic were purchased from J.T. Baker (Philipsburg, NJ). Bacto tryptone and Bacto yeast extracts were purchased from Becton, Dickinson and Company (Sparks, MD).

Acetonitrile (HPLC grade) and hydrochloric acid (37%) were purchased from EMD Chemicals (Gibbstown, NJ).

*Bacterial Bioreporter*

The bacterial strain used in this study was *E. coli* strain MC4100/pTGM whole-cell bacterial bioreporter, which was constructed by inserting *tet*(M) gene (encoding tetracycline resistance by ribosomal protection) into plasmid pTGM. The plasmid pTGM contained a transcriptional fusion between a *tetR*-regulated $P_{tet}$ promoter and flow cytometry-optimized *gfp* gene (*gfp*mut3) encoding green fluorescence protein (GFP) (Bahl et al. 2005). The *E. coli* bioreporter was cultured in a low-salt Luria-Bertani (LB) media which contained 10.0 g of tryptone, 5.0 g of yeast extracts and 0.5 g of NaCl in 1.0 L of 0.22 μm-filtered MilliQ-water. The pH of the media was adjusted to 7.0 using 50 mM of MOPS buffer. The LB media were autoclaved at 121 ℃ for 30 min prior to use. The *E. coli* bioreporter was inoculated and cultivated in 25 mL of LB media amended with 100 mg $L^{-1}$ ampicillin, and incubated on a horizontally-moving shaker at 150 rpm and at 30 ℃. When the bacterial culture grew to mid-log phase as indicated by optical density at 600 nm ($OD_{600}$) of the culture approaching ~ 0.7, bacteria culture was 100-fold diluted into freshly prepared LB media also containing 100 mg $L^{-1}$ ampicillin.

*Preparation of Tetracycline-Loaded Clays*

Tetracycline sorption to Mg-smectite clay from aqueous solution was measured using a batch sorption method. The prepared clay powder (10 mg) was weighed in glass centrifuge tubes (Corning Inc., Corning, NY) with PTFE-lined screw caps, and mixed with 25.0 mL of tetracycline solution with a series of concentration from 0.0 to 400 μg $L^{-}$

$^{1}$ in Milli-Q deionized water. The tubes were placed on a rotatory shaker at 40 rpm for 4 h at 30 ℃. The preliminary study indicated that sorption approached to equilibration within 4 h. The tubes were subsequently centrifuged at 1900 g for 20 min. Experimental controls consisted of the initial tetracycline solutions in the absence of clay. Based on the mass balance, the clay-sorbed concentration was calculated from the difference between the tetracycline in the clay-free controls solution and the amount remained in aqueous solution at equilibration. All experimental samples were prepared in duplicate.

After the centrifugation and supernatant sampling, the remaining solution was immediately decanted. This clay sample is referred to as Clay Sample 1 in this study. The residual supernatant that could not be removed prior to desorption was determined gravimetrically, and tetracycline concentration in the residual solution was assumed to be the same as that measured in the bulk supernatant. The similar procedure was applied after each desorption cycle. The clay residue was then resuspended in 25.0 mL of LB media, and centrifuged at 1900 g for 20 min. An aliquot of supernatant was collected and analyzed for tetracycline concentrations. After this desorption step, the Clay Sample 1 was further mixed with 25.0 mL freshly prepared LB media containing 100 μg $mL^{-1}$ of ampicillin. The tubes were shaken for 4 h at 30 ℃, and the suspensions were centrifuged at 1900 g for 20 min, and the supernatants were removed and replaced with another 25 mL of freshly prepared LB media. This desorption step was repeated for another cycle, and the obtained clay sample is referred as Clay Sample 2. Both prepared clay samples were utilized in the following bioavailability experiments.

*Bioavailability of Clay-Sorbed Tetracycline in Suspension*

Clay Samples 1 and 2 (10 mg) was added in 25.0 mL of freshly prepared LB media containing 100 μg $mL^{-1}$ of ampicillin in which the *E. coli* bioreporter was inoculated. The suspension was placed on a horizontally-moving shaker for 12 h at 30 ℃. As the bacteria grew to stationary phase, an aliquot of bacteria-clay suspension was centrifuged at 15000 g for 30 min at 4 ℃. The tetracycline in the supernatant (5.0 mL) was extracted using a preconditioned hydrophilic-lipophilic balanced (HLB) cartridge (Waters Corporation, Milford, MA). Prior to use the cartridge was sequentially rinsed with 3.0 mL of methanol, 3.0 mL of 0.1 M HCl, and 6.0 mL of water (6 mL). After passing through 5.0 mL of the suspension, the cartridge was then washed with 1:9 (v/v) methanol/water solution (5.0 mL). Tetracycline on the HLB cartridge was eluted with 1:1 (v/v) methanol/water solution (5.0 mL) containing 150 mg $L^{-1}$ of EDTA, then with additional 5.0 mL of methanol containing 1% (v/v) formic acid. The eluted solutions were combined and analyzed for tetracycline concentration using a Shimadzu high-performance liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS). Tetracycline was quantified in multiple reaction monitoring mode with precursor/product ion pair m/z 445.4/410.0. The extraction recovery for tetracycline from the LB media was 86% with a standard deviation of 10%. In addition, the bacteria-clay suspension was fixed and dried on a glass coverslip, and characterized using a JEOL JSM-7500F scanning electron microscope (JEOL Ltd, Tokyo, Japan).

In order to analyze the expressed intensity of activated antibiotic resistance gene, the *E. coli* bacterial cells were detached from clay particles, and the emitted fluorescence was quantified using a BD LSR II flow cytometry. To do so, bacteria-clay suspension (~100 μL) was diluted in 0.5 mL of detachment solution in 2-mL Eppendorf tube. The

detachment solution was prepared by dissolving sodium pyrophosphate (0.1%) and Tween 20 (0.5%) in phosphate buffered saline (PBS) solution, and passed through a 0.22-μm membrane. The suspension was shaken at 150 rpm for 30 min at 30 ℃. Then 0.5 mL of nonionic density gradient medium Histodenz was carefully spiked beneath the suspension in each tube, then centrifuged at 14000 g for 30 min in an Eppendorf centrifuge 5415D (Eppendorf, Hauppauge, NY, USA). The entire supernatant was collected and vortexed for 1 min. Fifty microliters of the mixture was diluted in 3.0 mL of PBS solution, and analyzed for the fluorescence intensity using the BD LSR II flow cytometry. The flow cytometry system was equipped with a solid-state 488 nm coherent sapphire blue laser for *gfp* excitation and a FITC detector for the subsequent detection and quantification of green fluorescence. A threshold of 400 was set on the SSC detector in order to eliminate small non-bacterial particles. Only bacterial cells of predefined size determined from the results of control study were analyzed for *gfp* expression. The reported result of the mean fluorescence detected per single cell within the operating range of the FITC detector was used to quantitate the expressed intensity of antibiotic resistance genes.

For selected samples, tetracycline concentration in bacteria was also analyzed. To do so, bacteria and clay particles were separated from culture media (25 mL) by centrifugation at 15000 g and at 4 ℃ for 30 min. The pellet was resuspended in 25 mL of PBS solution, and 5.0 mL Histodenz was carefully spiked beneath the suspension then centrifuged at 14000 g for 30 min. The upper layer suspension containing bacteria was entirely collected, and then well mixed for the measurement of optical density at 600 nm. The tetracycline concentration in bacteria was measured following the procedure

described in Chapter II. Briefly, after centrifugation at 15000 g, the bacterial cell pellets were sequentially rinsed twice with 25 mL of PBS solution. Then 10 mL of McIlvain buffer (12.9 g of citric acid monohydrate, 10.9 g of $Na_2HPO_4$ and 37.2 g of EDTA dissolved in 1 L of water) was used to suspend the cell pellets and remove tetracycline from the cells. The mixture was vortexed (1 min), sonicated (10 min), and centrifuged (15000 g for 15 min). This extraction step was repeated and the supernatants were combined prior to solid phase extraction. The cell extracts containing tetracycline (20 mL) was passed through a preconditioned HLB cartridge and eluted as described above. The extraction recovery of tetracycline was measured at 108% with a standard deviation of 14%.

*Bioavailability of Tetracycline on Clay Film*

The *E. coli* bioreporter was cultivated on clay films with sorbed tetracycline in order to evaluate the effects of close contact between bacterial cells and clay surfaces on bioavailability of clay-sorbed tetracycline. The prepared Clay Samples 2 (10 mg) was suspended in 1.0 mL of freshly prepared LB media containing 100 μg $mL^{-1}$ of ampicillin. The 100-fold diluted bacterial culture was inoculated to the suspension, and filtered through pre-sterilized 0.22-μm Millipore membrane (Millipore Corporation, Billerica, MA, USA) to form mixed bacteria and clay film. The clay film, along with the membrane, was transferred into a desiccator and cultivated at 30 ℃ at the exposure to 100% relative humidity for 12 h. The film (~5 mg) was placed into 0.5 mL of detachment solution to prepare a bacterial suspension. The bacteria were separated from clay particles using the density gradient centrifugation method described above, and the emitted fluorescence intensity was measured using the flow cytometry. In addition, the association of bacteria and

clay minerals was imaged by the scanning electron microscopy using the procedure described above.

## RESULTS AND DISCUSSION

*Preparation of Tetracycline-Loaded Clay Samples*

Tetracycline sorption by, and desorption from, Mg-smectite in LB media are presented in Figure 4.1. Sorption isotherms relating the concentrations of sorbed and aqueous phase tetracycline were sufficiently manifested linear within the studied aqueous concentration that the data could be represented by a simple linear equation. Sorption of tetracycline by Mg-smectite was very strong resulting in sorbed concentrations of 92.9, 228.0, 423.3, 849.3 μg $g^{-1}$ at the equilibrium aqueous concentration of 2.6, 5.5, 14.7, and 33.7 μg $L^{-1}$ (referred to hereafter as Clay Sample 1). After the first cycle of desorption into the LB media, approximately 70% of clay-sorbed tetracycline desorbed into solution resulting in clay-sorbed tetracycline concentrations of: 30.0, 86.0, 133.6, and 248.1 μg $g^{-1}$, and corresponding tetracycline concentrations in LB media of: 32.0, 65.3, 128.9 and 280.5 μg $L^{-1}$ (Figure 4.1). The clay samples were subject to two additional desorption cycles using LB media. A minimum amount of tetracycline was observed to desorb from the clay samples, and the corresponding aqueous tetracycline concentration in LB media after the final (third) desorption step was < 10 μg $L^{-1}$. The clay-sorbed tetracycline concentrations were reduced to 18.8, 70.6, 111.7, 211.2 μg $g^{-1}$ after three cycles of desorption (from Clay Sample 1) into LB media (referred to hereafter as Clay Sample 2). These tetracycline-loaded clay samples were used to investigate the bioavailability of

sorbed tetracycline for bacterial uptake as indicated (and quantified) by the expression of antibiotic resistance genes.

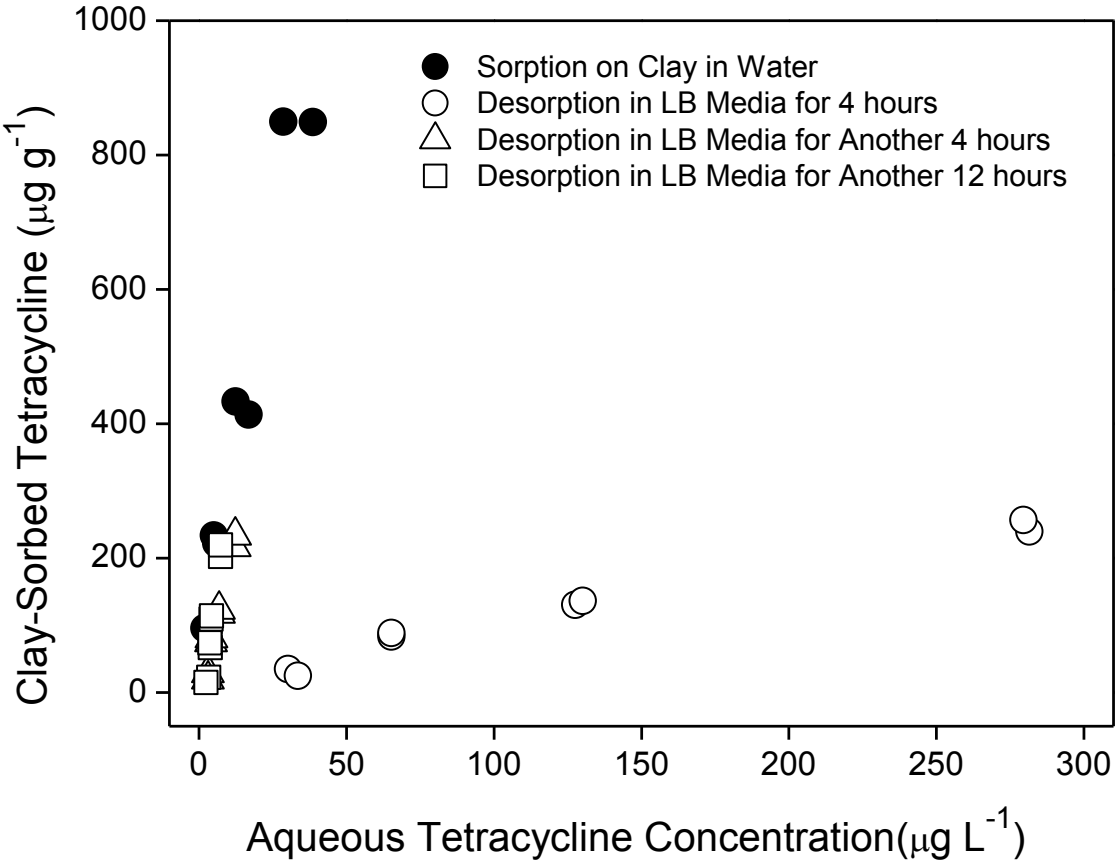


Figure 4.1. Tetracycline sorption from water and desorption in LB media by Mg-smectite clay.

*Bioavailability of Sorbed Tetracycline to the E. coli Bioreporter in Clay Suspensions*

Clay Sample 1 was added into *E. coli* bioreporter culture to form a clay-water (i.e. LB media) suspension into which clay-sorbed tetracycline could desorb. In this system the *E. coli* bioreporter bacteria were exposed to tetracycline sorbed by clay and that dissolved in LB media. In general, tetracycline that entered the *E. coli* cells evoked the expression of antibiotic resistance genes in this study, indicating its potential to exert selective pressure on microbial communities (Chapter II). The tetracycline desorbed from clay into LB media and the tetracycline associated with bacteria were measured; the tetracycline that remained sorbed by the clay was calculated by difference between tetracycline initially sorbed by clay (i.e. Clay Sample 1) and the sum of tetracycline present in the aqueous phase and that taken up by bacteria. The distributions of tetracycline in the resulting three phases (i.e. clay-sorbed, dissolved in LB media and that associated with bacteria) are compared to distributions in bacteria-free controls where tetracycline either desorbs from Clay Sample 1 into LB media devoid of bacteria, or remained sorbed. The mass of tetracycline that desorbed from clay (with different initial tetracycline loadings) into LB media in the absence of *E. coli* was 1.0, 2.2, 3.2 and 5.0 μg which corresponds to approximately 48.3%, 52.0%, 51.6% and 62.3% of masses of tetracycline originally sorbed by the clay. In the clay suspensions inoculated with the *E. coli* bioreporter and incubated for 12 h, the amounts of tetracycline in LB media decreased by 0.7, 1.3, 1.5, and 2.7 μg, and the total masses of tetracycline desorbed from clay were 0.5, 0.6, 1.1, and 0.2 μg. The measured tetracycline mass associated with bacteria was 1.2 ± 0.2, 1.9 ± 0.6, 2.6 ± 0.0 and 3.0 ± 0.1 μg at the corresponding clay-sorbed tetracycline concentrations and given in Figure 4.2. This analysis indicates that the

tetracycline mass associated with bacteria emanated primarily from the aqueous phase and to a lesser extent from that previously sorbed by clay. The significant reduction of aqueous tetracycline concentration following inoculation likely induces further desorption from the clay, resulting in additional tetracycline that is bioavailable to the bacteria.

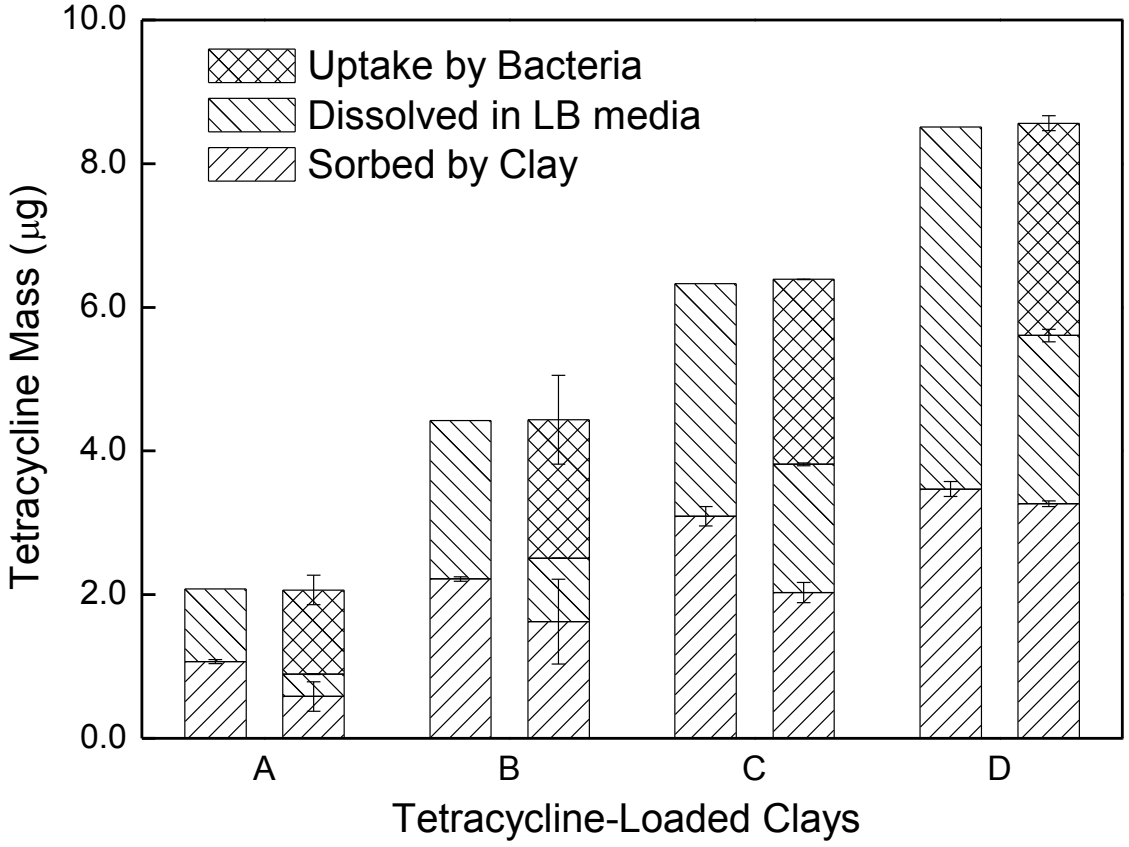


Figure 4.2. Distributions of tetracycline mass in bacteria-water-clay suspension (clay: 10 mg, clay-sorbed tetracycline concentration: A: 207.3 ± 3.3, B: 422.8 ± 0.7, C: 620.1 ± 19.5 and D: 815.5 ± 10.3 μg $g^{-1}$).

To further evaluate the bioavailability of clay-sorbed tetracycline to the *E. coli* bioreporter, similar experiments were conducted in which the bacterial reporter was inoculated in freshly prepared LB media amended with 10 mg of clay containing sorbed tetracycline concentrations ranging from 182 to 850 μg $g^{-1}$. After incubation for 12 h at 30 ℃, tetracycline that desorbed from the clay into the LB media was measured, and found to range from 6.7 to 102.1μg $L^{-1}$. The corresponding mean fluorescence intensity of the bioreporter, as measured by flow cytometry, increased from 108 to $1.05\times10^{5}$ per cell (Figure 4.3). The emission of fluorescence from the *E. coli* bioreporter indicated that tetracycline entered the *E. coli* cells. In a previous study, we demonstrated that only tetracycline entering the bacterial cells could activate the antibiotic resistance genes (Chapter II). To further test the hypothesis that tetracycline which effectively activated the antibiotic resistance genes originated from aqueous solution versus directly from the clay-sorbed form, a series of tetracycline solutions were prepared in LB media with concentration ranging from 0 to 200 μg $L^{-1}$. The *E. coli* bioreporter was inoculated into these solutions, without the addition of clay, and cultivated for 12 h at 30 ℃. The tetracycline concentration in the LB media was measured directly using LC-MS/MS and plotted against the emitted fluorescence from the bioreporter (Figure 4.3). In the clay-free control, the decrease in tetracycline concentration in the LB media was attributed to bacterial uptake (as opposed to sorption by clay). The tetracycline concentrations in the (clay-free) LB media were within a similar range as those resulting from tetracycline desorption from the clay (Figure 4.3). Plots of fluorescence intensity vs. aqueous tetracycline concentrations from the clay-free controls were similar in shape but generally

showed stronger fluorescence with data from the incubations containing clay (Figure 4.3). In general the mass of sorbed tetracycline was similar to the corresponding mass in water (Figure 4.2). This suggest that in dilute clay suspensions tetracycline in the aqueous phase is the major source for uptake by bacteria, and that sorbed tetracycline did not enhance the fluorescence intensity indicating that it was not bioavailable to the *E. coli* bioreporter. Desorption of tetracycline from clay into LB media with subsequent bacterial uptake and activation of antibiotic resistance genes is the major exposure pathway in dilute clay suspensions.

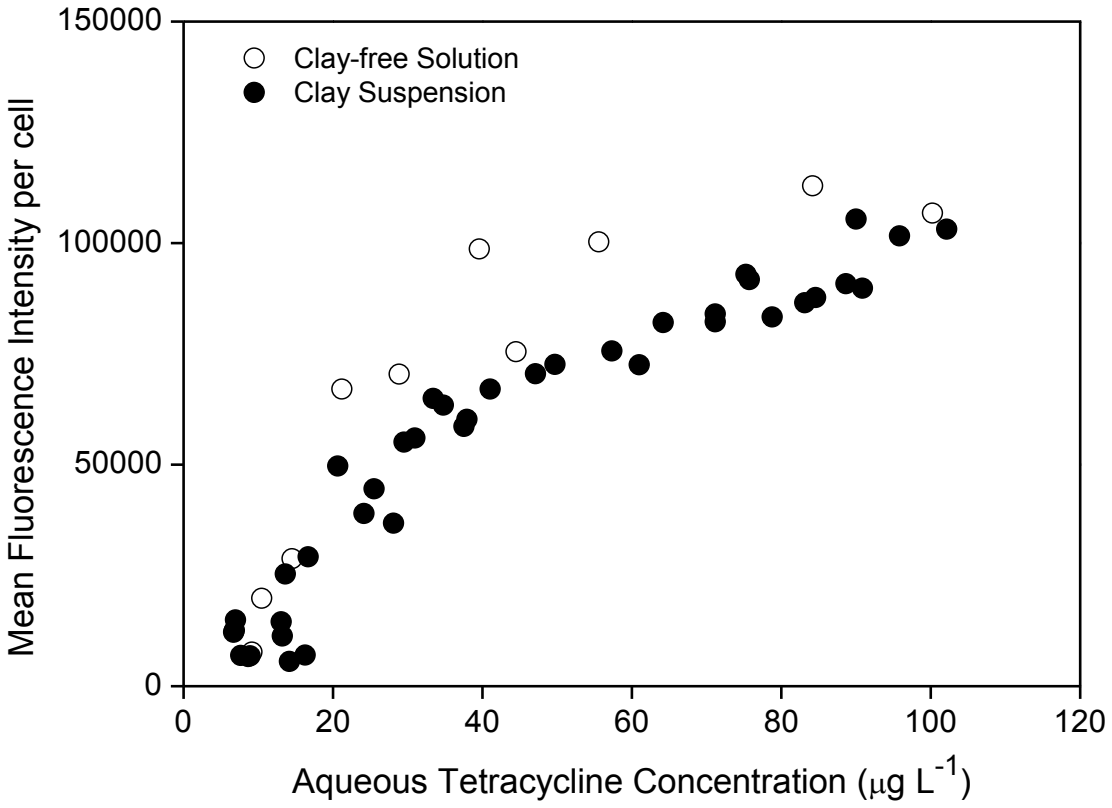


Figure 4.3. Comparison of mean fluorescence intensity emitted from *E. coli* bioreporter in the LB media amended with tetracycline with and without clay (clay-sorbed tetracycline).

Considering the bioavailability of clay-sorbed tetracycline, it seems apparent that bacteria must establish close contact with, or attachment to the surfaces of clay minerals where sorbed tetracycline resides. The bioavailability of tetracycline sorbed to Mg-smectite was evaluated using clay samples (Clay Sample 1) with sorbed tetracycline at concentrations of 203.9 $\pm$ 26.5, 513.9 $\pm$ 15.5, 707.7 $\pm$ 6.9 and 849.8 $\pm$43.3 mg $g^{-1}$. These samples were carefully enveloped in dialysis tubing having a molecule weight cut off (MWCO) of ~ 12000 to 14000 Da, and immersed in LB media containing the *E. coli* bioreporter. This experimental system allows desorbed tetracycline to freely pass through the membrane, but prevents the direct contact of bacteria with clay-mineral surfaces. The measured tetracycline concentration in LB media, and activity of the bioreporter in response to uptake of tetracycline, were compared with the results obtained from an otherwise identical system in which tetracycline-loaded clays were mixed with LB media to form suspensions consisting of 10 mg clay in 25 mL LB media (Figure 4.4). After 12 h incubation, both systems manifested similar tetracycline concentrations in LB media which ranged from 6.7 to 84.6 μg $L^{-1}$; the corresponding mean fluorescence intensity increased from $5.5\times10^{3}$ to $8.8\times10^{4}$ per cell (Figure 4.4). At the lowest clay-sorbed tetracycline concentration tested (203.9 $\pm$ 26.5 μg $g^{-1}$), the desorbed tetracycline concentrations in LB media were 12.7 $\pm$ 4.5 and 6.8 $\pm$ 0.1 μg $L^{-1}$. For systems with and without the dialysis tubing, the corresponding bioresponse (fluorescence intensity) was 6.47 $\pm$ $0.81\times10^{3}$ and 1.32 $\pm$ $0.15\times10^{4}$ per cell, respectively. The slightly higher bioresponse observed in the system lacking dialysis tubing suggests that direct contact

between the *E. coli* bioreporter and clay surfaces facilitated transfer of clay-sorbed tetracycline to bacteria. However, when all samples are considered, difference in bioresponse for the two experimental systems is hard to discern. Perhaps the diluted nature of the clay suspensions (i.e.10 mg clay in 25.0 mL LB media) sufficiently mitigates contact between bacteria and mineral surfaces, leading to no apparent distinction in the emitted fluorescence for the two types of tetracycline exposures to bacteria; this is particularly notable at aqueous tetracycline concentration > 40 $\mu g\ L^{-1}$.

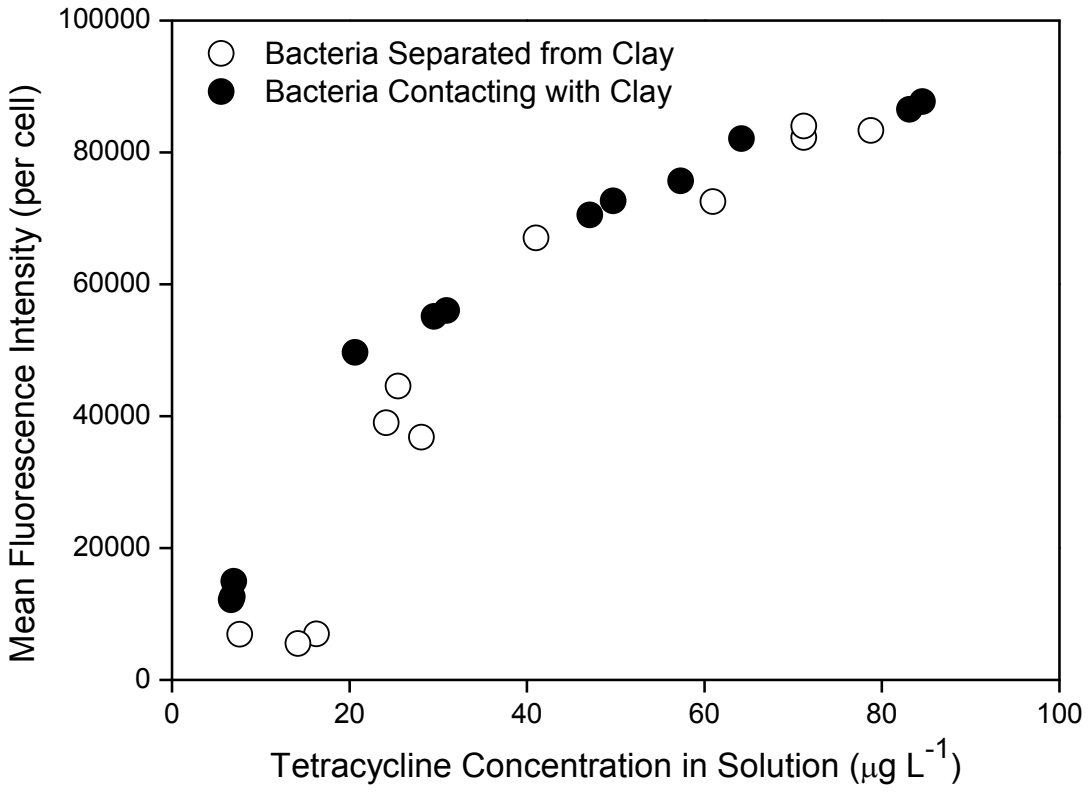


Figure 4.4. Relationship between tetracycline concentration in solution and mean fluorescence intensity of the *E. coli* bioreporter when the bacterial cells were mixed directly with or separation from (via dialysis tubing) clays with sorbed tetracycline.

*Bioavailability of Tetracycline in Clay Suspensions with Varying Clay to Solution Ratios*

To further explore the bioavailability of clay-sorbed tetracycline, the prepared clay samples with sorbed tetracycline were subjected to multiple cycles of desorption (referred to as Clay Sample 2). These samples with residual desorption-resistant tetracycline were used to more rigorously evaluate bioavailability since minimum amounts of tetracycline would desorb into solution. In this study, the clay sample with the highest concentration of residual sorbed tetracycline ($220 \pm 27$ μg $g^{-1}$) was mixed with freshly prepared LB media containing the *E. coli* bioreporter. To enhance the potential for contact between bacteria and clay surfaces, the increasing ratios of clay (10 mg) to water content varied from 25 mL to 100% relative humidity (no bulk water) (Table 4.1). The sample containing 10 mg clay and 25 mL LB media manifested a desorbed tetracycline concentration of $10.0 \pm 0.1$ μg $L^{-1}$ in fresh LB media, and the corresponding emitted fluorescence was $183 \pm 4$ per cell. As the solution volume decreased, the concentration of desorbed tetracycline increased, though the total mass of desorbed tetracycline did not increase. As the solid to liquid ratio increased, the corresponding fluorescence intensity significantly increased, indicating more tetracycline became bioavailable and entered the bacterial cells. For example, when the volume of LB media reduced from 25.0 mL to 10.0 mL, the aqueous tetracycline concentration increased from $10.0 \pm 0.1$ to $12.9 \pm 0.8$ μg $L^{-1}$. Interestingly, the intensity of fluorescence manifested (in the 10.0 mL system) increased 13 fold compared to the same system with 25.0 mL of LB media. When the bioreporter was cultivated with a clay film at 100% relative humidity, the bacteria could effectively access the clay-mineral adsorbed tetracycline. Very strong

fluorescence intensity was measured, approaching $6.82 \times 10^4$ per cell for the *E. coli* bioreporter, which is approximately 372 times greater than the fluorescence emitted from the *E. coli* bioreporter in the sample containing 10 mg of clay and 25 mL LB media. At an even lower relative humidity of 30%, the bioreporters could not survive due to unfavorable culture conditions (Table 4.1), viz, the lack of available water. Clay Sample 2 used in these experiments had been subjected to several desorption cycles in LB media. Although the readily desorbed tetracycline had been removed during prior desorption steps, the residual tetracycline remaining with the clay was still desorbed to some extent, and hence, as the volume of LB media was reduced, the aqueous phase concentration increased (Table 4.1). The reduced volume of LB media which manifested increased tetracycline concentrations in this manner also evoked higher expression of antibiotic resistance from the bioreporter. It is still not clear whether the enhanced bioresponse was due to close contact between bacteria and clay surfaces (i.e. bioavailability of sorbed tetracycline) and/or an increase in the aqueous phase tetracycline concentrations. However, the very strong fluorescence emitted from bioreporter in the culture with clay films at 100% relative humidity strongly suggests that the clay-sorbed tetracycline is bioaccessible to *E. coli*, and this does not require desorption into bulk solution.

Table 4.1: Bioavailability of clay-sorbed tetracycline under different culture conditions.

| | Culture conditions | Tetracycline in solution ($\mu g\ L^{-1}$) | Mean Fluorescence Intensity (per cell) |
|---|---|---|---|
| Clay suspension | 10 mg clay in 25 mL LB media | $10.0 \pm 0.1$ | $(1.83 \pm 0.04) \times 10^2$ |
| | 10 mg clay in 10 mL LB media | $12.9 \pm 0.8$ | $(2.35 \pm 0.91) \times 10^3$ |
| | 10 mg clay in 5 mL LB media | $21.1 \pm 0.8$ | $(5.59 \pm 0.49) \times 10^3$ |
| | 10 mg clay in 1 mL LB media | $113.5 \pm 2.1$ | $(3.94 \pm 1.44) \times 10^3$ |
| Clay film | 100% relative humidity at 30 ℃ | N/A | $(6.82 \pm 0.87) \times 10^4$ |
| | 30% relative humidity at 30 ℃ | N/A | < 100 |

The *E. coli* bioreporter demonstrated a significant bioresponse to tetracycline when cultured on clay films at 100% relative humidity. A density gradient approach was used to separated bacteria from clay particles comprising the clay film, which could obtain > 90% of the bacteria in the culture (Amalfitano and Fazi, 2008; Amalfitano and Puddu, 2009). This ability to obtain virtually the entire bacterial culture ensured that the flow cytometry analysis of the *E. coli* bioreporter was representative of the entire bacterial culture. The tetracycline-loaded clays had been subjected to several cycles of desorption, and the resultant clay-sorbed tetracycline concentrations were 86.0, 133.6, 190.9 and 248.1 $\mu g\ g^{-1}$. The corresponding measured *E. coli* bioreporter-emitted fluorescence ranged from $3.00\times10^{4}$ to $8.01\times10^{4}$ per cell (Figure 4.5). The emitted fluorescence increased with increasing clay-sorbed tetracycline, suggesting that more tetracycline became bioavailable for bacterial uptake at higher sorbed concentrations. When the *E. coli* was cultured on the clay films for 36 h, the measured fluorescence showed no significant difference from the values measured after the 12-h cultivation.

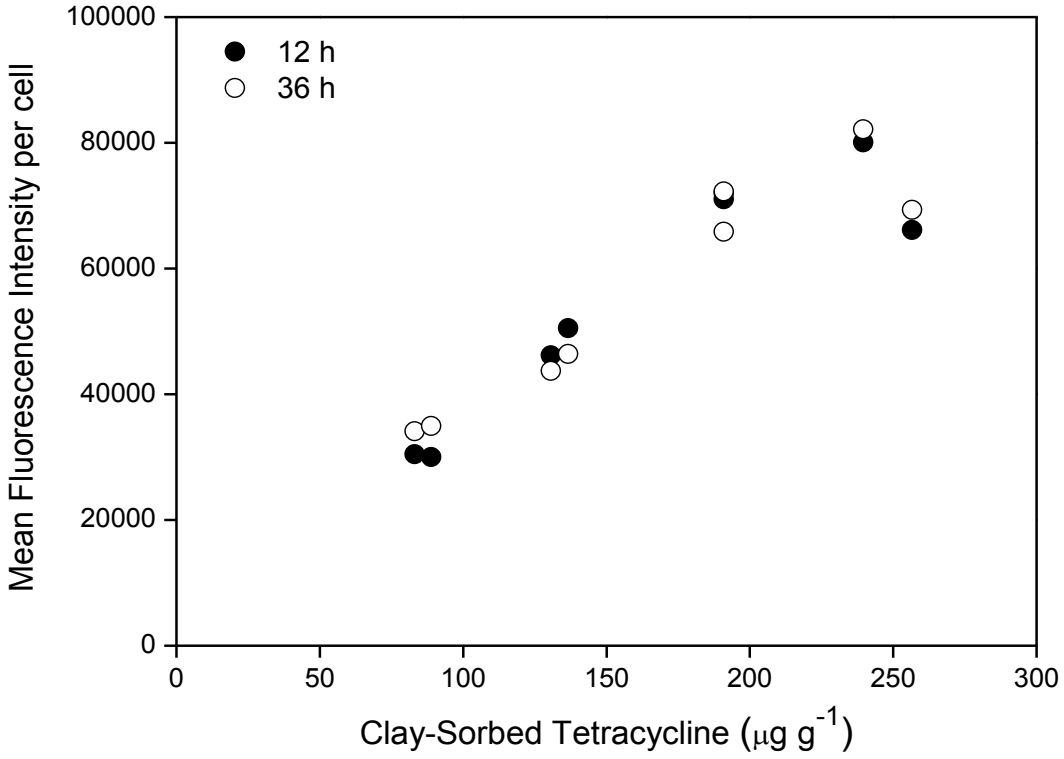


Figure 4.5. Emitted mean fluorescence intensity of per *E. coli* bioreporter cell after cultivated after 12 h and 36 h on clay films with varying sorbed tetracycline concentration.

*Effects of Close Contact of Bacteria with Mineral Surfaces on Bioavailability of Sorbed Tetracycline*

Clay Sample 2, which had been subjected to three cycles of desorption into LB media manifested a sorbed tetracycline concentration of $222 \pm 27$ μg $g^{-1}$, and when 10 mg of this clay was added to 25 mL of LB media, the desorbed tetracycline concentration was < 10 μg $L^{-1}$. The corresponding fluorescence intensity was very low i.e. $136 \pm 20$ per cell. However, the *E. coli* bioreporter cultivated on the films of this clay at 100% relative humidity (and no bulk water) demonstrated a comparatively high fluorescence intensity $6.82 \times 10^4$ per cell, which was of the same magnitude ($\times 10^4$) as that obtained from the *E. coli* culture in a suspension (25 mL) containing 10 mg of Clay Sample 1 with an initial sorbed concentration of $813 \pm 12$ μg $g^{-1}$. So in these two systems the *E. coli* bioreporters were exposed to same mass of tetracycline on clays. These results suggest that the close contact of bacteria with mineral surfaces significantly enhanced the bioavailability of clay-sorbed tetracycline to bacteria. The *E. coli* bacteria cultivated on clay films under field-type conditions of 100% humidity demonstrated a strong capability to access the desorption-resistant tetracycline that remained sorbed by clay.

When examining the association of bacteria and clay particles using scanning microscopy, it was found that in the suspension culture, most bacterial cells aggregated in the LB media (Figure 4.6A) and only a few cells were directly attached to clay surfaces (Figure 4.6B). The clay particles (10 mg) were mostly suspended in the LB media (25 mL) affording the bacterial cells a minimal opportunity to attach on mineral surfaces. Therefore, it is reasonable to postulate that the *E. coli* bioreporter primarily assimilated

the dissolved tetracycline from the LB media, and only a small (if any) amount of tetracycline associated with clays entered the bacterial cells. This observation is consistent with our observation that tetracycline in the aqueous phase (due to desorption from clay) was the major source for bacterial uptake from dilute clay suspension. In contrast, bacteria were in close contact with mineral surfaces when cultivation with clay films at 100% relative humidity and no bulk water (Figures 4.6C and 4.6D). In this case, smectite clay sheets formed relatively condensed layered structures (tactoids), and bacterial cells grew both on clay surfaces and between clay tactoids (Figure 4.6C). Upon close examination, it was apparent that the clay surfaces were covered with significant amounts of extracellular polymeric substances (EPS) excreted by bacterial cells (Figure 4.6D). These slimy, glue-like substances excreted by the bacteria serve as the main cements for bacterial adhesion to mineral surfaces. EPS is composed predominantly of polysaccharides, proteins, nucleic acids and lipids, and can form biofilms on mineral surfaces (Donlan, 2002). Multiple types of ionic functionalities (carboxyl, phosphoryl, amide, amino, hydroxyl) are present in the EPS, which could interact strongly with tetracycline via electrostatic attractions, facilitating their release from mineral surfaces and transfer into bacterial cells. In general, the size of the *E. coli* bioreporter is about 1.0×0.5 μm, which prohibits the bacterial cells from entering smectite interlayers (~0.4-0.6 nm) to access any intercalated tetracycline. However, bacterial cells in attached biofilms could plausibly approach the external surfaces of clay tactoids where high local concentrations of sorbed tetracycline exist. When a local equilibrium exists between the biofilms and water associated with clay surfaces, and bacterial uptake rate of tetracycline sufficiently exceeds its desorption rate in the local equilibrium, a steep concentration

gradient could be established which would drive the release of additional tetracycline from clay surfaces making it available for bacterial uptake. As a result, the *E. coli* bioreporter in close proximity to mineral surfaces could significantly assimilate tetracycline, and evoke a strong bioresponse of emitted fluorescence as observed. The released of EPS from bacteria and formation of biofilm on mineral surfaces could thereby facilitate transfer of tetracycline from mineral surfaces to bacteria, hence manifesting the bioavailability of clay-sorbed tetracycline (Singh et al., 2006; Wunder et al., 2011; Zhang et al., 2013).

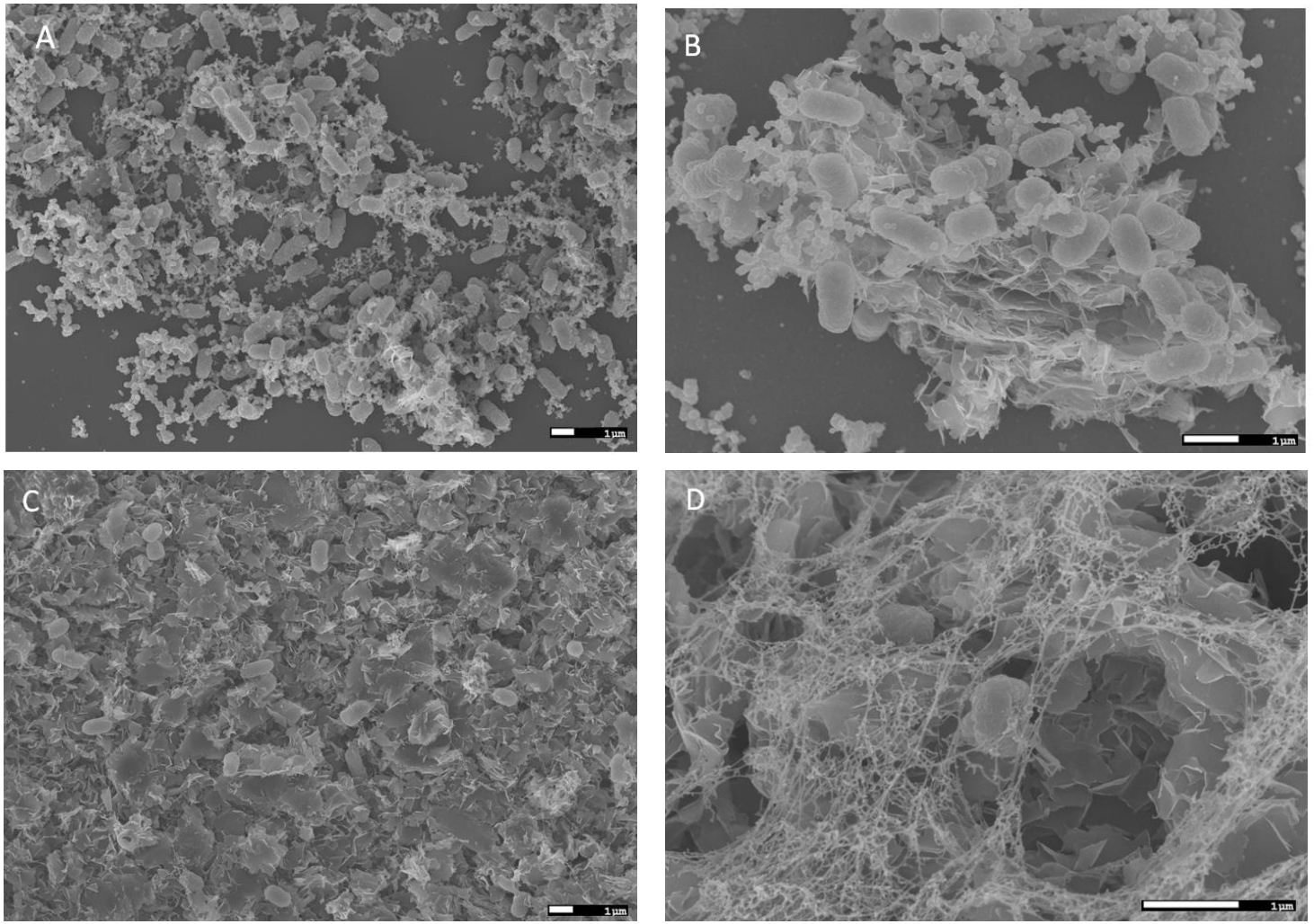


Figure 4.6. Images of scanning electron microscope (SEM) of the *E. coli* bioreporter growing with Mg-smectite clay in LB media (A and B) and with Mg-smectite films at 100% relative humidity (C and D).

## CONCLUSION

In clay suspension, tetracycline sorbed by Mg-smectite was desorbed to aqueous phase, and became bioavailable to the whole-cell bioreporter. Desorption of tetracycline from clay to solution is the major exposure pathway for bacterial uptake and subsequent activation of antibiotic resistance in the diluted clay suspensions. Direct contact of the *E. coli* bioreporter with clay surfaces plausibly facilitated the transfer of clay-sorbed tetracycline to bacteria, but to a much less extent. For tetracycline-loaded clays after multiple cycles of desorption, reducing volume of cultured solution was observed to evoke higher expression of antibiotic resistance from the bioreporter. Strong emitted fluorescence from bioreporter in the culture with clay films clearly indicated that clay-sorbed tetracycline is still bioaccessible to *E. coli*, and formation of biofilms facilitates bacterial uptake of tetracycline. Close contact of bacteria with soil surfaces, which are the most common in the environment, could significantly enhance bioavailability of soil-sorbed tetracycline, hence exposure and risks to the surrounding microbial communities.

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