Title
Planktonic Co-selection of Oxytetracycline Resistance via Arsenic pre-exposure in E. coli O55

Permalink
https://escholarship.org/uc/item/1gk1p3g3

Author
Flores, Ariel

Publication Date
2014

Peer reviewed|Thesis/dissertation
Planktonic Co-selection of Oxytetracycline Resistance via Arsenic pre-exposure in *E. coli* O55

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Civil Engineering

by

Ariel Flores

2014
With increased antibiotics usage over recent decades, there has been an emerging concern about
the accelerated development of antibiotics resistance in the environment. This development
poses several public health concerns, such as, the higher frequency of multiple drug resistant
bacteria both in the environment and in clinical settings. Exposure to metals can increase
antibiotic resistance through co-selective pressure; however, this non-traditional route of
antibiotic resistance acquisition has not been extensively studied. A high-throughput culture
based method was developed to assess the co-selective pressure of arsenic pre-exposure to
oxytetracycline resistance in planktonic cultures of \textit{Escherichia coli} O55. A glucose minimal
salts (GMS) media was used for the study. \textit{As}_2\text{O}_3 and oxytetracycline hydrochloride solutions
were made ranging from 1-100 ppb and 10-40 ppm, respectively. Lastly, the geochemical
equilibrium model PHREEQC was used to determine the bioavailable concentrations of the

metals and antibiotics in the defined GMS media. The co-selective relationship was investigated by comparing the net growth rates of the cultures pre-exposed to varying levels of arsenic to the unexposed arsenic cultures at various concentrations of oxytetracycline.
The thesis of Ariel Flores is approved.

Shaily Mahendra

Michael K. Stemstrom

Professor Jennifer A. Jay, Committee Chair

University of California, Los Angeles

2014
Table of Contents

Abstract .............................................................. ii
Committee Page ......................................................... iv
List of Figures ........................................................ vi

Background .............................................................. 1
  Arsenic Occurrence in the Environment ......................... 1
  Microbial Arsenic Toxicity & Resistance ......................... 2
  Oxytetracycline Background, Usage, & Occurrence in the Environment .......... 4
  Microbial Oxytetracycline Toxicity & Resistance ................. 6
  Co-selection ......................................................... 8

Methods ................................................................. 12
  Chemical & Culture ................................................ 12
  Experimental Setup ............................................... 12
  PHREEQC Batch Speciation ...................................... 13

Results ................................................................. 16
  Arsenic Pre-Exposure ............................................. 16
  Oxytetracycline Growth ......................................... 17

Discussion .............................................................. 20

Conclusion .............................................................. 24
  Future Work ....................................................... 25

Appendix ................................................................. 26
  Appendix A: PHREEQC Input File ................................ 26
  Appendix B: Zero and First Order Rate Law Eqs. .................. 28
  Appendix C: $R^2$ Values for Best-Fit Lines of Oxytetracycline Growth Rate .......... 28
  Appendix D: Co-selective Pressure Eq. .......................... 28

Bibliography .......................................................... 29
List of Figures

Figure 1: Arsenic Cycle 2

Figure 2: Oxytetracycline Structure 4

Figure 3: Overview of Co-selection in Microorganisms 10

Figure 4: PHREEQC Arsenic Speciation 15

Figure 5: PHREEQC Oxytetracycline Speciation 15

Figure 6: Arsenic Exposed *E. coli* Growth Curve 16

Figure 7: Arsenic Exposed *E. coli* Growth Rate 17

Figure 8: Arsenic Pre-exposed *E. coli* Growth Curve 18

Figure 9: Arsenic Pre-exposed *E. coli* Growth Rate 19

Figure 10: Arsenic Pre-exposed *E. coli* Growth Rate Comparison by Arsenic 19

Figure 11: Arsenic Pre-exposed *E. coli* Growth Rate Comparison by Oxytetracycline 20

Figure 12: Growth Rate Ratio 21

Figure 13: Growth Rate and Zero Order -k Comparison 22

Figure 14: Growth Rate Ratio and First Order -k Comparison 23
Background

Arsenic Occurrence in the Environment

Arsenic is a toxic metalloid element that occurs in various valence states (As(V), As(III), As(0), and As(-III)) and whose sources into the environment are both natural and anthropogenic. The predominant form of inorganic arsenic in aqueous, aerobic environments is arsenate (As(V) with $pK_a$s of 2.19, 6.94, and 11.5, whereas arsenite (As(III)) is more prevalent in anoxic environments with the first $pK_a$ occurring at 9.2 [30, 44, 48,73]. Typically, naturally occurring arsenic concentrations range from 0.1 - 1000 ppm in soils and approximately, 2.6 ppb in seawater [44]. Moreover, arsenic levels are governed by local geology, hydrology, and geochemical characteristics of an aquifer, as well as anthropogenic alterations to aquifers. Principally, the geochemical characteristics of an aquifer material and the interactions with the aqueous media play a critical role in determining retention and mobility of arsenic within the subsurface [30, 20, 23, 61]. Arsenic mobility in aquifers is also affected by the biogeochemistry within the aquifer. Mobility may occur due to the alteration of redox potential or acidity affecting the surface chemistry of the aquifer [24, 66]. However, local arsenic levels can vary according to anthropogenic inputs. Typical anthropogenic sources of arsenic are smelter slag, coal combustion, runoff from mine tailings, hide tanning waste, and pesticide usage. In fact, arsenic was used in pesticides during the early 1930s until the 1980s, which resulted in approximately 10,000 metric tons per year discharged into the environment [48]. Overall, the occurrence in the environment varies due to local geology and biogeochemical processes that affect the overall mobility of arsenic. A general overview of the arsenic cycle can be seen in Figure 1.
Microbial Arsenic Toxicity & Resistance

Arsenic toxicity and effects vary depending on the organism, organism order and arsenic speciation; moreover, exposure levels affect overall toxicity. The LD$_{50}$ of various organic and inorganic species ranging overall from 2 to over 5,000 mg As/kg [28]. It should be noted that this range was reported for various organisms and exposure. The acute and chronic toxicity effects of the various arsenic species differ; however, it is generally accepted that As(III) is more toxic than As(V). The toxicity of As(III) to humans is about 60 times higher than that of As(V), while inorganic forms of arsenic are two orders of magnitude more toxic than organic forms [30]. General chronic exposure symptoms in humans include skin lesions, peripheral neuropathy, diabetes, bone marrow metabolism, etc. [28]. However, of significant importance is the carcinogenicity of arsenic in humans. Cancer has developed in individuals exposed to arsenic either orally by drinking water contamination and occupationally. Skin hyperpigmentation and
hyperkeratosis have long been known to be signature signs of chronic arsenic exposure; yet, the most common arsenic-induced skin cancers are Bowen’s disease (carcinoma in situ), basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [28,74]. Moreover, organ arsenic induced cancers have been observed to occur in the kidneys, liver, and bladder. The carcinogenic mechanism of arsenic is not clearly delineated, but possible mechanism pathways may include genotoxicity, cell proliferation, altered DNA repair and DNA methylated oxidative stress, co-carcinogenesis, and tumor promotion [28,74].

Due to the differences in arsenic speciation, As(III) and As(V) have different mechanisms of toxicity. Arsenite toxicity stems from its broad specificity binding capabilities. Primarily, it impairs enzymatic activity by binding to thiol and vicinal sulfhydryls. It also affects respiration by binding to the vicinal thiols in pyruvate dehydrogenase and 2-oxo-glutarate dehydrogenase [28, 48]. On the other hand, arsenate toxicity results from phosphate mimicry in biochemical processes. Particularly, arsenate reacts in vitro with glucose and gluconate to form glucose-6-arsenate and 6-arsenogluconate, respectively. These compounds resemble glucose-6-phosphate and 6-phosphogluconate, respectively. Glucose-6-arsenate is a substrate for glucose-6-phosphate dehydrogenase and can inhibit hexokinase, as does glucose-6-phosphate [28, 48]. Thus, arsenate toxicity short circuits the respiration process.

Although arsenic is rather toxic, there are resistance mechanisms in bacteria to cope with arsenic toxicity, principally by efflux pump [9]. The primary operon in dealing with arsenic resistance is the ars operon and is found both in Gram-positive and Gram-negative bacteria [48, 57, 58]. The ars operon usually consists of a minimum of three co-transcribed genes, arsR, arsB, arsC, found
chromosomally in *E. coli*. The *ars*R gene is the regulatory repressor, *ars*B is the membrane transport pump, and *ars*C the determinant of a small intracellular arsenate reductase. Occasionally two additional genes, *ars*A and *ars*D, are found in *ars* operons of Gram-negative bacteria, so the gene order is *ars*RDABC and is found in plasmids, such as R733 in *E. coli* [48]. *Ars*A is an intracellular ATPase protein that binds as a dimer to the membrane *ars*B protein, converting its energy coupling from the membrane potential to ATP. The arsenite membrane efflux pump is unique in that it can function either chemiosmotically (with *ars*B alone) or as an ATPase (with the *ars*AB complex). However, the function of the *ars*D gene is less clear in the *ars*RDABC operon, but is thought to act as a co-repressor and chaperone protein [43, 57, 58]. Although there are other resistance mechanisms for anaerobic environments, such as arsenate reductase and arsenite oxidase mechanisms, these processes will not be discussed.

**Oxytetracycline Background, Usage & Occurrence in the Environment**

Oxytetracycline is a broad-spectrum tetracycline with chemical formula C\textsubscript{22}H\textsubscript{24}N\textsubscript{2}O\textsubscript{9} and molecular weight of 460.434 g/mol. As most tetracyclines, oxytetracycline is a polyketide with a napthecene ring, whose structure can be seen in Figure 2. Oxytetracycline has three pK\textsubscript{a}s, .3, 7.3, and 9.1 [54, 40, 62, 67, Wu], a log K\textsubscript{ow} of -1.22, a log K\textsubscript{oc} ranging from 1.2-5, and a K\textsubscript{d} ranging from 0.3 - 1030 [11, 63].
In general, tetracyclines are widely used for livestock and human bacteriostatic treatment. Generally, tetracycline compounds are used therapeutically and prophylactically to treat bacterial infections such as respiratory, bowel, and genital infections, along with more systemic infections such as acne [13]. Variances in consumption deal with availability of the antibiotics, such as solely prescription or over the counter availability. In the United States alone, approximately 3,200 metric tones of tetracycline compounds per year are used in animal operations, this approximation does not distinguish between therapeutic and sub-therapeutic dosing. However, Europe uses approximately 2,300 metric tones of tetracycline compounds per year for therapeutic purposes only [11, 13]. Moreover, Australia uses approximately 12 metric tones and 77 metric tones of tetracyclines per year for human and farm animal use, respectively. Tetracycline compounds are widely used for swine, poultry, and beef cattle; particularly, oxytetracycline has been used for extensive calf farming [11, 16]. In confined animal feeding operations (CAFOs), the livestock are densely grouped until slaughter. Although some CAFOs operate from birth to slaughter, others serve as a middle step prior to slaughter. In either case, antibiotics are generally administered to the livestock in the feed or drinking water. The use of tetracyclines as growth promoting feed additives, particularly oxytetracycline, began in the 1949
in poultry livestock then rapidly adapted and implemented in swine and cattle livestock, which, ultimately, resulted in oxytetracycline being approved by the Food and Drug Administration (FDA) as a feed additive in 1953 [13, 69]. The growth promoting effects of tetracyclines in livestock were increased growth and improved feed utilization, leading to a cost drop of feed per head used in CAFOs. Yet, CAFOs have been identified as sources of the dissemination of antibiotic resistance genes and antibiotic resistant bacteria, as well as hospitals [41, 53, 55]. Although the FDA is attempting to curb current use of antibacterial feed additives [71], other nations have stopped using feed additives such as the United Kingdom, who have not used feed additives since the 1970s [13]. In general, oxytetracycline concentrations in the environment vary depending on the environmental matrix. In aquatic environments, oxytetracycline concentrations are generally in the µg/L range. In U.S. surface waters, oxytetracycline occurs at 0.34 µg/L, while concentrations in manure may range between 4 - 40 mg/kg and concentrations in soils may range just under the limit of detection <10 µg/kg [11, 16, 27, 39, 42]. The relatively high $K_{oc}$ elucidates the observed higher concentrations in manure and biosolids from activated sludge. As most antibiotics, oxytetracycline undergoes metabolism and degradation [21]. However, oxytetracycline has been observed to have an unchanged excretion rate of approximately 80% [27, 33, 35, 36].

**Microbial Oxytetracycline Toxicity & Resistance**

Generally, antibiotics function by eliminating the viability of a cell in the host organism. The overall pathway used by tetracyclines is to alter the translation process by binding to bacterial
ribosomes and inhibiting the incorporation of amino acids in the ribosomal bound protein, post activation stage [25]. This is accomplished through binding of tetracycline compounds to the 30S subunit of the ribosome [11, 13, 25]. The antimicrobial properties of the tetracycline compounds are derived from their structure. Most importantly, the linearly fused tetracycle, the alpha stereochemical configuration at the 4a and 12a junctions (located in the A and B rings), the presence of a ketoenol functional group in the D ring, and the four dimethylamino groups [13]. The mode of action of tetracyclines requires the compounds to traverse at least one cellular membrane. Once within the cytoplasm of the cell, tetracycline compounds will either already be chelated with soluble metals like Mg$^{2+}$ or will chelate with such a metal within the cell due to the internal chemical equilibrium. It is probable that the active tetracycline compound that binds to the ribosome is a magnesium tetracycline complex; moreover, the association of tetracycline with the ribosome is a reversible process, which helps account for the bacteriostatic properties of the antibiotic [13, 25].

Oxytetracycline resistance is conferred by several genetic elements located on mobile elements [37]. However, these elements can be grouped by general mechanism: efflux pump, ribosomal protection, or enzymatic alteration. However, since E. coli most commonly uses the tetracycline efflux pump [3, 5, 13, 15, 63], along with lack of sequence data for E. coli O55, the tetracycline efflux pump and regulation will be briefly discussed. The efflux tet genes are by far the most widely seen and studied, generally efflux proteins in E. coli are coded by genes tetA, tetB, tetC, tetD, tetE, tetI, and tetY [13]. All the tet efflux genes code for membrane-associated proteins which export tetracycline from the cell. Export of tetracycline reduces the intracellular drug concentration and thus protects the ribosomes within the cell. Efflux genes are found in both
gram-positive and gram-negative bacteria [13]. In terms of regulation, the efflux determinants consist of two genes, one coding for an efflux protein and one coding for a repressor protein, tetR, both of which are regulated by tetracycline. However, induction in the system occurs when a magnesium tetracycline complex enters the cell and binds to the repressor protein. Drug binding changes the conformation of the repressor so that it can no longer bind to the DNA operator region [13, 34]. Kinetically, the reaction rate of only tetracycline binding to tetR is described by pseudo-first order kinetics, binding of a magnesium-tetracycline complex is to require several consecutive reversible reactions [13, 32, 34].

Co-selection

A result of the existence of both metal and antibiotics resistance in the bacterial genome has exposed a recent bacterial genomic synergism called co-selection. Although bacterial exposure to metals predates human history, anthropogenic-derived sources of metals represent a major source of contamination in the environment [8, 56]. Moreover, aquatic environments contain sublethal concentrations of toxic metals, selecting for metal resistant bacteria [2]. Since metals are more persistent than antibiotics in the natural environment, metals have the potential to exert a longer selective pressure for resistant microbiota. Generally, the presence of multiple stressing compounds provides a greatly antagonistic environment for bacteria to grow; multiple stressors have different modes of action or may interact synergistically [14]. However, microorganisms have recently shown the ability to cope with such antagonistic environments though co-selection. For example, increased antibiotic resistance gene abundances had high correlations with five trace metals found in the soil, mainly chromium, copper, nickel, lead, and iron [19, 22, 38].
There are three major mechanisms of co-selection. First, *co-resistance* is the mechanism where genes that encode for resistance for different stressing compounds are located next to each other on the same genetic element. This linkage of antibiotics and metals resistance determinants is more common on plasmids [8, 10, 22, 47, 51, 52]. Genetic resistance determinants can be contained within larger elements (e.g. integrons that exist within transposons) so that the transfer of the larger element also leads to the transfer of integral elements. For example, the genetic linkage of copper resistance encoded by *tcrB*, macrolide [*erm*(B)] and glycopeptide resistance (*vanA*) were observed on a *Enterococcus faecium* strain, when tested, the isolates were resistant to the copper and antibiotics in tandem [8]. Similarly, *E. coli* O25:H4-ST131 contained plasmid pEK204, which harbors the genetic elements for arsenic resistance (*arsA* and *arsB*) adjacent to tetracycline resistance elements (*tetA*, *tetR*, *tetC*, and *tetD*). A similar relation was found in the same *E. coli* species for sulfonamide resistance genes (*sul1*) being located down stream of mercuric ion resistance genes (*mer*) on the plasmid pRSB107 [70]. Thus, co-resistance as a mechanism for the co-selection of antibiotic and metal resistance and the extent of these linkages is related to the abundance of transposons in clinically relevant and environmental bacteria [8].

A second form of co-selection is *cross-selection*. Cross selection describes the process of resistance synergism from two stressors. This synergism may occur when different antimicrobial agents attack the same target, initiate a common pathway to cell death or share a common route of access to their respective targets [8, 10]. Thus, the stress of one inhibiting compound results in the resistance of another stressing compound. One common example is the efflux of dissimilar compounds from a cell through the same mechanism, such as a multi-drug resistant pump [8].

Lastly, *co-regulation* is the genetically coordinated response of an organism to a set of stressing
compounds. More specifically, it consists of a range of transcriptional and translational responses to metal or antibiotic exposure can be linked to form a coordinated response to either stress [8, 56]. Since the existence of metals resistance has been more pronounced in the bacterial genome over a longer time, the influence of existing metal contamination may be selecting for overall more resistant/fit bacterial communities. It should be noted that biofilm mass transfer and horizontal gene transfer are key elements in the mobilization and proliferation of the bacterial resistome [6, 8]. Resistance gene mobility, ultimately, results in differing fate and transport of key genetic elements, such as key resistance determinants migrating to biofilms rather than remaining suspended in the water column and the effect of genetic elements remaining “active” after the cell host has lost viability [7, 8,11,18, 68]. A schematic of co-selection in a bacterial cell is provided in Figure 3.

![Figure 3: Overview of Co-Selection in microbes [8]](image)

For example, in China, a study done on manure and manure amended soil found that the abundance of antibiotic resistance genes were more closely associated to the presence of metals than to antibiotics [31]. This points to the notion that influence on resistance expression from antibiotics is a much more transient process, due to their high degradation rates. Similarly, when tested for metals and antibiotics resistance, acinobacters demonstrated multiple resistances
towards metals and antibiotics [17]. Although it should be mentioned that these bacteria do produce exopolysaccharides. This extracellular component acts a pseudo-biofilm mechanism and provides a resistance of mass transfer to the stressing compounds. Furthermore, Fernández-Calviño and Bååth demonstrated that bacteria from copper polluted soils had increased pollution induced community tolerance to major antibiotics, not necessarily increased tolerance to copper, when compared to non copper polluted soil bacteria [19]. Similarly, freshwater microcosm experiments amended with toxic metals and antibiotics conducted resulted in 50-100% of the isolates expressing multiple resistances after 7 day incubation periods [60]. The link between metals and antibiotics resistance is seen not only in stationary or batch samples, the relationship between multiple stressor resistance is consecutive over spatial fields as well [55, 65, 71]. Hellweger and Asce describe antibiotic resistance along the Poudre River in Colorado as determined by metal impaction, not only antibiotic presence [26]. On the other hand, antibiotics may also coselect for other antibiotics, as seen by fluoroquinolone resistance in poultry isolates in Australia in which fluoroquinolone has never been used [29]. Overall, the area of co-selection is still relatively new and proper methods to hone in on method to properly test co-selection have yet to be demonstrated and modeled. However, the microbial phenomena of co-selective pressure underscores the need to better understand the acquisition, development, and proliferation of the resistome to improve regulation and modern medicine to ensure environmental and public health.
Methods

Chemical & Culture

The bacterial strain used for the study was E. coli O55 (ATCC 12014), which is an O antigen stereotype [49, 59]. Cultures were grown in a Glucose Minimal Salt (GMS) media at pH 6.8 (45). The media consisted of 7g/L glucose, 7g/L K$_2$HPO$_4$, 2g/L KH$_2$PO$_4$, 1g/L (NH$_4$)$_2$SO$_4$, 200 mg/L MgSO$_4$, and 20mg/L CaCl$_2$•H$_2$O. Arsenic (As$_2$O$_3$) solutions were made at the following concentrations: 1, 2.5, 5, 10, 25, 50, and 100 ppb. Also, solutions of Oxytetracycline hydrochloride were made at the following concentrations: 10, 20, 30, and 40 ppm.

Experimental Setup

Prior to experiments, bacterial cultures were grown for at least 24 hours and to an optical density of approximately 0.57 at 600nm wavelength. 384-well plates were used to conduct high throughput optical density measurements, which were conducted in two phases. First, optical density was measured at 600nm wavelength for a plate with wells containing 90% GMS, 5% arsenic solution, and 5% bacterial culture for a total of 100 uL per well. Each arsenic solution was performed in triplicate and a growth curve describing the culture's growth with respect to arsenic was constructed to identify exponential growth. Simultaneously, 1.5 mL Eppendorf tubes were setup exactly like the 384-well plate i.e. 90% GMS, 5% arsenic solution, and 5% bacterial culture. Upon reaching exponential growth, samples were aliquotted from the Eppendorf tubes into different 384-well plates filled with 100 uL containing 90% GMS, 5% oxytetracycline solution, and 5% bacterial culture. Each arsenic pre-exposure concentration was performed in
triplicate. Ultimately, optical density at 600nm was determined to similarly construct a growth curve and determine the growth rate with respect to oxytetracycline. All high-throughput plates were protected from light exposure and evaporation by wrapping the plate with aluminum foil.

**PHREEQC Batch Speciation**

The USGS geochemical modeling software PHREEQC ver. 3.0 [50] was used to determine arsenic and oxytetracycline speciation in the GMS. Speciation was performed using the MINTEQ database, amended with oxytetracycline speciation reactions. PHREEQC speciation models were used to determine the bioavailable concentrations of arsenic and oxytetracycline. Oxytetracycline is an amphoteric compound with acidity constant (pKa) values of 3.3, 7.3, and 9.1[54, 40, Wu, 62, 67]. Over the range of environmentally relevant pH values, OTC exists predominately as a zwitterion (pH 5–7) or a monoanion (pH 8–9). Hence, tetracycline compounds, such as oxytetracycline, are subject to complex chemistry that affect its bioavailability. For example, chelating interactions influence the chemistry and fate of tetracyclines in aquatic environments. Chelation is the process of a metal ion establishing multiple bonds with ligands, typically organic molecules. For example, tetracyclines are strong chelating agents in the environment. They act as ligands with di- and trivalent metal ions such as Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, and Al$^{3+}$ [12, 42]. The chelating activity of tetracyclines is a direct result of its chemical structure and components. Complexation involving the C-11 – C-12 ß-diketone system is the major form of chelation in alkaline mediums, and that the dimethyl amino group position at C-4 becomes involved in the complexation as pH increases [42]. Tetracycline’s complex chemistry allows it to also interact with humic acids and other
anionic groups within environmental matrices. Although most antibiotics do not require metal ion coordination to exert biological action, tetracycline has prerequisites for binding of metals ions to function properly; sorption of tetracycline in clays, where intercalation of metal complexes occur, may provide suitable conditions for the drug to exert a biological effect [11].

In the PHREEQC input file, oxytetracycline deprotonation and complexation reactions for oxytetracycline were entered to determine bioavailable oxytetracycline. The concentrations of arsenic and oxytetracycline added to the batch reactor were converted to molarities and inputted to the REACTION block in PHREEQC. In particular, complexation reactions between Ca$^{2+}$ and Mg$^{2+}$ were considered because these major cations are present in the GMS. Complexation with oxytetracycline is dependent on the protonation of oxytetracycline [32, 46]. Reported log equilibrium constants for the double deprotonated oxytetracycline species for Ca$^{2+}$ and Mg$^{2+}$ 6.44 and 5.77, respectively [46]. To simulate aerobic conditions, the redox potential of the system was equilibrated to an atmospheric partial of O$_2$. Moreover, system temperature was assumed to be 25°C with an initial pH of 7. Chemical concentrations were determined from the GMS components and calculated for the individual components. Arsenic and oxytetracycline relative speciation curves are shown in Figure 4 and Figure 5. The PHREEQC input for arsenic and oxytetracycline speciation is found in Appendix A.
Figure 4: PHREEQC Arsenic Speciation

Figure 5: PHREEQC Oxytetracycline Speciation
Results

Arsenic Pre-exposure

Figure 6 shows the optical density of *E. coli* over a 24 hour period, while being exposed to arsenic.

![Figure 6: Arsenic Exposed E. coli Growth Curve](image)

The growth rate under arsenic exposure was determined during exponential phase. Exponential phase was found to occur during the 8\textsuperscript{th} through 16\textsuperscript{th} hour of growth. The growth rate was determined by using the SLOPE function in Numbers, a spreadsheet software. The SLOPE function calculates the slope of the best-fit line for a given set of data. As demonstrated by Figure 6 and Figure 7, the largest growth rate was observed at 0 ppb arsenic pre-exposure. Also, the growth rate for arsenic exposure demonstrate an inhibitory pattern. There are two
local maxima in the growth rate corresponding to 0 ppb arsenic and 25 ppb arsenic. Similarly, there are two local minima corresponding to 1 ppb and 100 ppb arsenic.

![Figure 7: Arsenic Exposed E. coli Growth Rate](image)

*Oxytetracycline Growth*

The optical density of each arsenic pre-exposed *E. coli* is shown in Figure 8. Similar to the arsenic culture, growth rates were determined for the each pre-exposed concentration. Exponential phrase occurred during different intervals depending on the oxytetracycline concentration. For 0 ppm oxytetracycline, exponential phase occurred during the 4th to 10th hour. For 10 and 20 ppm, oxytetracycline, exponential phase occurred during the 20th to 28th hour; while true exponential phase intervals for 30 and 40 ppm could not be determined, rates were determined during the same time interval of the 10 and 20 ppm rates. The growth rate for the entire range of oxytetracycline concentrations tested are shown in Figure 9.
Figure 10 describes the growth rate where each set is a particular arsenic pre-exposure and bar for each oxytetracycline concentration. On the other hand, Figure 11 shows the growth rates of the pre-exposed arsenic growth rates per antibiotic concentration. There was similar growth pattern for each arsenic pre-exposed culture for 0 ppm oxytetracycline. However, notable differential growth was observed for 10 and 20 ppm, along with some differential growth observed at 30 and 40 ppm.

Figure 8: Arsenic Pre-exposed E. coli Growth Curve
Figure 9: Arsenic Pre-exposed *E. coli* Growth Rate

Figure 10: Arsenic Pre-exposed *E. coli* Growth Rate Comparison by Arsenic
Discussion

Although differential growth was observed over the entire range of oxytetracycline arsenic pre-exposed *E. coli*, notable changes in the growth rate occur in the 10 and 20 ppm oxytetracycline ranges. There is similar growth observed for all arsenic concentrations at the 0 ppm oxytetracycline concentration; yet the magnitudes of the observed growth rate at 0 ppm was higher than the initial growth rates observed in Figure 7 during arsenic conditioning. However, at both 10 and 20 ppm oxytetracycline concentrations, all arsenic pre-exposed growth rates were generally orders of magnitude higher than growth rates without arsenic pre-exposure. To gauge the magnitude of resistance conferred by arsenic exposure, a ratio of the arsenic pre-exposed growth rate to the unexposed growth rate was determined for each set of oxytetracycline
concentration. Figure 12 demonstrates the growth rate ratio, where $\mu_{\text{As}}$ is the growth rate of the arsenic pre-exposed *E. coli* and $\mu_0$ is the growth rate of the unexposed *E. coli*. For 0 ppm oxytetracycline, there is relatively consistent growth, indicated by the growth rate ratio remaining relatively constant at 1. However, at 10 ppm oxytetracycline, the ratio of the pre-exposed and unexposed growth rates demonstrates pseudo inhibitory function in the range of 0 to 25 ppb, where after 25 ppb the ratio steadily increase. Similar ratio patterns are seen for 20 ppm oxytetracycline. Given the growth rate trends (Figures 9, 10, and 11), one may consider the influence of oxytetracycline on our pre- and unexposed growth rates to vary according to some rate law, either zero or first order with respect to oxytetracycline, as seen in Appendix B, where $\mu$ is the growth rate of the culture and $k$ is a decreased growth ratio coefficient that takes the units according to the order of the equation. Since co-selection is a phenomena that depends on the influence of several factors, the rate laws
must be written as partial differentials. Best fit curves were applied to integral forms of the rate law and correlation values for each form are listed in Appendix C with the imbedded trend line function in Numbers. Correlation values suggest that there is a higher correlation for the growth rate varying exponentially rather than linearly with respect to oxytetracycline. The inhibition coefficients for both zero and first order equations were retrieved from the best fit line equations provided by Numbers. The zero order inhibition coefficient was calculated from the slope of the line, while the first order coefficient was retrieved from the coefficient in the exponential. Figure 13 compares the growth rate at each oxytetracycline concentration and the derived zero order inhibition coefficient to the arsenic concentration. Similarly, Figure 14 compares the ratio of pre-exposed to unexposed growth rates and the derived first order coefficient to the arsenic concentration.

Figure 13: Growth Rate and Zero Order -k Comparison
First, it is notable the different inhibition coefficients for the different order assumptions are orders of magnitude different. However, both orders have the same general trend in their with a global minimum occurring at 5 ppb arsenic. Moreover, the distribution of inhibition coefficients demonstrates a reduction in magnitude of the decay coefficient after 10 ppb arsenic. In other words, as the arsenic concentrations increase, the decay coefficient tends to be less negative. Furthermore, the inhibition coefficient for both reaction orders is inversely related to the respective growth rate, whose pattern can be seen in 10 and 20 ppm. For the zero order assumption, the inhibition coefficient is compared to the growth rate in oxytetracycline as a function of arsenic concentration. On the other hand, for the first order assumption, the inhibition coefficient was compared to ratio of pre-exposed arsenic to unexposed growth rates. One may conclude that the inhibition coefficient, regardless of the particular
order assumed, is a function of the arsenic concentration or, more generally, of metals
ccentration. Ultimately, a governing equation can be formulated to describe the co-selective
pressure exerted on an antibacterial growth rate by toxic metal exposure, as seen by Appendix D,
where the selective pressure of arsenic for oxytetracycline resistance, $F$, is described as a second
order partial differential equation of the bacterial growth rate with respect to arsenic and
oxytetracycline. Although validation of the co-selective pressure equation is still required, it has
conceptual merit to elucidate the complexities of nontraditional antibiotic resistance in the
environment, in particular co-selection. Yet, it is reasonable to consider the co-selective pressure
of a toxic metal impacted aquatic system, similar to considering the ionic strength of an aquatic
system. The total co-selective pressure of an aquatic system would be the sum of all the co-
selective pressures imparted by the toxic metals found in the system to a particular antibiotic, for
example.

Conclusion

A high-throughput culture based methods was used to assess the co-selective pressure resulting
from arsenic pre-exposure on the expression of oxytetracycline resistance. Speciation of arsenic
and oxytetracycline were determined under aerobic conditions determined that system speciation
affects the overall bioavailability of oxytetracycline. Increased differential growth was observed
for cultures pre-exposed to arsenic rather than unexposed cultures, in concentrations 1000 times
higher than aquatic environmental concentrations. Moreover, the differential growth acquired by
the bacterial cultures is described by the inhibition coefficient of the pre- or unexposed culture in
oxytetracycline. Ultimately, the co-selective pressure of arsenic improves the survivability of E.
coli in oxytetracycline concentrations over 1000 times higher than concentrations found in aquatic environments.

Future Work

Recommended future work on co-selective pressure would be to perform more mechanistic studies on co-resistance and cross-resistance, as well as co-selective pressure phenomena in biofilms. Possible forms of evaluating co-resistance is determining presence and element composition of plasmids in bacteria. Similarly, cross-resistance evaluation may be studied by qPCR or gene expression measurement and correlation of metal and antibiotic resistance genes. Moreover, to assess the co-selective pressure within biofilms, special attention must be given to the mass transfer of stressing compounds, which can affect total exposure of the biofilm culture [4], particularly, observing proliferation of resistant bacteria as function of biofilm depth or dislodgment. Of equal importance when considering co-selective pressure in biofilms and planktonic cultures is the magnitude of horizontal gene transfer [1, 11, 8]. The rate of horizontal gene transfer may increase the proliferation of plasmids conferring metal and antibiotic resistance.

On the other hand, validating models of coselective pressure may inform managerial and regulatory decisions. Coselective pressure has the potential to serve as a measure of biological impact of contamination with extended implications on public health. Validation of such models may lead to key indicator metals, antibiotics, resistance genes, or bacteria. For example, prediction of resistance determinants in natural environments, hospitals, or water and wastewater treatment plants may allow for mitigation of such determinants in effluent waters.
Appendix

Appendix A: PHREEQC Input File

SOLUTION 1
   temp    25
   pH      7
   pe      12 O2(g) -0.67
   redox   pe
   units   ppm
   density 1
   Ca      5.44
   Cl      9.51
   K       3714.75
   Mg      403.11
   N(-3)   272.43
   P       5171.85
   S(6)    2319.06
   -water  1 # kg

SOLUTION_MASTER_SPECIES
Oxy   H3Oxy+   0.0   C22H24N2O9 460.434

SOLUTION_SPECIES
Glucose = Glucose
log_k 0
H3Oxy+ = H3Oxy+
log_k 0
H3Oxy+ = H2Oxy + H+
log_k -3.22
H2Oxy = HOxy- + H+
log_k -7.46
HOxy- = Oxy-2 + H+
log_k -8.94
Oxy-2 + Mg+2 = OxyMg
log_k 5.77
Oxy-2 + Ca+2 = OxyCa
log_k 6.44

EQUILIBRIUM_PHASES 1
   O2(g)   -0.67 10

END

USE solution 1
REACTION 1
   As2O3   1
   0 0.005054 0.012636 0.025272 0.05054 0.12636 0.25272
0.5054 micromoles

\[
\frac{\partial \mu}{\partial Oxy} = -k
\]

\[
\frac{\partial \mu}{\partial Oxy} = -k\mu
\]

\[
\begin{aligned}
\text{molalities} & \quad \text{H}_3\text{AsO}_3 \quad \text{H}_2\text{AsO}_3^- \quad \text{HAsO}_3^-_2 \quad \text{H}_4\text{AsO}_3^+ \quad \text{AsO}_3^-_3 \\
& \quad \text{HAsO}_4^-_2 \quad \text{H}_2\text{AsO}_4^- \quad \text{AsO}_4^-_3 \quad \text{H}_3\text{AsO}_4 \quad \text{As}_2\text{O}_3
\end{aligned}
\]

END

USE solution 1

REACTION 2

\[
Oxy \quad 1
\]

\[
0 \quad 0.021718 \quad 0.043437 \quad 0.065155 \quad 0.086874 \quad \text{millimoles}
\]

SELECTED_OUTPUT

- file coselOxy.xls
- simulation true
- ph true
- pe true
- reaction true

- molalities Oxy H2Oxy HOxy- Oxy-2 OxyMg OxyCa

END

USE solution 1

REACTION 2

\[
Oxy \quad 1
\]

\[
0 \quad 0.021718 \quad 0.043437 \quad 0.065155 \quad 0.086874 \quad \text{millimoles}
\]

SELECTED_OUTPUT

- file coselOxy.xls
- simulation true
- ph true
- pe true
- reaction true

- molalities Oxy H2Oxy HOxy- Oxy-2 OxyMg OxyCa

END

\[
F_{As,Oxy} = \frac{\partial^2 \mu}{\partial As \partial Oxy} = \frac{\partial}{\partial As} \left( \frac{\partial \mu}{\partial Oxy} \right)
\]
Appendix B: Zero and First Order Rate Law Eqs.

Appendix C: $R^2$ values for best-fit line of Oxytetracycline Growth Rate

<table>
<thead>
<tr>
<th>As(V) Concentration</th>
<th>$R^2$ Linear</th>
<th>$R^2$ Exponential</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ppb</td>
<td>76.46%</td>
<td>89.28%</td>
</tr>
<tr>
<td>1 ppb</td>
<td>75.93%</td>
<td>77.63%</td>
</tr>
<tr>
<td>2.5 ppb</td>
<td>81.21%</td>
<td>91.24%</td>
</tr>
<tr>
<td>5 ppb</td>
<td>45.76%</td>
<td>24.83%</td>
</tr>
<tr>
<td>10 ppb</td>
<td>85.60%</td>
<td>99.35%</td>
</tr>
<tr>
<td>25 ppb</td>
<td>80.66%</td>
<td>90.33%</td>
</tr>
<tr>
<td>50 ppb</td>
<td>84.50%</td>
<td>95.65%</td>
</tr>
<tr>
<td>100 ppb</td>
<td>90.32%</td>
<td>98.02%</td>
</tr>
</tbody>
</table>

Appendix D: Coselective Pressure Eq.
Bibliography


