Existing and Inducible Antibiotic Resistance in Worcester, Massachusetts

A Major Qualifying Project

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by

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Abstract

This project investigated existing and inducible antibiotic resistance (AR) present at several public sites in and around Worcester, MA. Bacteria from the soil were screened for ampicillin resistance and tested for multidrug resistance. AR was induced through the bacterial metabolite 2,3-dihydroxybenzoate (DHB) in *E.coli.* Liquid cultures of *E. coli* were exposed to DHB, then cultured on antibiotic plates over time. Optical density measurements provided data on *E.coli's* proliferation to infer the fitness cost of expressing AR genes. Our soil isolates showed that multidrug resistant organisms are present at many locations around Worcester. Our DHB experiments supported previous findings that exposure to DHB induces antibiotic resistance in *E. coli.*

Introduction

Antibiotic resistance is a major challenge to global health and has been for a long time, with some of the first cases documented in the early 1940's (Abraham & Chain 1940). Today, antibiotic resistance is considered to be at "dangerously high levels in all parts of the world" (World Health Organization 2020). As of 2021, antibiotic resistance is cited as the source of over 2.8 million infections and 35,000 deaths annually in the US (Centers for Disease Control and Prevention, 2021 November 30). Studies show that over the course of the latter half of the 20th century and the 21st century, antibiotic resistance has grown more common all over the world (Figure 1) and cases of resistance to most known antibiotics have been documented (Klein et al., 2018). This scenario poses an incredible risk to the future public health. Effective treatments for the worst of bacterial infections appear to be running out, as resistance appears to develop faster than new antibiotic drugs can be discovered (PEW Charitable Trusts, 2016). The threat posed by these circumstances make the study of antibiotic resistance and the molecular mechanisms responsible all the more important.

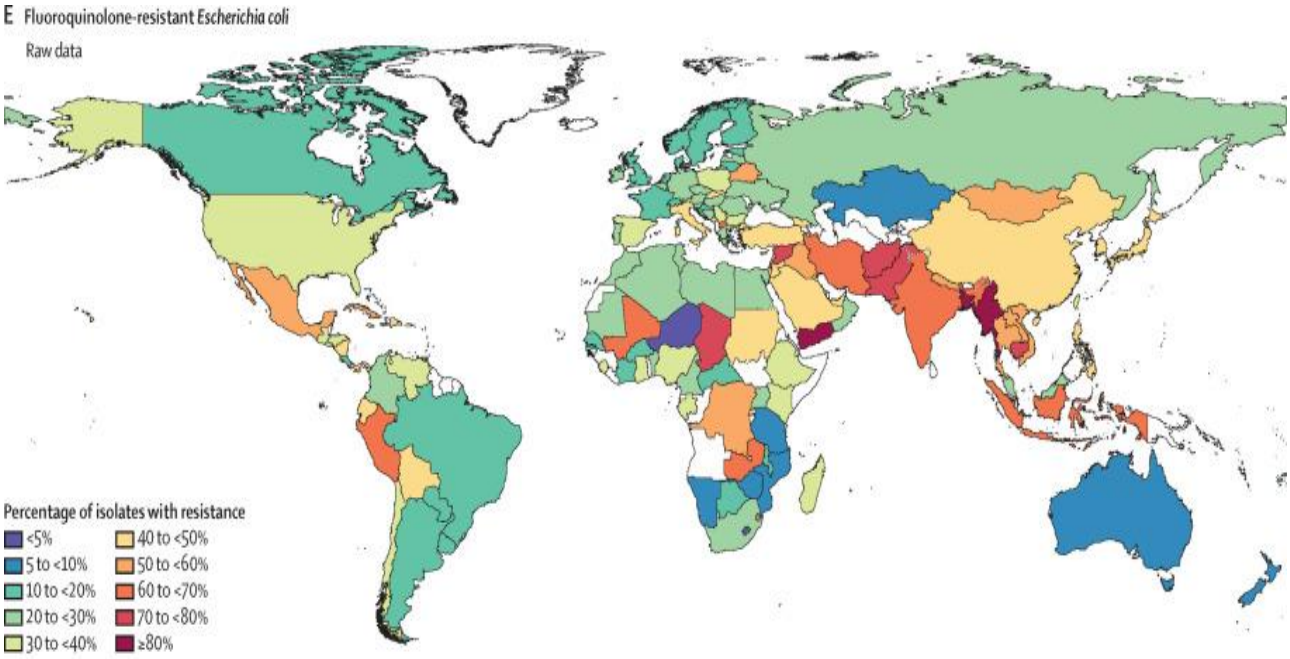

Figure 1. Global Distribution of Fluoroquinolone-resistant *E.coli*

In 2019, authors Murray et al used publicly available data to estimate the percentage of *E. coli* isolates with fluoroquinolone resistance. Fluoroquinolone is a broad spectrum antibiotic used to treat bacterial infections in humans and animals. This figure shows that a majority of *E.coli* isolates in the Middle East are resistant to fluoroquinolone (Murray et al., 2022).

Antibiotics are substances that are able to inhibit growth or kill bacteria and are vital to treating life-threatening bacterial infections, as only antibiotics can kill bacteria. Although antibiotics have successfully treated bacterial infections for nearly a century, they are not a silver-bullet for bacteria. In certain conditions, bacteria are able to develop resistance to antibiotics, called antibiotic resistance (AR) or antimicrobial resistance. AR describes the phenomenon when bacteria and other microbes develop the ability to survive and grow in the presence of substances used with the intention of killing or inhibiting growth of these organisms..

Antibiotic resistance can occur naturally as an evolutionary defense for microorganisms against other microorganisms (Calero-Cacares & Muniesa, 2016). For example, in an environment with limited resources, it is advantageous for a microorganism to eliminate resource competitors by secreting antibiotics. While some baseline-level of natural antibiotic resistance is expected, extensive human use of antibiotics in agriculture and medical care, as well as their

release into the environment, has increased the exposure of bacteria to such substances (World Health Organization, 2020).

The need to identify new antibiotics has led researchers to discover non-antibiotic molecules, called metabolites, produced by bacteria (Perry et al., 2021). At the time of discovery these molecules were thought to be waste products, so resources were not dedicated to characterizing them. However, the need to understand AR has caused researchers to revisit metabolites. In this MQP, the metabolite 2,3-dihydroxybenzoate (DHB) was studied. Previous research has shown that DHB interacts with regulators of an efflux pump found in *E.coli* to make this bacteria less susceptible to fluoroquinolones, tetracycline, and beta-lactams. This metabolite is of particular interest because it is a byproduct of human metabolism of aspirin (Grootvelt & Halliwell, 1988). In a clinical setting, this is concerning as a patient undergoing antibiotic treatment who is also ingesting aspirin could mitigate the efficacy of the antibiotics. Additionally, human waste containing the byproducts of aspirin could be released into the environment and induce antibiotic resistance in naturally occurring bacteria.

In this MQP project, we investigated the prevalence of AR organisms in several different locations around Worcester, MA. In addition, we conducted experiments to see if it is possible to induce antibiotic resistance in *E. coli* using a compound that is a byproduct human's metabolism of aspirin.

Background

This chapter will describe how antibiotics were discovered, sources and mechanisms of antibiotic resistance, and the current state of knowledge about AR.

Historical Background

In 1928, Scottish scientist Alexander Fleming first identified a "mould juice" from his staphylococcal bacteria cultures that would revolutionize medicine (Tan & Tatsumura, 2015) . Fleming noticed that a petri dish growing bacteria, but contaminated by mold growth, was killing the bacteria. Fleming knew the magnitude and life-saving meaning of his "bacteria killer" discovery as infections in the 19th and 20th century usually led to death (Tan & Tatsumura, 2015). Researchers today attribute many of the deaths in the United States, United Kingdom, and New Zealand during the 1918 influenza epidemic to secondary bacterial infections (Brundage et al., 2007). The importance of Fleming's scientific contribution was recognized in 1945, where he received a Nobel Prize in Medicine for the discovery of the antibiotic penicillin. His work would lead to future researchers identifying over 100 types of antibiotics in the ensuring century later (Lewis, 2021). However, Fleming's discovery had unforeseen consequences. In 1940, researchers Abraham and Chain identified an enzyme from *Staphylococcus aureus* that caused penicillin to "entirely [lose] its growth-inhibiting activity" (Abraham & Chain 1940) . Over the remainder of the 20th century, increasing numbers of resistant bacteria were observed. Additionally, after the discovery of a new antibiotic, cases of resistance would emerge (Lobanovska & Pilla, 2017, 135-145).

Importance of Understanding Antibiotic Resistance

Antibiotic resistance has the potential to cause significant harm to human health and leaves us vulnerable to mortalities from otherwise treatable bacterial infections. The need to prevent unnecessary fatalities as well as limited new antibiotics is why AR has been an increasingly popular research field in recent decades (Ventola, 2015, 277-283).

When antibiotics become ineffective against bacterial infections, the consequences are serious and quickly felt. Not only is the well-being of a patient put at risk, but medical costs can

rise because more expensive drugs are often required to treat the resistant bacteria and the side effects of the infection. These factors lead to longer hospital stays and a higher mortality rate. Increased prevalence of antibiotic resistance in human pathogens also introduces the risk that medical procedures that need to occur in a sterile environment cannot happen, which is not safe for doctors or patients. Without effective antibiotics, the ability to treat and prevent bacterial infections is severely diminished (World Health Organization, 2020).

In its most recent antibiotic resistance threats report, the CDC estimates 2.8 million antibiotic resistant infections occur in the United States every year, and greater than 35,000 of these cases result in death annually (Centers for Disease Control and Prevention et al., 2021 November 23). Another alarming statistic is that one bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for more deaths in the United States than emphysema, HIV, Parkinson's, and homicide combined (Llor & Bjerrum, 2014, 229-241).These figures become particularly alarming when considered alongside the fact that difficulties in antibiotic discovery and development have contributed to a drastic decrease in antibiotic discovery programs in the pharmaceutical industry. Many of the antibiotics in use today were discovered during "the golden age" of antibiotic discovery, lasting from 1940 to 1960 (Aminov, 2014), and no new class of antibiotics has been discovered since the late 1980s . Even if a new class of antibiotic was discovered or invented, that antibiotic would eventually not be useful. While it is possible to mitigate resistance through antibiotic stewardship programs and protocols, these protocols are only meant to postpone resistance and not completely prevent it (Plackett, 2020). The cycle of antibiotic discovery, clinical use, and resistance has and will occur for all antibiotics, which is why it is so important to understand what factors promote AR (Waglechner & Wright, 2017).

How Does Antibiotic Resistance Arise?

There are a number of different ways through which antibiotic resistance may arise, including through human activity as well as natural sources.

Natural Sources

Antibiotic resistance is a natural phenomenon and exists absent of human activity. Antibiotic resistant genes (ARGs) have been identified in remote areas of the world, such as Antarctica, indicating that resistance predates human-made antibiotics and that resistance can develop independently of antibiotics (Goethem, 2018). AR can be broadly categorized into natural and acquired resistance (Reygaert, 2018, 482-501). Natural resistance can be sub-grouped into intrinsic resistance and induced resistance. Intrinsic resistance is AR that microorganisms express due to mechanisms or pathways present regardless of exposure to naturally occurring or man-made antibiotic substances (Reygaert, 2018, 482-501). Induced resistance refers to AR where the genes necessary for the resistance mechanisms are already present in the bacteria, but the level of expression required for resistance is achieved through environmental pressures (Reygaert, 2018, 482-501). Acquired resistance refers to AR conferred to bacteria through uptake of ARGs in the environment.

Bacteria collect genetic material through a process called transformation, which allows genes to spread one from one bacterial cell to another (Figure 2). In times of environmental stress, bacteria can release their genetic material into the environment in order to preserve remaining resources but also maintain genetic diversity (Sigma-Aldrich, n.d.). Other methods of acquired resistance include conjugation and transduction (Figure 2). During conjugation, a structure called a pilus is extended from a donor bacteria cell to a recipient cell and connects the two cells together. DNA is then given from the donor to the recipient bacteria (Khan Academy, n.d.). In transduction, DNA is transferred from one bacterial cell to another through a virus, such as a bacteriophage, and does not require physical contact between the two cells. When a bacteriophage parasitizes a bacteria, it injects its genetic material into the bacteria in order to hijack the bacteria's genetic replication machinery. The bacteria will replicate the phage's genome and create new phages. However, some of the bacteria's own genetic material can be incorporated into the new phages. A process called lysis allows the new phages to leave the bacteria, giving the phages a new opportunity to spread genetic material and repeat the aforementioned cycle (Nature Education, n.d.).

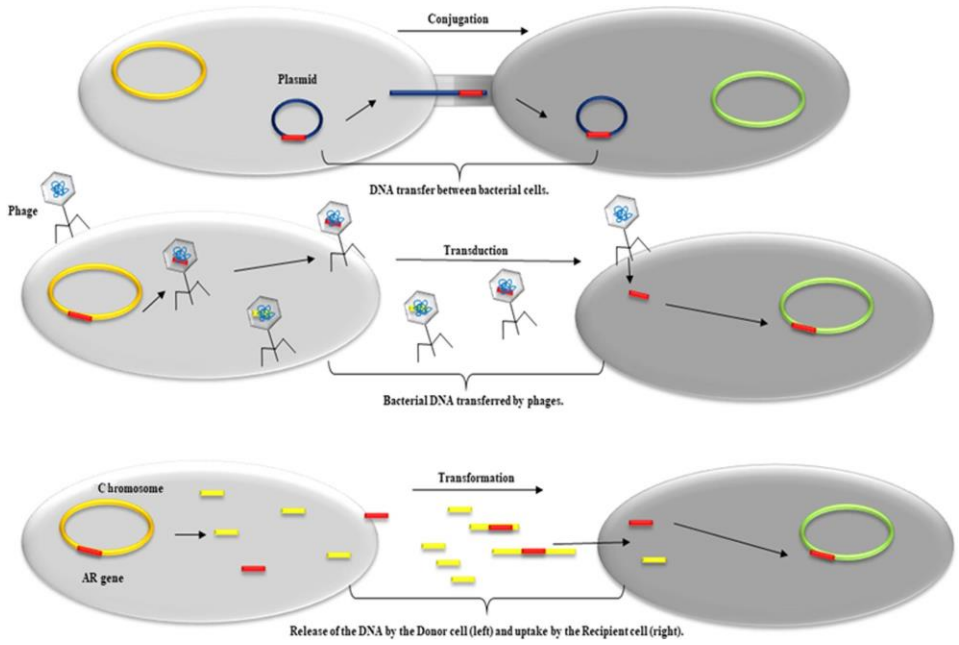

Figure 2. Genetic Material Transferred Among Bacteria

Bacteria are able to exchange genetic material and pass on advantageous resistance-causing genes through conjugation, transduction, and transformation (Dawadi, 2021).

Anthropomorphic Sources

Human activity has increased the prevalence of antibiotics in the environment and consequently AR (Stanton, 2020). The following pathways have been proposed as sources for antibiotics and AR in the wild: irresponsible use and abuse of antibiotics for human infections, aquaculture and animal husbandry, wastewater treatment facilities, and pollution (University of Birmingham, n.d.) (Figure 3). Irresponsible use of antibiotics includes self-medicating with antibiotics, over-prescribing antibiotics and prescribing antibiotics when not needed, and patients not finishing their antibiotic course (Llor & Bjerrum, 2014, 229-241). In the United States, the Center for Disease Control (CDC) estimates that inappropriate use of antibiotics approaches 50% in outpatient use (Center for Disease Control and Prevention, n.d.). In other countries, such as Vietnam, antibiotics are legally available without a prescription and individuals may self-medicate a viral illness with antibiotics (Wertheim, 2021). Not only will antibiotics not kill the virus, but that individual may induce AR in the bacteria naturally present within their body.

There is a body of research describing the link between AR and animals, but the contribution of aquaculture and animal husbandry to antibiotic resistance in the environment is

still questioned (Marshall & Levy, 2011, 718-733). Antibiotics are used to treat active diseases, but are also used inappropriately as a preventative medication in healthy animals and to increase animal size. It is thought that antibiotic resistance spreads from animals to humans by handling meat contaminated with resistant bacteria, coming into contact with animal waste, and also touching animals without sanitization (Centers for Disease Control and Prevention, n.d.). The environment can also be contaminated by antibiotics through agricultural runoff, which includes water, soil and waste that can carry antibiotics and ARGs to secondary locations where potential ARB can easily proliferate and spread (Manyi-Loh et al., 2018).

Another pathway for environmental contamination is from wastewater treatment plants (WWTPs). WWTPs clean polluted water to recycle it back into the water cycle. Water pollution includes bacteria and antibiotics, which WWTPs cannot completely eliminate. WWTP often has a high density of bacteria present, which makes ARG easily transmittable between bacteria. A Polish study from 2020 examining four different WWTPs found that "considerable quantities" of ARGs and the antibiotic doxycycline was detected in treated wastewater, which could result in AR spreading geographically from the WWTP (Osinska, 2020).

Antibiotic resistance is also facilitated through the release of antibiotics from pharmaceutical manufacturing sites. Pharmaceutical manufacturing is often contracted overseas because there are financial incentives to manufacture pharmaceuticals in countries with different environmental standards (Nijsingh et al., 2019). Although cheaper manufacturing benefits consumers, it does not benefit the environment. A 2009 study from a region in India that is a hub for pharmaceutical manufacturing analyzed surface, ground, and drinking water for the presence of antibiotics. The study detected 11 antibiotics in effluent taken from one WWTP that is supposed to clean water from the various pharmaceutical manufacturers. Antibiotics were detected in surface, ground, and drinking water at concentrations up to a 1 million times found in the United States, which could impact not only bacteria and select for AR, but also adversely impact the individuals who live in this region (Fick, 2010).

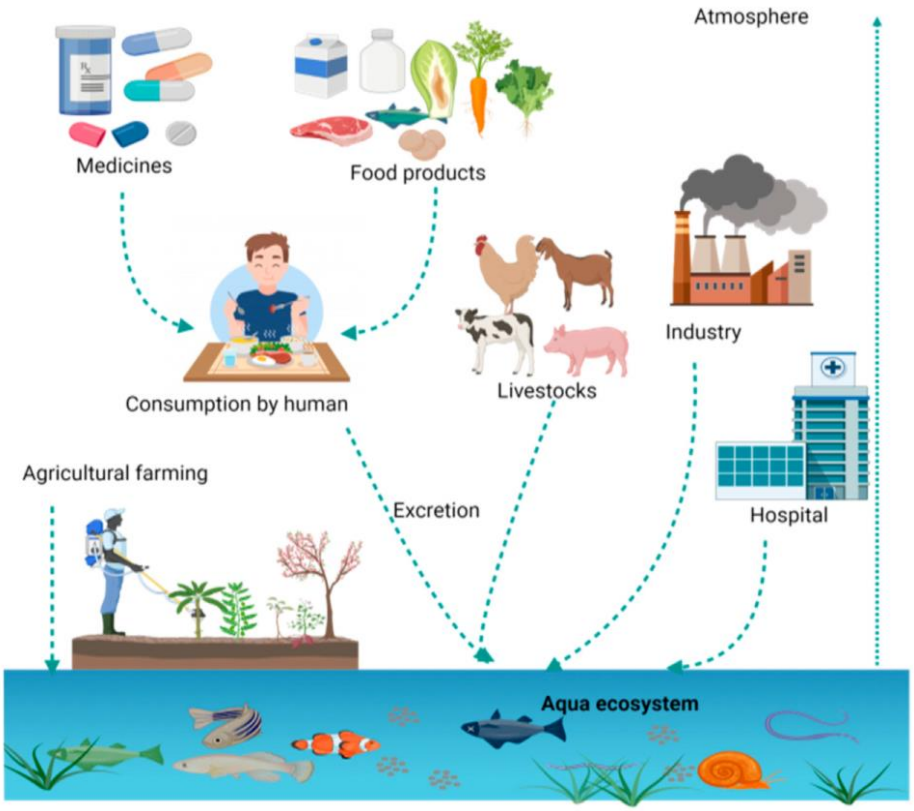

Figure 3. Anthropomorphic Sources for Antibiotics

Antibiotics are able to enter and diffuse through the environment through direct and indirect pathways as shown (Dawadi, 2021).

Mechanisms of Antibiotics and Antibiotic Resistance

The bactericidal (bacteria-killing) and bacteriostatic (bacteria-growth prevention) properties of antibiotics are enabled through the following mechanisms: ability to inhibit cell wall synthesis of bacteria, disrupting the cell membrane of bacteria, inhibiting protein synthesis of bacteria, inhibiting nucleic acid synthesis of bacteria, or metabolic pathways of bacteria (Reygaert, 2018). Inhibiting the cell wall, specifically the peptidoglycan layer, is lethal to bacteria because this layer is necessary for structural integrity of the cell wall. Antibiotics such as beta-lactams and glycopeptides interfere with peptidoglycan precursors which results in a damaged bacterial cell wall. Damage to the cell wall activates the cell death pathway and the bacteria is lysed (LibreTexts, 2021). Bacteria cell membrane disruption typically occurs through depolarizing the cell membrane in Gram-positive bacteria with the antibiotic group lipopeptides (Reygaert, 2018). Membrane disruption is deadly to bacteria cells because it results in the release

of intracellular ions and cell death. Protein synthesis inhibition is lethal to bacteria cells because proteins necessary to life are not able to be created. Inhibition happens by antibiotics such as tetracyclines and aminoglycosides binding to various sites in the ribosome, which prevents the binding of tRNAs (LibreTexts, 2021). Inhibition of nucleic acid is typically achieved through Quinolone antibiotics. Quinolones are able to inhibit topoisomerase, an enzyme necessary to DNA replication. Without topoisomerase, bacterial mRNA and DNA cannot be synthesized . The last way bacteria are prevented from proliferating is through inhibiting metabolic pathways. Antibiotics belonging to the sulfonamide group prevent folic acid production, which is needed to synthesize nucleic acids for bacterial DNA replication (DrugBANK, n.d.).

Although the above-described antibiotic mechanisms are effective against sensitive bacteria, they are not effective if the bacteria is resistant to the antibiotic. As previously described, bacteria can have natural resistance as well as acquired resistance to antibiotics. Antibiotics act as a selective pressure to a bacteria population, as only those that have resistance and the ability to reproduce in the presence of antibiotics will survive. Those survivors will pass on their resistance to their offspring, allowing for antibiotic resistance to propagate (Davies & Davies, 2010, 417-433).

There are four primary mechanisms that bacteria have developed to neutralize or evade the lethal effects of antibiotics: limiting drug uptake, modification of an antibiotic target, inactivation of an antibiotic, and pumps to efflux antibiotics (Figure 4). Limiting drug uptake describes a broad mechanism through which bacteria are able to prevent an antibiotic from permeating its membranes. Differences in hydrophobicity between bacterial membranes and antibiotics, the presence of a cell wall and porins, as well as the ability to produce a biofilm are all techniques to limit uptake of an antibiotic. Drug target modification reduces the effectiveness of antibiotics as their original target within the bacteria may be altered or removed. Bacteria are known to modify the structures topoisomerase and ribosomes, which prevents the antibiotic from recognizing its target and preventing bacteria reproduction. Drug inactivation prevents the antibiotic from affecting the bacteria and is made possible through either degrading the antibiotic or transferring a chemical group, such as an acetyl or phosphoryl group, to the antibiotic. The last mechanism of resistance is the presence of efflux pumps, which are able to transport antibiotics out of the cell. Pumps are not antibiotic-structure specific, so some pumps are able to

remove antibiotics that are structurally diverse. These types of pumps can confer multidrug resistance since antibiotics are structurally different

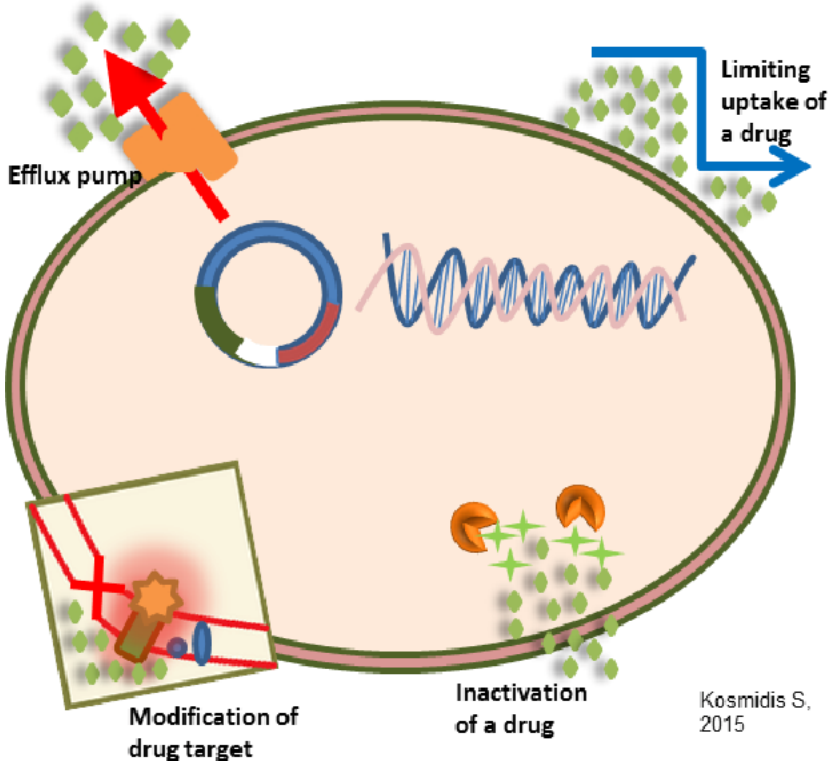

Figure 4: Antibiotic Resistance Mechanisms

Bacteria are able to remove antibiotics from the inside of the cell, inactivate the antibiotic, modify the drug target, or limit uptake of the drug. Each of these four mechanisms reduce or completely eliminate the bactericidal or bacteriostatic effects of the antibiotic (Dawadi, 2021).

Mitigating Antibiotic Resistance and its Challenges

The lack of new antibiotics and the inevitable cycle of development, clinical use, and resistance means that the best tools for delaying resistance are preventing bacterial infections and good stewardship of antibiotics. The CDC recommends preventing bacterial infections through immunizations, preparing food safely, and handwashing (Centers for Disease Control and Prevention, 2013). But, these preventative measures may not be preventive in regions where sanitation is lacking. For example, the 2009 study described above detected antibiotics in drinking water, which is presumably the same water an individual would wash their hands with. There is a chance that if someone had a wound and washed it with this water, they could induce resistance in bacteria already in the wound.

Prevention of AR should also include international standards and data collection, since AR is a global issue. Data collection on the presence of AR is not uniform or equal. Some-high income countries have tracked antibiotic usage, whereas historical data from low-income countries is scarce. Antibiotic sales and usage in both humans and animals for a given country is not always available. This gap in available data presents challenges for researchers to understand the geographic and temporal spread of AR. Antibiotic resistance is even harder to monitor because there is not an internationally accepted definition of "resistance" (Kirchhelle, 2020). Resistance in general describes a lower susceptibility to antibiotics, but what that lower threshold is in mg/uL or another unit is not defined for all countries. If there was a quantity that defined resistance, researchers would be able to identify and agree if a location had resistance.

In situations where preventative measures are not possible or appropriate, antibiotics should be used under the guidelines of antibiotic stewardship programs (ASPs). ASPs are typically implemented in hospitals as "superbugs" -life-threatening microorganisms that are resistant to commonly used antibiotics-are prone to spread in a hospital setting (National Institute of Health, 2014). ASPs broadly work to reduce unnecessary antibiotic use and protect patients from ARB (Center for Disease Control and Prevention, n.d.). However, there is not a singular recommended ASP, so antibiotic use guidelines are not consistent across all healthcare facilities.

In the future, advancements in biotechnology and genetic engineering may allow for the development of antibiotic alternatives. Bacteriophages, CRISPR therapy, and lysins are proposed treatments to work with or replace antibiotics. The three aforementioned treatments have promise, but require additional research in order to overcome cost and toxicity concerns in patients (Ghosh, 2019, 323-338). While alternate treatments are being explored, the abilities of antibiotics must be conserved.

Inducible Antibiotic Resistance

The need to identify new antibiotics has led researchers to discover non-antibiotic molecules, called metabolites, produced by bacteria (Perry et al., 2021, 129-142). At the time of discovery these molecules were thought to be waste products, so resources were not dedicated to characterizing them. However, the need to understand AR has caused researchers to revisit metabolites. Surprisingly, it was found that metabolites are more than waste products as they have roles in the bacteria's metabolic pathway and cell to cell signaling, among other roles

(Perry et al., 2021, 129-142). Recently, researchers at Tufts University found that the metabolite 2,3-dihydroxybenzoate (DHB) is capable of inducing expression of the AcrAB-TolC efflux pump by interacting with the pump's protein regulators. The AcrAB-TolC pump is assembled from the AcrA, AcrB, and TolC proteins (M. Rai & K. Kon (Eds.) 2016, 19-35). Its function in *E. coli* is to expel molecules toxic to the cell, such as antibiotics. Some antibiotics known to be expelled from the cell by this pump include tetracycline, carbenicillin, and ciprofloxacin. If the pump were to stop performing its function, DHB would accumulate and increase expression of AcrA and/or increase expression of its transcriptional regulators, *soxS* and *marA* (Figure 5).

Metabolites such as DHB are thought to be expelled from the cell by the acrAB pump when they accumulate in the cell (Ruiz & Levy, 2014, 390-399). This change in the concentration of DHB causes the up-regulation of *acrAB* to replace the nonfunctional AcrA and return the cell to homeostasis. As discussed in the above section (Figure 4), efflux pumps are a mechanism of antibiotic resistance as they remove antibiotics from the cell. The ability of *E.coli* to regulate the expression of its own efflux pumps through a metabolite (whether produced by the cell or present in its environment) implies that *E. coli* can induce antibiotic resistance. *E.coli* is capable of producing its own DHB in an iron deficit environment (Page, 2019, 529-537). It is also able to uptake DHB from the environment. Exposure to DHB is possible as it is a product of human aspirin metabolism (Grootveld & Halliwell, 1988, 271-280). Additionally, human waste containing the byproducts of aspirin could be released into the environment and induce antibiotic resistance in naturally occurring bacteria. DHB is not a well-studied metabolite and our experiment seeks to identify which antibiotic(s) DHB induces resistance to, as that information is not available.

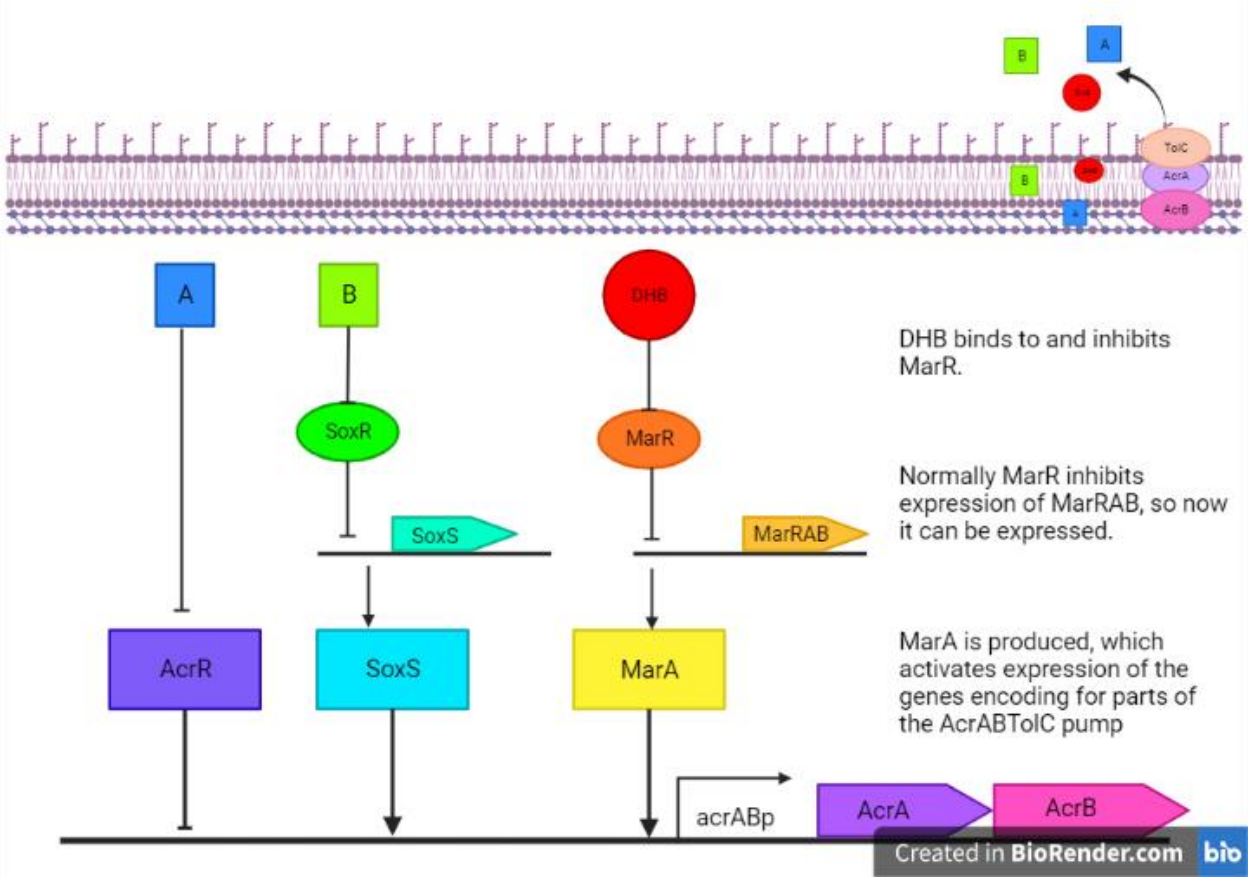

Figure 5: Proposed DHB Pathway in *E.Coli* and Interactions

DHB is thought to directly bind to MarR, a repressor of the marRAB operon. By binding to the repressor, DHB enables MarA expression. The MarA gene gives *E. coli* resistance to structurally different antibiotics (Ruiz & Levy, 2014, 390-399).

Methods

Soil Collection

The Worcester area was first evaluated for the types of sites which tend to have an increased presence of antibiotic resistance, including agricultural establishments, public bodies of water, and current and former industrial sites. A total of fourteen properties were selected for outreach to owners or managers in order to express interest in sampling from these properties. A survey was created to gain additional background information about the properties, intended to be sent to those who would allow soil collection from their property. The survey questions are available electronically here or in Appendix 1. Of these properties, only Cordelia's Farm responded affirmatively, so other samples were taken from the local bodies of water Indian Lake, Coes Reservoir, and Bell Pond, as well as from the former industrial site Gateway Park for a total of five properties.

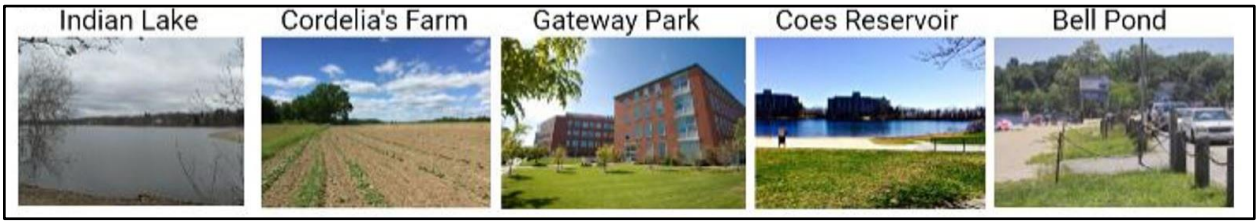

Figure 6. Soil Sample Sites. Soils samples were taken from the banks of three bodies of water, one former industrial site, and a family farm.

Two half-cup soil samples were taken from three digging sites on each property, resulting in a total of 15 digging sites from five properties and 3 cups of soil per property. Soil from each digging site was kept in separate gallon-Ziploc bags for storage. For each selected digging site, soil was taken about 15 cm (6.0 inches) from the surface level from spots within a five foot radius of an initially designated point to try to gain a sample as representative of the microbiome and chemical contents of this area as possible. These sites were chosen in order to attempt to represent soils with different characteristics from each location (e.g. found at the base of a tree, directly next to the water's edge, from fields with different crops, etc). See Appendix 2.1 for further information on soil collection.

Bacterial Culturing

In order to establish a baseline of the quantity and qualities of bacteria present in the soil, 1.0 g of soil from each property was diluted in 10 mL of sterile water, from which five more serial dilutions were created ($\frac{1}{10^2}, \frac{1}{10^3}, \frac{1}{10^4}, \frac{1}{10^5}, \frac{1}{10^6}$). 100 µL of each dilution was plated on LB growth medium agar plates with no antibiotic and spread across the plate by using sterile glass beads. The plates were incubated at 37°C and observed after two days of growth. It was determined that the $\frac{1}{10^3}$ dilution grew the optimal number of colonies, between 30 and 300 on a plate, which allows for enough colonies to grow without overcrowding.

Antibiotic Screening

100 µL of each of the $\frac{1}{10^1}, \frac{1}{10^2}$, and $\frac{1}{10^3}$ dilutions were plated on LB agar plates with 16 µg/mL ampicillin as a general screening process to eliminate bacteria that were not resistant to any antibiotics. 32 µg/mL is the threshold as defined by Clinical and Laboratory Standards Institute (CLSI) for which bacteria that grow in this concentration of ampicillin are considered resistant (Kidsley, 2018). The growth on these plates was also used to determine if any of these dilutions would yield a bacterial growth distribution suitable for harvesting single colonies. A suitable distribution sample has approximately 30 to 300 colonies. When plates exceeded 300 colonies after incubating for two days, the same dilutions were plated on 32µg/mL ampicillin in 100 µL samples. Additionally, $\frac{1}{10^4}, \frac{1}{10^5}$, and $\frac{1}{10^6}$ dilutions were plated on 32µg/mL plates in 100 µL samples. The same six levels of dilutions were plated on 16µg/mL ampicillin plates in 100 µL samples to determine if there may also be bacteria present that meet the threshold for intermediate antibiotic susceptibility that do not meet the threshold for antibiotic resistance. All plates were incubated for two days before significant growth became visible. The results from these plates showed the optimal plating dilution for our purposes was $\frac{1}{10^3}$ on the 32 µg/mL plates.

Colonies of bacteria from each of the five properties were chosen from the $\frac{1}{10^3}$ dilution, 32 µg/ml Ampicillin plates. Four colonies were selected by dividing the Ampicillin plate into a quadrant and selecting one colony from each quadrant that was not in contact with nearby

colonies. A total of twenty isolates were selected from the five properties. Each isolate was spread on an LB plate with no antibiotic, and the plates were incubated for two days to grow a stock of each isolate for experimental use. Additional samples taken from each stock were used to grow a lawn of each isolate on LB media. Each lawn was divided into equal quarters, marked with a permanent marker on the back of the plate. An antibiotic disc of 30 µg Chloramphenicol, 30 µg Vancomycin, 30 µg Tetracycline, and 5 µg Trimethoprim were placed in the center of a different quadrant of each lawn in order to test the isolate's response to different antibiotic drugs. These four antibiotics were chosen because each antibiotic interrupts a different process of the bacteria, such as cell wall or protein synthesis. Each zone of inhibition was measured from four radii from the antibiotic disc. The average of these four values was calculated, then doubled to obtain an average diameter in millimeters.

DHB Experimentation

Collecting OD_{600} Data

Cultures of lab strain *E. coli* (ATC Strain No. 11775) were grown in four different conditions and their OD_{600} absorbance was monitored over the course of eight hours in order to evaluate differences in rate and extent of growth. The cultures were grown in LB broth medium in the presence of either 0.2 mM or 5.0 mM 2,3-dihydroxybenzoate (DHB) or equal volumes of methanol. Methanol was used as a solvent for the 100x µg/mL DHB stock solution, so two cultures with methanol only were added as negative controls in order to account for any effect the addition of methanol may have had on the growth of the bacteria. Each of the DHB cultures had three technical replicates for a total of six cultures. In total, there were 8 cultures (controls were not grown as replicates). Cultures were grown in 15 mL conical tubes at 37℃ and shaken at 250 rpm. OD_{600} measurement were taken from each culture every two hours in order to track growth. This data showed growth decreasing by the eighth hour, so data was not collected after this time.

Susceptibility and resistance thresholds were derived from the Clinical and Laboratory Standards Institute (Kidsley, 2018). 100 µL of one culture from each set of technical replicates was plated on LB agar with the six antibiotic concentrations listed in Table 1. To ensure that the ATC strain 11775 of *E. coli* was initially susceptible to any concentration of antibiotic, samples

were plated on LB plates containing 50 µg/mL tetracycline, which yielded no growth. These plates were incubated at 37°C for 93 hours. The growth rate was calculated from the OD_{600} measurements using an R package. Resistance was quantified by the number of colony forming units visible on the plate where applicable.

Table 1: Antibiotic Plate Concentrations

	Tetracycline	Carbenicillin	Ciprofloxacin
Sensitive Threshold	4 µg/mL	8 µg/mL	1 µg/mL
Resistant Threshold	16 µg/mL	32 µg/mL	4 µg/mL

E. Coli Screening

Tests were performed on bacterial isolates grown from soil samples in order to select for those with as many characteristics consistent with *E. coli* as possible. The initial screening process for these isolates was to grow soil dilutions ($\frac{1}{10^3}$ serial dilution of 1g soil in 10 mL deionized water) on MacConkey agar plates to select for Gram-negative and lactose-fermenting bacteria. Isolates taken from these plates were then tested for the presence of oxidase with 1% tetra-methyl- p-phenylenediamine dihydrochloride, and catalase with hydrogen peroxide. PCR for *E. coli's* 16S ribosomal subunit was conducted on seven isolates consistent with the characteristics of *E. coli* and gel electrophoresis confirmed its presence. The primer used was 1492R (5'-TAC GGG TAC CTT GTT ACG ACT T-3') and 27F (5'-AGA GTT TGA TYM TGG CTC AG-3', 5'-AGA ATT TGA TCT TGG TTC AG-3', 5'-AGA GTT TGA TCC TGG CTT AG-3',5'-AGG GTT CGA TTC TGG CTC AG-3'). Cycling conditions are described in Appendix 2.2. These samples were then sent to Quintara Biosciences in Cambridge, MA for sequencing. Unfortunately, results were not received in time to record in this report.

Results

This project followed two main lines of experimentation: one identified the types of antibiotic resistance in Worcester, MA and another determined if it is possible to induce antibiotic resistance in *E. coli* through a bacterial metabolite. It was hypothesized that antibiotic resistance will exist in bacteria cultured from water samples, industrial sites, and public parks and that DHB will induce resistance in *E.coli* to tetracycline, carbenicillin, and ciprofloxacin with a fitness cost to the bacteria. In this section, the results will be divided into these two respective sets of experiments.

Multi-Drug Resistant Isolates are Present in Worcester, MA

In order to understand the types of resistance present in bacteria from the five collection sites (Bell Pond, Coes Reservoir, Cordelia's Farm, Gateway Park, and Indian Lake), four isolates were chosen from each site as described (see Methodology). . Three of these sites, Bell Pond, Coes Reservoir, and Indian Lake, contained bodies of water, while Gateway Park is a formal industrial site and present-day home of a WPI research building, and Cordelia's Farm is a family farm that grows fruits and vegetables.

A Kirby-Bauer disc diffusion assay was performed to identify isolates that were susceptible, intermediately resistant ("intermediate"), and resistant to Chloramphenicol, Vancomycin, Tetracycline, and Trimethoprim based on their zone of inhibition (ZOI) as described in Table 2. These four antibiotics were chosen because they each target a different necessary structure or process of the bacteria (Table 3).

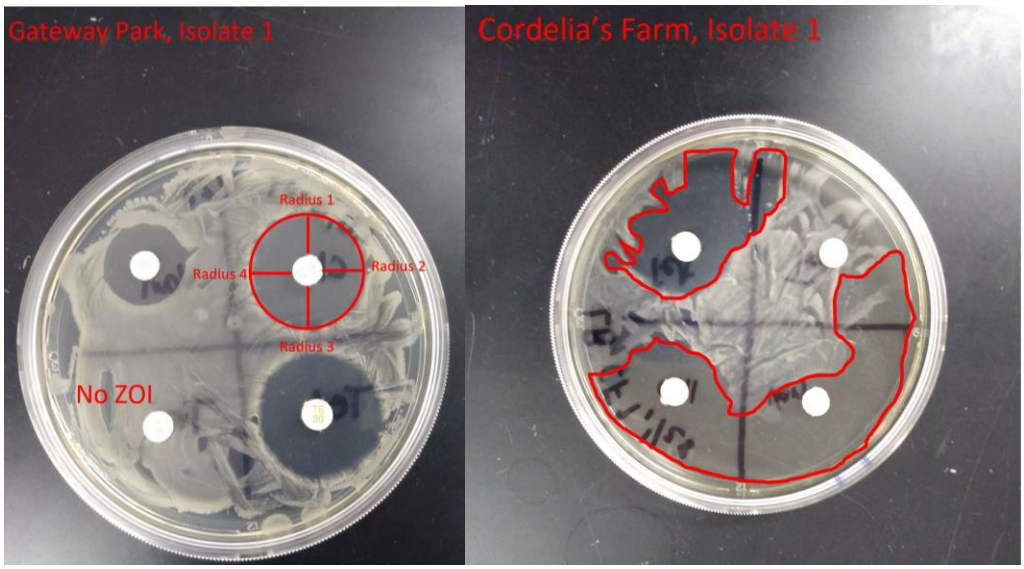

Figure 7. **Expected and Irregular Example Zone of Inhibitions.**

An expected ZOI has a circular, empty circle surrounding the white antibiotic disc (Figure 7). The ZOI was obtained by measuring four radii from the antibiotic disc, averaging their values together, and doubling the average radii to obtain an average diameter in millimeters. The antibiotic discs, beginning in the top-left quadrant and moving clockwise, are as follows: Vancomycin, Chloramphenicol, Tetracycline, and Trimethoprim. It was not possible to perform this calculation on an irregular ZOI (Right). Irregular ZOIs are recorded as "NA" in subsequent tables.

Table 2: Zone of Inhibition Breakpoints

	Chloramphenicol (mm)	Vancomycin (mm)	Tetracycline (mm)	Trimethoprim (mm)
Resistant Diameter	≤12	≤9	≤14	No threshold
Intermediate Diameter	13-17	10-11	15-18	No threshold
Susceptible Diameter	≥18	≥12	≥19	No threshold

Table 3: Mechanism of Action of Selected Antibiotics from Kirby-Bauer Disc Diffusion

	Chloramphenicol	Vancomycin	Tetracycline	Trimethoprim
Bacterial Target	Inhibits the 50S Ribosomal subunit	Inhibits the bacterial cell wall from forming	Inhibits the 30S Ribosomal subunit	Inhibits an enzyme necessary for bacterial DNA synthesis

Site Summary

For all five sites, all isolates yielding valid results (17) displayed susceptibility to trimethoprim. The remaining isolates had no ZOI around the trimethoprim antibiotic discs, indicating that all bacterial isolates displayed resistance to trimethoprim. While the levels of susceptibility for other antibiotics tested vary, 'susceptible' is the least common (Figure 8, 9). There is a comparable distribution of levels of resistance to vancomycin and chloramphenicol, but there appears to be substantially less full resistance to tetracycline. Most isolates displayed intermediate resistance to tetracycline and there were more isolates displaying susceptibility to tetracycline than to any other antibiotic, though there was only one more instance of susceptibility here than with other antibiotics.

As shown in Figure 8, isolates from Bell Pond and Indian Lake soil samples both displayed some level of resistance to all four antibiotics. Isolates from Coes Reservoir and Gateway Park had varying levels of resistance, including susceptibility to antibiotics. Cordelia's Farm sampled isolates also had different levels of resistance, but only two samples had distinct ZOI's.

	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
BP1	R	I	I	R
BP2	I	I	R	R
BP3	I	R	I	R
BP4	R	R	R	R
IL1	I	R	R	R
IL2	I	R	R	R
IL3	I	R	R	R
IL4	N/A	N/A	N/A	N/A
CF1	N/A	N/A	N/A	N/A
CF2	I	R	I	R
CF3	N/A	N/A	N/A	N/A
CF4	S	S	I	R
CR1	R	S	I	R
CR2	S	R	S	R
CR3	S	S	R	R
CR4	I	S	R	R
GW1	I	R	I	R
GW2	S	I	S	R
GW3	S	R	S	R
GW4	I	I	S	R

Legend	
R	Resistant
I	Intermediate
S	Susceptible

Figure 8. Summary of Antibiotic Resistance of Bacterial Isolates. Level of resistance against different antibiotics (tetracycline, vancomycin, chloramphenicol, and trimethoprim) is displayed by each bacterial isolate. Our sites are abbreviated as Bell Pond (BP), Indian Lake (IL), Cordelia's Farm (CF), Coes Reservoir (CR) and Gateway Park (GP). Possible levels of resistance include: R (resistant), I (intermediate resistance), and S (susceptible). Isolates with the status "N/A" Indicates there was not a distinct ZOI from which an accurate resistance level could be determined.

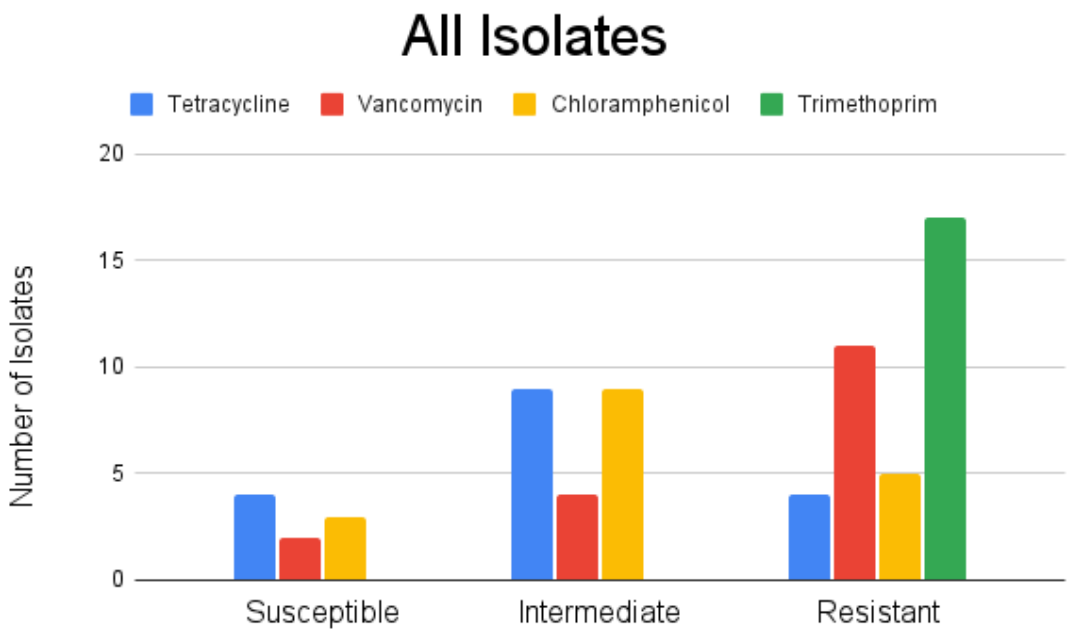

Figure 9. Distribution of Antibiotic Resistance Severity Found Across All Isolates

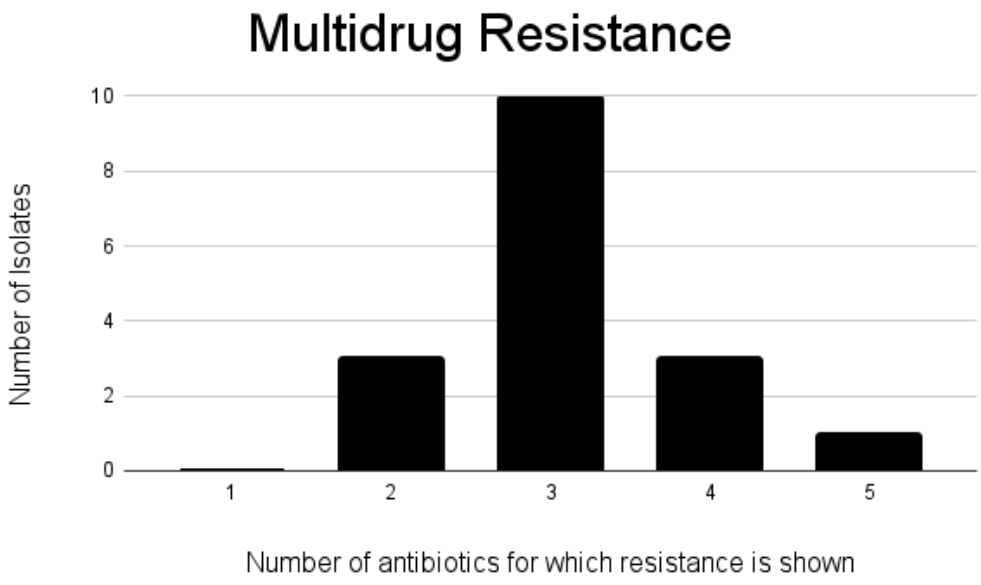

Figure 10. **All isolates showed multidrug resistance**

Data shown include ampicillin resistance used to screen isolates from soil samples.

When the ampicillin resistance used to screen bacteria from soil samples was accounted for, all bacterial isolates showed multidrug resistance (Figure 10). Three isolates showed only resistance to ampicillin and trimethoprim, while all others displayed resistance to at least one other antibiotic. These results speak to the potential harm these bacteria could cause if they were to become pathogenic, or if they were to transfer the genes for these resistance mechanisms to other, more dangerous bacteria.

Potential Induction of Resistance in *E. coli* by DHB

In order to determine whether the bacterial metabolite DHB can induce antibiotic resistance in *E.coli,* the bacteria were grown in four different conditions and plated on three types of antibiotic plates. A spectrophotometer was used to collect OD_{600} data every two hours as a metric for bacterial density. An R program, "growthcurver", was used to calculate the growth rate and predict the growth curves of the experiment.

Preliminary Results Suggest That *E. coli* Colonies Grown on Antibiotic Plates Yielded Assorted Results

Samples of *E. coli* cultures at various time points following DHB exposure were plated on plates with various concentrations of antibiotics. The 4 µg/mL (low level) tetracycline plates yielded the expected result - an increasing number of resistant colonies for each progressive time interval (Fig. 11). This result was expected, as DHB interacts with transcriptional regulators of the AcrAB-TolC pump to increase antibiotic resistance. The decrease in colonies at Hour 8 could mark the *E. coli* entering the stationary or death phase. However, the initial number of colonies was not zero. *E. coli* exposed to 5.0 mM DHB at Time 0 yielded five colonies, and *E. coli* exposed to 0.2 mM DHB and 0.0 mM DHB at Time 0 each yielded one colony. The colonies at Time 0 could indicate contamination of those plates.

The five other antibiotic concentrations tested were 16ug /mL Tetracycline, 8 µg/mL and 32 µg/mL Carbenicillin, and 1 µg/mL and 4 µg/mL Ciprofloxacin. Cultures grown on the 1 µg/mL ciprofloxacin plates yielded between 0-4 colonies for each time interval for each experiment, with no discernible increasing trend (Appendix 4). No bacterial colonies were present on the 4 µg/mL ciprofloxacin plates for any time interval. The 8 µg/mL and 32 µg/mL

carbenicillin plates presented the most difficult to distinguish colonies, as for any time period and DHB concentration, there were many small bacterial colonies, and some of the plates were covered by a film (Appendix 5). In order to attempt to find an explanation for this unexpected result, the genome of our strain of *E. coli* was analyzed by a database that matches sequences of a bacteria of interest with known resistance genes. The 16 µg/mL tetracycline plates did not indicate possible induced antibiotic resistance, as no colonies grew on the plates between Hour 2 and 8. However, at Hour 0, nine colonies were present on the 0.0 mM plate and one colony was present on the 5.0 mM plate. The colonies at Time 0 and the lack of colonies on subsequent plates could indicate contamination of the Time 0 plates.

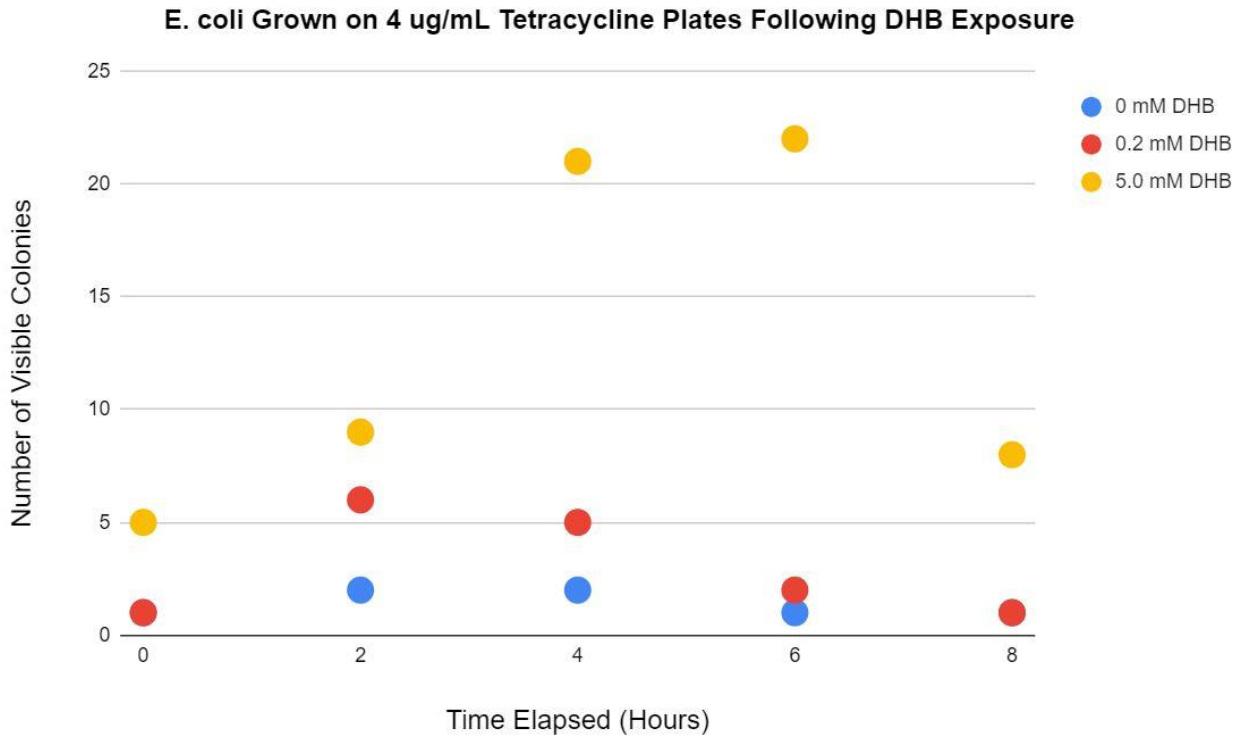

Figure 11: *E. coli* Grown on Low-Level Tetracycline Plates following DHB Exposure

Samples of *E. coli* cultures at various time points following DHB exposure were grown on 4 µg/mL of tetracycline to determine susceptibility to a low-level threshold. Plates were grown for 93 hours and visible colonies counted.

Non-Significant Difference Between Growth Rate of Experimental Conditions

In order to understand if DHB affected the growth of *E. coli*, the growth rate was calculated from the R program "growthcurver" using OD_{600} data collected every two hours (see Methodology). The growth rate quantifies the reproduction of a population of *E. coli* (Britannica, n.d.). It was expected that bacteria exposed to 5.0 mM DHB would experience the slowest growth rate and the control would experience the fastest growth rate due to the induction of antibiotic resistance. To test this hypothesis, *E. coli* were grown in four different conditions (Figure 12).

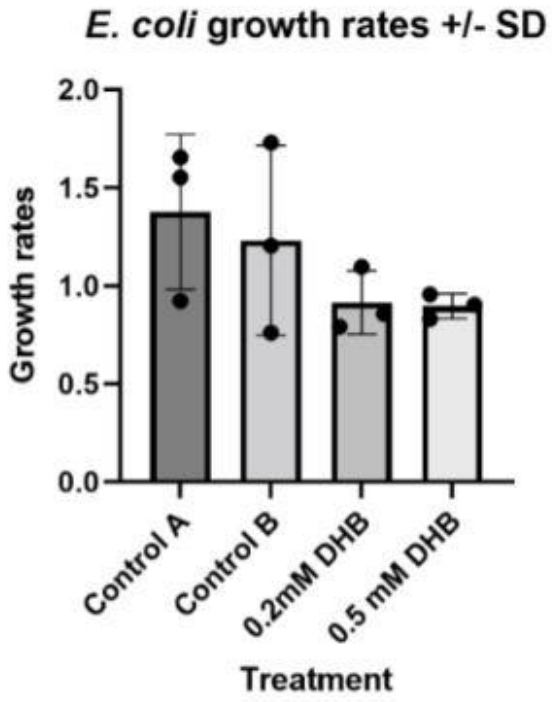

Figure 12. Growth Rate of *E. coli* exposed to DHB

The growth rate of *E. coli* grown in the control and experimental conditions was calculated from the R package, growthcurver. Control A corresponds to the 0.2 mM DHB condition and Control B corresponds to the 5.0 mM DHB.

There was not a significant difference in the means of the growth rates observed between the four conditions (Figure 13). It is possible that this experiment could have benefitted from more trials, as there was large variation in the experimental data, especially for the controls.

Non-Significant Difference Between Growth Curves of Experimental Conditions

E. coli (ATC Strain 11775) were grown in two different concentrations of DHB, 0.2 mM and 5.0 mM. 0.2 mM was chosen as this is the value reported in the literature that salicylates (a type of NSAID) was detected in human plasma (Creamer, 2017). 5.0 mM was chosen from past bacterial research (Wang, 2019, 563-571). Because induction of antibiotic resistance requires bacteria to transcribe and translate resistance genes, we hypothesized that resistance would be linked to a reduction in growth rate of the bacteria, with the highest fitness cost observed in *E. coli* grown in 5.0 mM DHB. Existing literature exploring the fitness cost of inducing antibiotic resistance through salicylate exposure confirms this trend of decreasing growth with increasing metabolite concentration (Wang, 2019, 563-571).

An R package, "growthcurver" fits experimental OD_{600} values to an accepted logistic equation used in ecology and evolution to determine population size. This is important as growthcurver allowed our MQP group to compare parameters, such as growth rate, more easily between different experiments. Given experimental OD_{600} values, the best values for carrying capacity, growth rate and initial population size are found to determine expected population size at any time (Sprouffske & Wagner, 2016).

Figure 13 shows a growth curve of *E. coli* over an eight hour period. OD_{600} measurements were used as a proxy for bacterial growth and taken every two hours for eight hours as described in Wang, 2019. Data was not collected after eight hours as *E. coli* transitioned into the stationary phase. Experimental values are represented as a dotted line with circular data points and the predicted values are a smooth line.

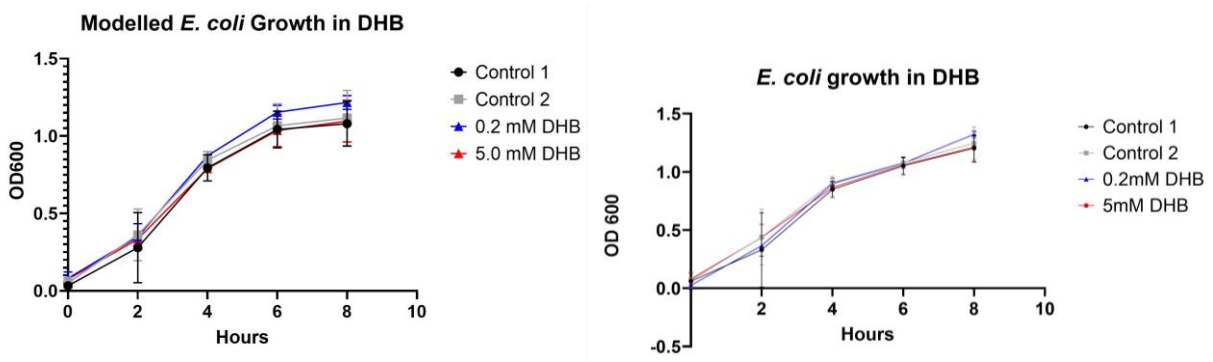

Figure 13. Growth of *E. coli* Exposed to DHB

E. coli were grown in LB broth and two concentrations of DHB for eight hours. Methanol was used as the negative control.

It was expected that the growth rate of both controls would increase at a higher rate than those of the experimental data due to the fitness cost of maintaining resistance mechanisms. Our sampled data did not confirm the hypothesis as there was not a consistent trend of decreasing OD_{600} values for increasing DHB concentration. The predicted 0.2 mM and 5.0 mM DHB data (Figure 14) also diverge from their respective experimental growth curves (Figure 14); however, given the variability of the data, these small differences do not appear significant.

At Hour 0, it is expected the experimental values should be similar as the DHB and negative control was inoculated with a similar amount of bacteria. However, the growth curves were expected to diverge as soon as one hour elapsed (Wang, 2019).

Discussion

In this section, the discussion will be divided into our two respective lines of inquiry.

Multidrug Resistance Testing

Of the sampled isolates that were resistant and produced valid results, a high proportion of isolates were resistant to at least two drugs, but the majority of isolates were fully resistant to at least three different antibiotic drugs. Resistance alone does not make a strain of bacteria an immediate threat to human health, but if those bacteria become capable of infecting humans or transferring the genes for this resistance to more pathogenic bacteria, they can be very dangerous.

Bell Pond

Bell Pond, formerly Bladder Pond, was established in 1845 as a water reservoir, making it Worcester's oldest water reservoir (City of Worcester, n.d.). Bell Pond is considered one of Worcester's cleanest water supplies, but the accumulation of trash has led to a dirty appearance (Moulton, 2018). All isolates retrieved from Bell Pond were either intermediately resistant or resistant to Chloramphenicol, Vancomycin, Tetracycline, and Trimethoprim. The dearth of susceptible bacteria and the presence of resistance to bacteria that target biological processes of the bacteria indicate that Bell Pond has been exposed to different antibiotics in the past.

Indian Lake

Indian Lake is a lake in northwest Worcester, previously known as North Pond. In the 19th century it was a part of a system of small bodies of water used to control the flow of the Blackstone Canal, however this canal had a short duration due to its incompatibility with other public projects in the vicinity (City of Worcester, n.d.). Indian Lake has experienced some trouble with its bacteria levels in the past. The 2019 Recreational Water Quality Report for Bell Pond, Lake Quinsigamond, Coes Reservoir, and Indian Lake states that Indian Lake has not been subject to 'chronic' beach closures due to unacceptable levels of *E. coli*. However, it also discloses that there were four separate days during the summer of 2019 during which the

acceptable level was exceeded and the beach had to close, as well as four additional consecutive days of closure after the last instance in order to allow the 5-day average to return to an acceptable value (City of Worcester, 2019).

While these instances may not have led to any serious immediate consequences for human health, the fact that there are relatively high concentrations of bacteria in the area may increase the likelihood of those bacteria developing antibiotic resistance. Many antibiotics are developed from secondary metabolites bacteria produce when trying to survive in areas with high bacterial population density. They provide an advantage by killing competing bacteria and allowing the secreting colony to take additional resources that would have otherwise been used by the competing colony (Verran's, n.d.). This can then cause some bacteria to evolve resistance to these naturally produced antibiotics, which will often prove effective against man made antibiotics due to molecular similarities. A high population density of bacteria may also contribute to the prevalence of antibiotic resistance if the resistance genes in the population are capable of being transmitted by horizontal gene transfer.

Implications of Observed Antibiotic Resistance

Our sampled isolates showed resistance to Trimethoprim in all valid isolates (Figure 8). To validate this result, a bacteria with known susceptibility to Trimethoprim should be tested using the Trimehtoprim disc to confirm its potency. The abundance of Trimethoprim resistance across different sites may indicate that this antibiotic is already present in the environment and can persist. Trimethoprim has been identified as an antibiotic that does not degrade per standard biodegradation tests and degrades “slowly” in the presence of natural solar light, indicating that trimethoprim is capable of persisting in the natural environment (Straub, 2013, 115-162).

Further, existing literature reveals that Trimethoprim has treated human urinary tract infections (UTIs) caused by *E.coli* since 1962 (World Health Organization, n.d.).
The abundance of UTIs, up to 150 million diagnoses globally a year, and the clinical recommendation to prescribe trimethoprim is thought to have contributed to the prevalence of trimethoprim resistance in samples (American Urological Association, n.d.). For example, 18.6% of isolates from one study of 123,691 *E.coli* cultures obtained from human UTI infections were found to be resistant to Trimethoprim (Sahn, 2001, 1402-1406). The duration of Trimethoprim’s

clinical use, the abundance of UTI diagnosis, and its ability to persist in the environment is consistent with the ubiquitous resistance we found.

DHB Experimentation Insufficient to Determine its Effect on *E. coli*

DHB is a bacterial metabolite that is thought to induce antibiotic resistance in *E. coli* (Perry et al., 2021, 129-142). DHB interacts with transcription regulators to increase transcription of the AcrAB-TolC pump, which is able to efflux the structurally different antibiotics tetracycline, chloramphenicol, and ciprofloxacin (Ruiz & Levy, 2014, 390-399). Although antibiotic resistance allows bacteria to persist in environments that would otherwise be lethal, resistance can come at a fitness cost to the bacteria (Yokoyama, 2018). In the presence of antibiotics, the cost to maintain resistance is necessary for survival. In the absence of antibiotics, resistance bacteria are disadvantaged compared to their non-resistant peers. It was hypothesized that the growth rate of the *E.coli* exposed to 5.0 mM DHB would grow slower than either control.

Understanding the relationship between resistant and bacterial metabolites is important as existing research has identified a connection between certain metabolites and resistance (Cohen et al., 1993, 7856-7862). DHB is a product of the human metabolism of aspirin. Aspirin, a type of Non-steroidal anti-inflammatory drug (NSAID) drug, is recommended to treat inflammation, pain, and fevers, which are a symptom of a bacterial infection (Sepsis Alliance, 2022). NSAIDS are one of the most common medications to treat symptoms as a result of bacterial infections (Laudy, 2016). It is possible that concurrent treatment with NSAIDs and antibiotics could adversely affect patient recovery from bacterial infection as limited existing research shows that NSAID byproducts can induce bacterial resistance (Laudy, 2016). The proliferation of antibiotic resistant bacteria and the lack of new antibiotics means that the effects of existing antibiotics must be preserved.

The antibiotic plate experiments showed unexpected and inconsistent results. In order to investigate these results, the Comprehensive Antibiotic Resistance Database, and their Resistance Gene Identifier (RGI) tool was used to investigate known resistance genes in the strain of *E. coli* used in the DHB experiments and to determine whether it could possess genes that confer resistance to carbenicillin, ciprofloxacin, or tetracycline. RGI identified protein-

coding genes that possibly contribute to antibiotic resistance in an organism. If an antibiotic resistance gene is identified, RGI classifies which antibiotic it confers resistance to.

The genome of this specific *E. coli* strain (ATC 11775) was uploaded (GenBank CP033092) to the RGI. For sequence CP033092, there were no genes identified from RGI that may confer resistance to carbenicillin (CARD McMaster, n.d.). It is possible the carbenicillin antibiotic was contaminated or that the antibiotic plates were not made correctly, which would allow bacteria to grow on them.

The initial amount (Time 0) of colonies grown on the low-level tetracycline plates results in some growth. The result was not anticipated, so RGI was used to search for the presence of potential tetracycline resistance genes. The RGI confirmed a perfect match with three known bacterial genes correlated with tetracycline resistance: *evgA, H-NS,* and one of the genes of interest, *acrB* (CARD McMaster, n.d.). It is possible that the low level of tetracycline is able to be overcome by the presence of three above-mentioned genes. This possibility is also validated as for each time interval, the 0.0 mM DHB exposed *E. coli* grew one or two colonies (Appendix 4).

The 1 µg/mL ciprofloxacin plates resulted in inconsistent growth of 0-4 colonies for each time interval, including Time 0. There was no clear trend of the number of colonies increasing as time progressed or DHB concentration increased. RGI was used to search for the presence of potential ciprofloxacin (a type of fluoroquinolone) resistance genes. The RGI confirmed a perfect match with five genes correlated with tetracycline resistance: *gadW, evgA, H-NS, mdtH,* and one of the genes of interest, *acrB.* It is possible that the low level of ciprofloxacin is able to be overcome by the presence of five aforementioned genes. Three of these genes (*evgA, H-NS,* and *acrB)* are broadly responsible for regulating expression of multidrug efflux pumps. The 4 µg/mL ciprofloxacin plates did not produce any colonies, which potentially means that increased resistance was not induced. If this experiment is to be repeated, our MQP group recommends decreasing the ciprofloxacin concentration.

For the growth curve (Figure 14), each time interval did not yield the expected trend of decreasing OD_{600} values with increasing metabolite concentration. Similarly, the growth rates of each experiment did not yield the expected result of increasing growth rate with decreasing DHB concentration. However, our collected data does not definitely disprove the initial hypothesis due as the results of the growth curve are inconsistent with each other and the expected result. This

experiment could have benefitted from many more replicates since a greater pool of data would be more likely to yield consistent, reliable results. Existing metabolite-induced antibiotic resistance literature conducted at least four replicates, while our experiment conducted three (Wang, 2019). Another experiment utilizing the same R package created 937 growth curves from 33 different strains of *E. coli* and measured the OD_{600} values every 10 minutes for 24 hours (Sprouffske & Wagner, 2016). If this procedure is to be repeated, our MQP team recommends conducting it in 96-well plates instead of 15 mL centrifuge tubes as it will be easier to manage many replicates. Our MQP team does not think it is necessary to collect data every 10 minutes for 24 hours, for every trial, but instead conduct initial experiments to estimate when the bacteria enter the stationary phase and adjust the experiment duration from that estimate.

Future Direction and Work

Understanding the mechanisms of antibiotics, how bacteria are able to overcome those mechanisms, and if antibiotic resistance can be unintentionally induced is important as new antibiotics are seldom researched and approved. Therefore, the capabilities of existing antibiotics must be preserved so unnecessary illness and mortality may be avoided. Existing literature has shown that other medications, such as aspirin, can impact a bacteria's susceptibility to antibiotics, but this literature is sparse. Our MQP relied on three primary research articles: Cohen et al., 1993, Perry et al., 2021, Ruiz & Levy, 2014 and Wang, 2019. Our MQP team also had to reach out to the authors of one of these papers for further background information, as the necessary information was not readily accessible.

This project could be expanded in two possible directions. The multidrug resistant isolates could be sequenced to understand if resistance to one antibiotic implies decreased susceptibility or resistance to another different antibiotic. This possibility is worrisome, as if one, or few genes, are able to confer multidrug resistance to different antibiotics, then creating new antibiotics might be in vain. The DHB experiment could be repeated with many more replicates, as described above. Some potential directions for expansion on the achievements of this project are: 1) confirming that DHB can induce resistance in *E. coli* and, 2) Co-culturing DHB-exposed *E. coli* and wild-type *E. coli* to see if wildtype *E. coli* can outcompete its resistant counterparts in the absence of antibiotics.

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Appendix

Appendix 1

Soil Collection Survey Questions

Q0-Signature Thank you for allowing us to collect soil samples from your location. These samples will help us complete our senior research project. Below is an optional questionnaire about your property. You are welcome to answer some, none, or all of these questions. You may also answer “I do not know” or “unsure”. These answers will help contextualize our experiment results by providing background on the area from which the soil was taken. Your property's location data will be anonymized during the research process and not published in our findings. No personally identifiable information will be associated with our report or data. General property details will only be retrieved during the project if needed. Please sign below to confirm you agree to take this survey voluntarily.

Q1 Property Details What type of property do you have?

O Farm

O Industrial Site

O Other _____

Q2 Property Details If your property is an industrial site, can you give us more specific details regarding its current or past industrial use(s)?

O Please type here. _____

Q3 Soil History To your knowledge, are pesticides used on this property, or have they been previously? If so, what type?

O Yes (Please specify, brand name and/or type of pesticide is acceptable)

_____

O No

O Unknown

Q4 Soil History To your knowledge, are fertilizers used on this property, or have they been previously? If so, what type?

O Yes (Please specify, brand name and/or type of fertilizer is acceptable)

_____

O No

O Unknown

Q5 Soil History Have antibiotics been used on this property, or have they been previously (to your knowledge)? If so, what type?

O Yes (Please specify, brand name and/or antibiotic name is acceptable)

_____

O No

O Unknown

Q6 Soil History If you answered "Yes" to the above question, can you describe for what purpose the antibiotics are used and the frequency of administration/application?

_____

Q7 Soil History Do you know if antibiotics have been used for at least the last year?

O Yes-Please describe length of time

_____

O No

O Unknown

Q8 Soil History To your knowledge, are above natural levels of heavy metals, like iron, zinc, or copper, present on your property, or have they been previously?

O Yes (Present now / Present previously)

O No

O Unsure

Q9 Soil History If you answered "Yes" to the above question, what metal(s) is/was present on your property? For how long has this metal been present?

_____

Q10 Farm: What type of farm is your property?

O Large scale/industrial commercial farm

O Intermediate Commercial farm

O Small local commercial farm

O Residential farm

O Other _____

Q11 Farm Do you practice organic farming, per the Environmental Protection Agency's definition? ("...food grown and processed using no synthetic fertilizers or pesticides".)

O Yes

O No

O Unknown

Q12 Farm: Does the farm raise animals or grow crops?

O Animals

O Crops

O Both

Q13 Agriculture If your farm grows crops, what crops are grown on the farm?

_____

Q14 Animals: What animals are reared on the farm?

_____

Q15 Animals: Are the animals used for food? Check all that apply

O Yes, for meat

O Yes, for eggs

O Yes, for dairy products

O None of the above

O Other _____

Q16 Animals Are domesticated animals a frequent visitor to your property? For example: Do you have a dog or a cat?

_____

Q17 Can you provide any historical information about your property? Has the land use changed, for example, from a vegetable farm to an animal farm?

_____

Q18 May we contact you with follow up questions?

O Yes

O No

Q19 Do you have any questions or concerns for us?

_____

Q20 Do you want to be notified of the final results of the study? You may notify us at a later time if your decision changes.

O Yes

O No

Q21 Thank you Thank you for providing us with this information! We believe it will be very helpful to us in identifying patterns in what types of locations are more or less likely to have antibiotic resistant bacteria. If you would like to know anything about the study or would like to contact us for any other reason, we, or our advisor, can be reached at the contact information provided below.

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Rachel McBrine remcbrine@wpi.edu

Appendix 2

2.1 Dirt Collecting Procedure

Safety measures: wear gloves, masks, sanitize instruments between digging areas, keep soil in ziploc bags, transport instruments in a closed bag/container, keep all waste in a tightly closed trash bag.

Materials

- 1 Gallon ziploc bags
- Garden trowel
- Ruler
- 1 c. Measuring cup
- ½ c. measuring cup
- EtOH spray bottle
- Paper towels
- Nitrile/latex gloves
- Disposal bag

Procedure

1. Note in a spreadsheet of where the soil was generally collected from.
2. Choose a region to sample then assign it an area designator in the spreadsheet. Use a permanent marker to label a gallon ziploc bag with the date, location #, and area designator.
3. Measure and indicate with a marker (plastic knife?) a 10 ft diameter of the area from which to dig. Use another marker to indicate the center point of the digging area.
4. Clear off organic matter above the soil as much as possible.

5. Dig a small hole, approximately 4-5 inches in diameter to a 6 inch depth.
6. Mix the removed soil well to try to achieve homogeneity in soil type and contents.
7. Place ½ c. of the mixed soil in the previously labeled ziploc bag. Replace remaining disturbed soil back in its original spot.
8. Repeat 5-11 for the remaining 5 spots.
9. Shake up the bag to achieve as much homogeneity as possible.
10. Sanitize digging implements and ruler with EtOH and paper towels, and switch gloves before moving on to the next area from which to sample. Place disposed gloves and paper towels in a waste container.
11. Repeat 5-14 for all areas of a property from which samples are being taken.

2.2 Polymerase Chain Reaction for Bacteria Identification

Materials:

- Bacteria colonies
- 16S Ribosomal Primers
- dsDNA ladder
- OneTaq (“Taq”)

PROTOCOL

1. Obtain all PCR reagents except enzymes and keep them frozen. Thaw on ice. Label tubes with names of isolates or DNA samples, and if possible, primers used. Place on ice.
2. Determine the volume of each reagent to add to your reaction(s) and calculate amounts for master mix if appropriate.
3. Add each reagent to the master mix starting with water and ending with the DNA polymerase enzyme. Add everything but the template (bacterial cells or cell lysis) to your master mix;check off as added. Put Taq enzyme back in freezer.4
4. Aliquot master mix into individual tubes.
5. Using a micropipette tip, carefully touch the colony on the streak plate. A small dab that collects a small yet visible blob of cells will provide enough DNA template for the reaction.Or add 5 µL of the cell suspension to the mix.

6. Using a micropipette, mix the contents of each tube by gently swishing the solution up and down several times. Cap the tubes. If necessary, gently flick or vortex for a few seconds to attain a more uniform mix.
7. Transfer tubes to the thermal cycler.
8. Select the appropriate program† to start cycling (about 2 hours).
9. Once cycling is complete, remove tubes and keep in ice. Follow your instructor's instructions about storage, and follow up protocols to quality test the PCR products and prepare them for sequencing.

†PCR cycling program:
94℃ for 10 minutes - breaking down cells/ denaturation
94℃ for 30 seconds - denaturation
58℃ for 30 seconds - annealing
72℃ for 1 min 50 sec (1 minute per kb of DNA template) - elongation Cycle 30 times
72℃ for 10 minutes
https://drive.google.com/file/d/1FN04FPZPIgYSGbtPXBAJILXHECmdJBbo/view?usp=sharing

Appendix 3

Classification of Zone of Inhibitions as Resistant (R), Intermediately resistant (I), and Susceptible (S)

Bell Pond Isolate 1	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	13.4	10.8	14.2	0
Resistant, Intermediate , or Susceptible,	R	I	I	R

Bell Pond Isolate 2	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim

Average Diameter of ZOI (mm)	16.1	10.12	12.19	0
Resistant, Intermediate , or Susceptible,	I	I	R	R

Bell Pond Isolate 3	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	15.2	11.6	13.6	0
Resistant, Intermediate , or Susceptible,	I	R	I	R

Bell Pond Isolate 4	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	13.4	8	12	0
Resistant, Intermediate , or Susceptible,	R	R	R	R

Indian Lake Isolate 1	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	16.28	NA	0	NA

Resistant, Intermediate , or Susceptible,	I	R	R	R

Indian Lake Isolate 2	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	17.42	0	7.035	0
Resistant, Intermediate , or Susceptible,	I	R	R	R

Indian Lake Isolate 3	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	18.075	0	0	0
Resistant, Intermediate , or Susceptible,	I	R	R	R

Indian Lake Isolate 4	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	NA	NA	NA	NA
Resistant, Intermediate , or Susceptible,	S	S	S	S

Cordelia's Farm Isolate 1	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	NA	NA	NA	NA
Resistant, Intermediate , or Susceptible,	NA	NA	NA	NA

Cordelia's Farm Isolate 2	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	16.56	8.28	15.18	0
Resistant, Intermediate , or Susceptible,	I	R	I	R

Cordelia's Farm Isolate 3	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	13.135	9.57	15.005	0
Resistant, Intermediate , or Susceptible,	R	R	I	R

Cordelia's Farm Isolate 4	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	21.77333333	16.33	15.87	0
Resistant, Intermediate , or Susceptible,	S	S	I	R

Coes Reservoir Isolate 1	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	14.25	12.75	15.75	0
Resistant, Intermediate , or Susceptible,	R	S	I	R

Coes Reservoir Isolate 2	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	26.1	0	17.1	0
Resistant, Intermediate , or Susceptible,	S	R	S	R

Coes Reservoir Isolate 3	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	NA	0	NA	0
Resistant, Intermediate , or Susceptible,	N/A	N/A	N/A	N/A

Coes Reservoir Isolate 4	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	15.24	11.77	12.415	0
Resistant, Intermediate , or Susceptible,	I	S	R	R

Gateway Park Isolate 1	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	16.33	9.66	15.64	0
Resistant, Intermediate , or Susceptible,	I	R	I	R

Gateway Park Isolate 3	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	27.08	0	18	0
Resistant, Intermediate , or Susceptible,	S	R	S	R

Gateway Park Isolate 2	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	25.2	10.8	17.1	0
Resistant, Intermediate , or Susceptible,	S	I	S	R

Gateway Park Isolate 4	Tetracycline	Vancomycin	Chloramphenicol	Trimethoprim
Average Diameter of ZOI (mm)	17.445	11.45	17.72	0
Resistant, Intermediate , or Susceptible,	I	I	S	R

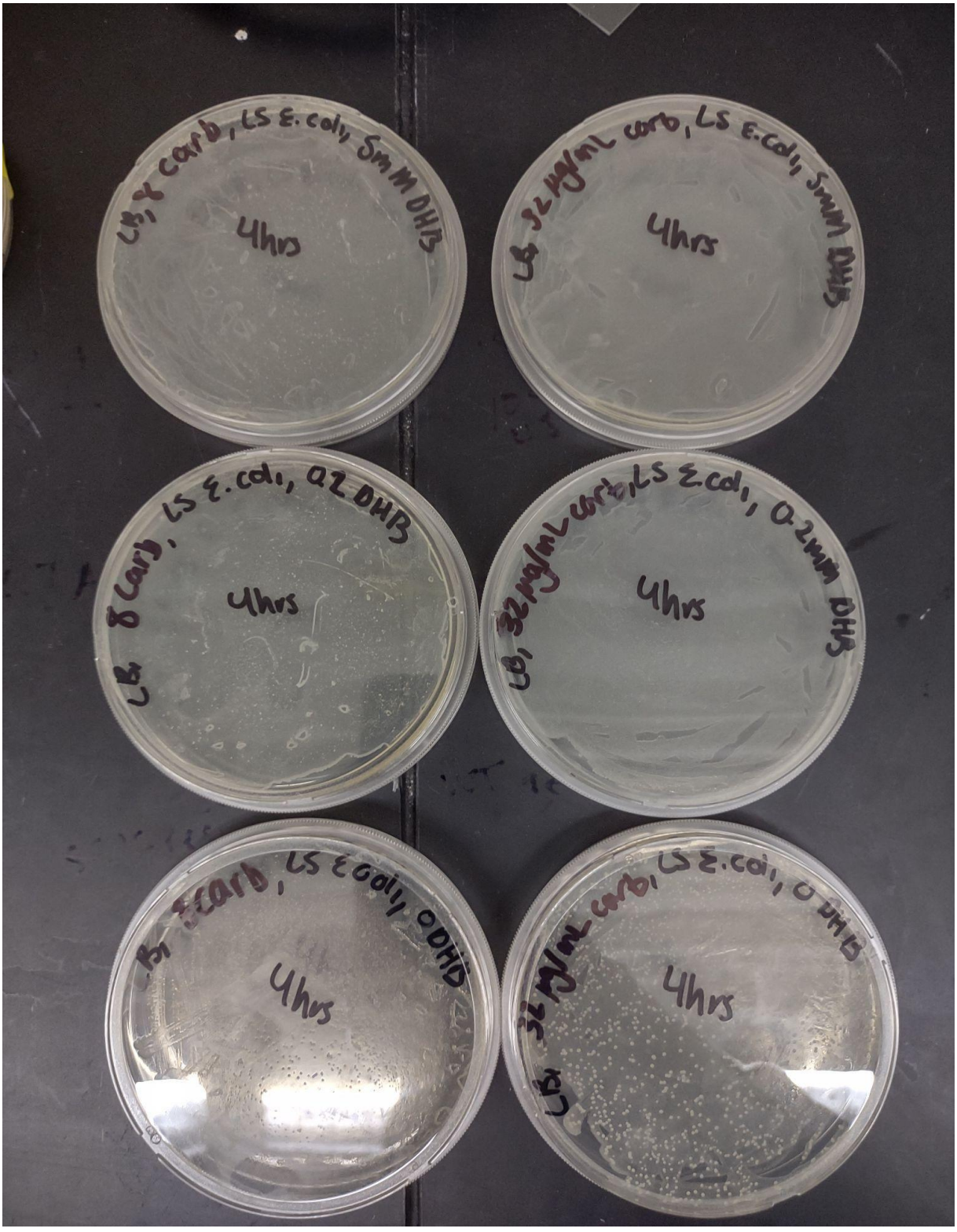
Appendix 4

Number of Visible Colonies Counted on the Antibiotic Plates

Antibiotic Plate			Hour 0	Hour 2	Hour 4	Hour 6	Hour 8
	4 ug/mL Tetracycline						
		0 DHB	1	2	2	1	2
		0.2 mM DHB	1	6	5	2	1
		5.0 mM DHB	5	9	21	22	8
	16 ug/ml Tetracycline						
		0 DHB	9	0	0	0	0
		0.2 mM DHB	0	0	0	0	0
		5.0 mM DHB	1	0	0	0	0
	8 ug/mL Carbenicillin						
		0 DHB	NA	NA	NA	NA	NA
		0.2 mM DHB	NA	NA	NA	NA	NA
		5.0 mM DHB	NA	NA	NA	NA	NA
	32 ug/ml Carbenicillin						
		0 DHB	NA	NA	NA	NA	NA
		0.2 mM DHB	NA	NA	NA	NA	NA
		5.0 mM DHB	NA	NA	NA	NA	NA
	1 ug/ml Ciprofloxacin						
		0 DHB	1	0	1	3	2
		0.2 mM DHB	0	1	3	0	1
		5.0 mM DHB	2	3	3	4	2
	4 ug/ml Ciprofloxacin						
		0 DHB	0	0	0	0	0
		0.2 mM DHB	0	0	0	0	0
		5.0 mM DHB	0	0	0	0	0

Appendix 5:

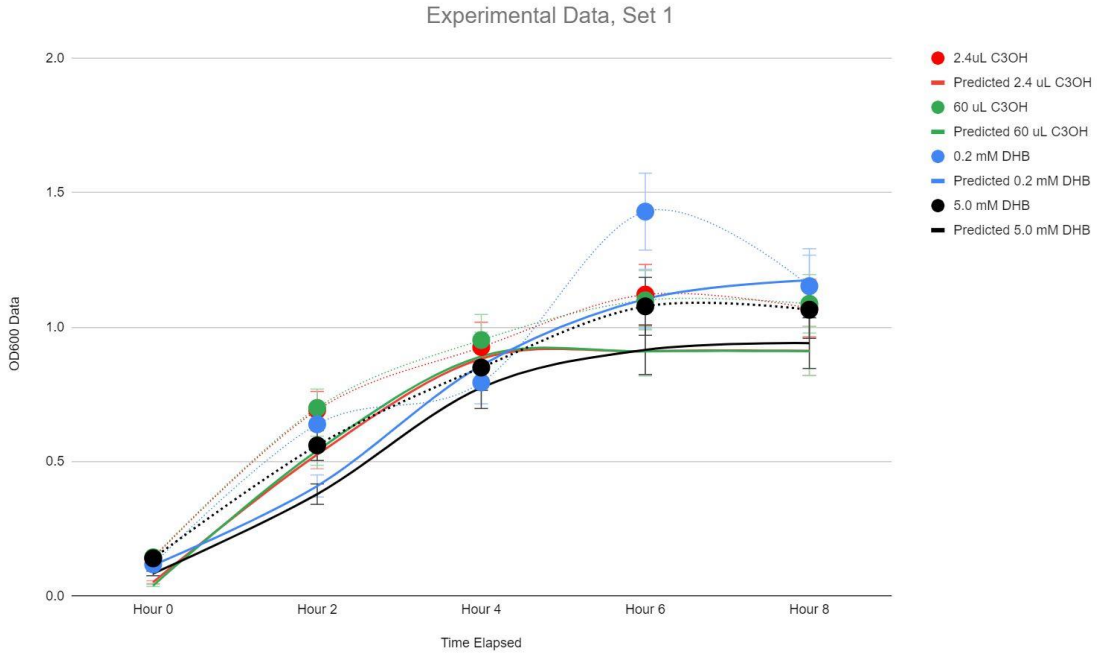
Example Carbenicillin Plate with Film



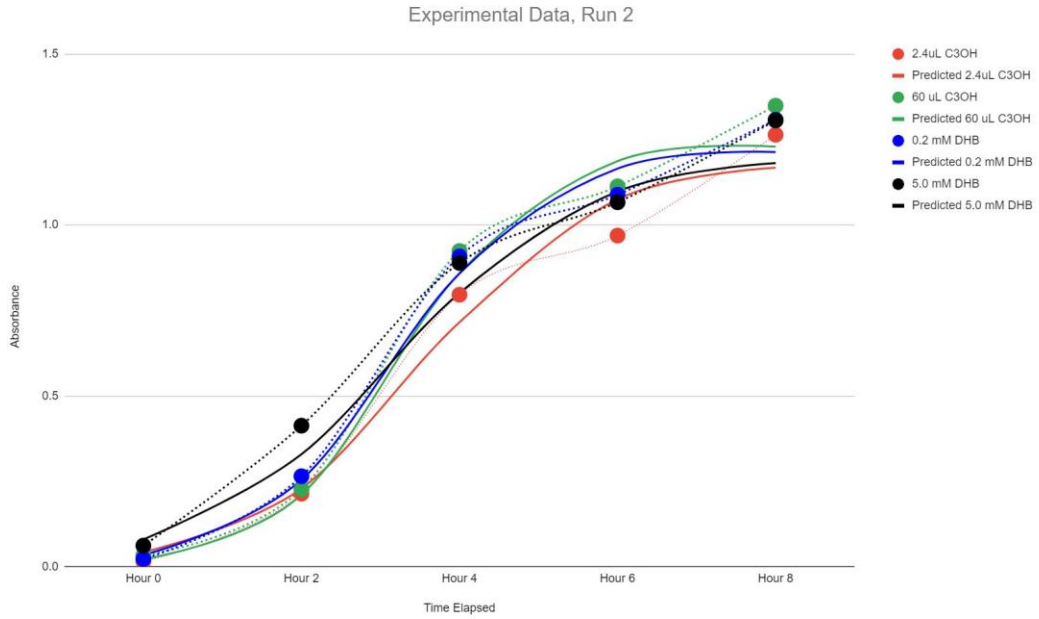
Appendix 6

Biological replicates of DHB experiments comparing experimental and predicted data

DHB Biological Replicate 1



DHB Biological Replicate 2



DHB Biological Replicate 3