Causes, Consequences, and Tests of Antibiotic Pollution in the Environment

Cedrick R. Dimaranan
Ocean Lakes High School
September 16, 2022

Introduction

Antimicrobials, or antibiotics[a], are compounds that either inhibit the growth of or kill bacteria altogether through a variety of different means, such as destroying the cell wall; these can either be completely synthetic, partially synthetic, or naturally-occurring. Despite being initially created and marketed as a revolutionary item in the field of medicine, antimicrobials are becoming increasingly problematic due to the widespread development of resistance to the drugs in bacterial populations. While improper usage of antimicrobials remains one of the leading causes, one of the emerging breeding grounds for antibiotic resistance is the environment, as antibiotics continue to contaminate soil and water ecosystems in a similar fashion to other common pollutants like plastics and pesticides. Seeing as research on environmental antibiotic pollution is still in its infancy, this literature review details the various causes, consequences, and testing methods for antibiotics in the environment in addition to information regarding commonly used and alternative antimicrobials, their persistence in the environment, and general results from recent studies in preparation to perform a personal research project on the issue.

Causes of Environmental Antibiotic Pollution and Common Environmental Antibiotics

Antibiotics are dispersed through the environment in a variety of ways. By knowing how antibiotics are introduced into the environment and where, certain locations and types of places can be pinpointed for specific study in regards to potential cases of environmental antibiotic pollution. This typically occurs due to organisms partially metabolizing the antimicrobials being given to them, thus lacing their waste products with unmetabolized antibiotic [1,4,10,15]; the improper disposal of antibiotics into the environment [5,10]; or the incapacity of treatment facilities to fully filter out any antibacterial compounds from incoming wastewater [5,10]. Each method of antibiotic pollution has the compounds typically introduced into soil before ending up in a nearby body of water through runoff [12]. In the case of partial metabolism, anywhere from 40-90% of the originally consumed antibiotic dosage will be excreted by the organism in their urine or feces as the drug's active form [1,4,10,15]. With the ways in which antibiotics find themselves introduced into the

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a. While antimicrobials and antibiotics are generally interchangeable, the term 'antibiotics' typically refers to those that are at least partially synthetic and taken for medicinal purposes in everyday usage. However, 'antimicrobials' is a broader term for any compound that exhibits properties that render bacteria inanimate, including heavy metals and plant matter. Both are acceptable usages[4], but this paper will use the former (synthetic and medicinal) definition for both terms unless otherwise noted.

environment in mind, there are three main sources that truly exemplify the means through which antimicrobials are polluted: livestock growth and medicinal therapy, human medicinal therapy, and veterinary medicinal therapy. Other sources include aquaculture [12] and the effluent of pharmaceutical factories [15].

Agriculture

In agricultural business practices and livestock cultivation, antibiotics are commonly used as a means to fend off disease or increase the growth rates of the animals as growth supplements [1,2,10,12,20]. They are fed to both diseased and healthy livestock alike [12], and as a result of these usage patterns, antibiotics are actually most commonly used in agriculture, as up to 80% of antibiotics in the United States and 73% worldwide are found in the feed of livestock as of 2009 with the expectation that this usage has either expanded or stabilized since then [1,12]. Because of this, livestock practices can be considered one of the major contributors to environmental antibiotic pollution. Unfortunately, there lies a lack of transparency with agricultural practices, including antibiotic use, making study of their usage patterns difficult [10]. Regardless, antibiotic pollution with agriculture is mainly seen through how livestock partially metabolize antibiotics, as so many are given to them on a regular basis. As a result, the waste products of the animals are ridden with either active antibiotics or compounds that can be transformed to become active antimicrobials again [7,10,12]. Their waste trickles into the surrounding soil and water environments, causing antibiotics to be introduced into the environment. However, this is not the only way through which antimicrobials are circulated by agricultural practices, as wastewater that may be laced with antibiotics can occasionally be used by farmers in growing crops or cultivating animal growth [12]. Common agricultural antibiotics include tetracyclines, erythromycin, sulfamethazine, lincomycin, and penicillin, but the specific antibiotics used vary based on the type of livestock being grown [10].

Human Medicinal Therapy

While farms represent the main way in which humans contribute to the problem of antibiotic pollution, humans themselves can pollute the environment with antimicrobials. The main problems surrounding human antibiotic usage are the unnecessary or improper intake and prescription of the drugs as well as the incorrect disposal of antibiotics [12,15,20]. In fact, 266.1 million antibiotics were prescribed out to patients in 2014, and 30% of those prescriptions may have been unnecessary [1]. These incorrect prescriptions could be caused by one of many reasons,

including patients' hasty expectations of immediately receiving a prescription, the inability to properly diagnose symptoms, or a lack of rules and guidelines regarding prescriptions [15], but ultimately, the effect of this practice is that antibiotics are being unnecessarily introduced into the environment. Unused antibiotics can often be flushed down toilets [15,20], and again, humans' waste products are riddled with antibiotic residues that have not been metabolized [4,10,15]. While wastewater treatment plants do attempt to filter out any antimicrobial compound found in wastewater, oftentimes, not all of the antibiotics can be taken out of the water, as most, if not all, treatment plants lack the equipment necessary to deal with the high volumes and variety of antibiotics in wastewater due to its high cost [15,20]. Hospitals can be considered a hotbed for this water treatment and disposal method, as they as well frequently lack the facilities necessary to deal with their wastewater appropriately [12]. The treated wastewater can then find itself in environmental bodies of water or in use in agriculture, as has been stated [12,20]. Common human medicinal antibiotics include amoxicillin, erythromycin, ciprofloxacin, sulfamethoxazole, and ciprofloxacin [10].

Veterinary Therapy

Lastly, antibiotics can be introduced into the environment through the waste of veterinary animals, such as dogs and cats. Unfortunately, similarly to humans, veterinary antibiotic usage is poorly regulated and researched as it currently stands, leading to unnecessary prescriptions and improper usage; a lack of standards across the veterinary industry predominates, and the preemptive prescription of antibiotics without a diagnostic test is common [22]. Again, domestic animals very poorly metabolize antibiotics, leading to their excretion in waste products [12]. While animals' feces are occasionally picked up to avoid pollution, urine is not and also contains antibiotics, making places such as dog parks places of particular interest in regards to antibiotic pollution. Common veterinary antibiotics include amoxicillin, sulfamethoxazole, lincomycin, tetracycline, and chloramphenicol [10,24].

Consequences of Environmental Antibiotic Pollution

While studying environmental antibiotic pollution is predominantly done for the potential microbiological effects, there exists the potential for disastrous ecological impacts if more action is not taken. Understanding the effects of environmental antibiotic pollution allows for a foundation as to the existence of studies in regards to antibiotics in the environment and

emphasizes the importance of why measures to curtail and study antibiotic pollution are imminently necessary.

Antibiotic Resistance

The main impact resulting from environmental antibiotic pollution is the development of antibiotic-resistant bacteria (ARBs), leading to pathogens that confer untreatable diseases. 10 million people are projected to be killed by ARBs by 2050 [7], and as of 2020, 33,000 people die every year in both the United States and European Union as a result of complications from ARBs [15,22]. Environmental antimicrobials are one of the major reasons that many people will be killed by antibiotic resistance and why the amount of ARBs will increase. In the environment, antibiotics are a 'selective pressure' that cause bacterial populations to trend towards becoming more resistant to antibiotics [1,12,15], as those populations that survive in the presence of antibiotics should naturally have some antibiotic resistance and will be allowed to reproduce, causing strains of ARB to become heavily predominant in the environmental populations of bacteria [12,15,20].

Antibiotic resistance is actually created as a result of sections in bacterial DNA coined antibiotic resistance genes (ARGs). These ARGs, initially created through mutations [6], cause bacteria to become resistant to antimicrobials through coding for the creation of proteinaceous enzymes that more effectively metabolize and dispose of antibiotics, pumping antibiotics out of the cell (specifically called efflux pumps), modifying the cell wall to be less susceptible to antibiotics, and creating alternate synthesis pathways for those that antibiotics may target [1]. ARGs may be present either on ARBs' singular chromosome or on transferable segments of DNA called plasmids. If an ARG is present on a bacterium's plasmid, it may be transferred to another bacterium through horizontal gene transfer under the right type of environmental conditions, such as antibiotics being present, causing antibiotic resistance to exponentially increase amongst the population [1,6,20]. This is the main danger of environmental antibiotics, as even if ARGs are initially developed mainly in environmental populations of bacteria, which tend to be non-pathogenic for humans, these genes could then be transferred to pathogenic bacteria from the environmental ARB [1,6,12,15,20]. The concern doubles when considering the many sources of antibiotic pollution, as because of how livestock, domestic pets, and humans use the same type of antibiotics [10,24], resistance for all pathogens that affect any type of lifeform will result [12,20,22]. Ultimately, an increase in the amount of pathogenic ARB for humans would result, leading to increased hospitalization times, taxing healthcare costs for both patients and hospitals,

and more deaths [15]. In fact, the United States is projected to lose $55 billion per year as a result of the healthcare and productivity complications and costs that arise from antibiotic resistance [15].

Environmental Impacts

While the microbiological side already underlines how essential studies for environmental antibiotic pollution are, the environmental impacts are also severe in their own right. Contamination and the disruption of natural processes are the main ecological impacts to worry about in regards to antibiotic pollution, and its effects are multiplied if antibiotics are together in mixtures as they commonly are in the environment [15]. As mentioned before, antibiotics could be found in the irrigation of crops with wastewater or in the natural groundwater under crop fields, leading to antibiotics being taken in by plants [12]. This contamination has several far-reaching effects, including the stunting of plant growth because of how antibiotics may interfere with plants' photosynthesis, cellular respiration, and other metabolic processes [15,20]. In addition to decreasing crop yields, humans may consume food products that include contaminated plants or animals, leading to disruptions in the digestive system and allergic reactions [15,20]. However, if antibiotics are not absorbed by plants, microbes that are resistant to antibiotics or have dysfunctional metabolisms due to the environmental presence of antibiotics [10,20] could be present on their surfaces instead. Because ecosystems and those organisms within it rely on microbes for decomposition of unusable matter into usable nutrients, environmental antibiotics would disrupt this relationship and cause habitat degradation and less species diversity [7].

Persistence of Antibiotics in the Environment

With that being said, the impact of antibiotics and their degree of severity will differ from one place to another. This is because different antibiotics will persist at different concentrations based on the local environment and the specific chemical and physical properties of the antimicrobial. Studying the factors that affect the concentration of antibiotics in the environment allows for an understanding of what to look out for in potential areas of study and during the transport of samples and gives an insight to possible mitigation strategies that could be used to combat environmental antimicrobial pollution. Certain environmental metrics and factors such as pH, temperature, water content, organic carbon content, weather conditions (e.g. acid rain) and the local microbial communities in close proximity will impact the concentration of antibiotics in the environment [4,10,15,20]. In addition to the environment, antibiotics themselves will impact how

well they persist in the environment, as their own chemical or physical properties in regards to their relative stability as a compound and half-life cause different antibiotics to persist at different rates under more stressful environmental conditions like elevated temperatures [15]. These factors influence the degree to which antibiotics in the environment will degrade or be removed through one of the following four main pathways: biodegradation, adsorption, photolyzation, and chemical transformation [10,15].

Biodegradation serves as the main form of biotic transformation for antibiotics and refers to how the local bacterial communities in the areas of antibiotic pollution can transform and degrade antibiotics themselves through normal metabolism [10,20]. Generally, this tends to occur in soil environments but can also occur in aquatic ones [10]. Again, the amount of biodegradation that occurs depends on the types of microbes present, pH, temperature, and humidity in the local environment [20]. Because of their intrinsic properties, antibiotics in the beta-lactam, fluoroquinolone, sarafloxacin, tetracycline, and sulfonamide families are generally more resistant to biodegradation than are some of those in the aminoglycoside and macrolide families [10].

The rest of the main transformation and degradation pathways of antibiotics are considered to be abiotic. Adsorption refers to how antibiotics may attach, or adsorb, to the surfaces of certain particles present in the environment, such as clay, sludge, and soil [10]. While this can impact the concentration of the antimicrobial in a certain area, adsorption may allow the antibiotics to persist for a longer period of time in the environment, as depending on their properties, they may become more stable when adsorbed onto a particle [10,20]. Antibiotics in the tetracycline family undergo the strongest adsorption of the antimicrobial families in most types of environmental conditions followed by the macrolide and fluoroquinolone families [10]. Sulfonamides are not strongly adsorbed to any type of particle in the environment [10]. Photolysis refers to the degradation of antibiotics by sunlight while in the environment, and while different amounts of sunlight an environment receives will impact how much an antimicrobial decomposes, fluoroquinolones and tetracyclines tend to degrade the most under sunlight [10]. UV light can also degrade antibiotics, especially those in the macrolide family [15]. Chemical transformations, namely hydrolysis, serve as the main vector for antibiotic transformation in the environment, as antibiotics can be oxidized or reduced, depending on their properties and the environment once again [10,15]. Beta-lactams, macrolides, and sulfonamides tend to be susceptible to this transformation pathway, but still, much information does not exist in regards to the impact

of redox reactions on antibiotics in the environment [10]. However, any stressful environmental conditions such as an acidic or basic pH, high environmental temperatures, and aerobic conditions will enhance the degradation of antibiotics through any of these pathways [15].

Of course, antibiotics that are most resistant to these degradation pathways will most likely be found in the environment as polluters [10]. In decreasing order, sulfonamides, fluoroquinolones, and macrolides are most likely to pollute the environment and can be considered 'indicators' for the antibiotic pollution in a particular location mainly due to their synthetic nature [10,20]. Simply put, some antibiotics will persist longer than others [12,15].

Other Antimicrobial Compounds in the Environment

Other non-pharmaceutical substances in the environment, such as heavy metals and naturally-occurring compounds, can serve as antimicrobial agents. These antimicrobials generally are found in the environment through the same sources as the traditional medicinal antibiotics. Because of this, knowing the different types of alternative antimicrobials is essential to truly understanding what else may be in samples taken from the environment nearby points of pollution that would cause bacteria to die or select for some type of antibiotic resistance. While there remains some points of conflicting evidence as to whether these alternative antimicrobials truly contribute to the problem of ARBs or instead merely assist bacteria in their metabolic processes [5,19], their types, sources, and effects still deserve to be studied.

Heavy Metals

In regards to the field of alternative antimicrobials that may be found in the environment, heavy metals such as copper, mercury, zinc, cadmium, chromium, nickel, iron, silver, and lead serve as the main point of study [19]. There exist many similarities between heavy metals and antimicrobials of the traditional type. To start, heavy metals are commonly introduced into environments through farms and agriculture, as they can be found in animal feed, fertilizers, pesticides, fencing, cages, and other farm equipment [19]; heavy metals are also found in sewage waste and manure [19]. While their concentration and exact presence affects their degree of their environmental effects, heavy metals' toxicity to bacteria is impacted by environmental conditions such as pH, the presence of other organic matter, and their own chemical properties such as redox potential similar to pharmaceutical antibiotics [19]. Bacterial resistance to heavy metals occurs through some familiar methods: absorbing heavy metals with the plasma membrane, cell wall, or biofilm; detoxifying heavy metals with enzymes; or using efflux pumps

[19]. Although heavy metal resistance appears inconsequential, considering their limited application for medicinal purposes, heavy metals may actually contribute to antibiotic resistance, especially in the environment, through cross-resistance and co-resistance. Cross-resistance refers to how resistance to pharmaceutical antibiotics may be executed through the same means as heavy metal resistance [19]. For example, an efflux pump used for heavy metals may actually be also capable of pumping medicinal antibiotics out of bacterial cells, thus conferring resistance. On the other hand, co-resistance refers to when heavy metal resistance genes and ARGs are found on the same plasmid, meaning that when only one of the selective pressures (pharmaceutical antimicrobials or heavy metals) are present in the environment, resistance to both is conferred to any bacterium receiving the plasmid [19]. However, as mentioned before, heavy metals' correlation to antibiotic resistance is not truly understood as of this time, as certain heavy metals such as zinc, nickel, and chromium are actually necessary for bacterial enzymatic activity in trace amounts [19]. Current signs point towards there being a minimum concentration of heavy metals that select for antibiotic resistance, but more evidence is needed to definitively prove this connection [19].

Naturally-Occurring Antimicrobials

Other possible alternative antimicrobial compounds that could be present in the environment are those that are either plant-based or naturally-occurring. Of those that are derived from plants, most should come from disposal of food waste, as the antimicrobials of phenols, quinones, saponins, flavonoids, tannins, and terpenoids are typically found in herbs and spices [8]. Plant organic matter such as pulp, seeds, husks, and kernels also contain phenols and therefore could potentially serve as antimicrobial agents [8]. This is because phenolic compounds generally contain hydroxyl groups that weaken the plasma membrane of bacteria and could potentially inhibit enzymatic activity [8]. Essential oils also contain hydroxyl groups, thus explaining their demonstrated efficacy against bacteria [8,17,21]. Excluding essential oils, these substances may also be found nearby livestock farms, as they are also used in feed to enhance growth similar to pharmaceuticals [2]. Naturally-occurring alternative antimicrobials include those polymers created by both animals and bacteria themselves, such as chitosan and lactoferrin; different species of algae; and mushrooms [8]. While research on these types of natural alternative antimicrobial agents is sparse in regards to their contribution to antibiotic resistance, it would be unsurprising to see a

connection between these agents and resistance, considering heavy metals' potential relationship to antibiotic resistance.

Common Tests to Assess Environmental Presence of Antibiotics

There are two common methods that recent research studying environmental antibiotic pollution uses to detect their presence: directly determining the concentration of antimicrobials in the environment by assessing water, soil, or sediment samples or sampling certain environmental bacterial strains to test for their resistance to antibiotics. While the general methodology differs between the two techniques, some commonalities do exist amongst the literature for both methods. One of those similarities is the fact that most studies center around measuring the seasonal environmental presence of antibiotics, choosing to take environmental samples from each season in their local area to determine any fluctuations and variations that may exist [1,4,7,16]. While not explicitly stated in some literature, most, if not all, studies follow some version of the Mackie and McCartney water collection standard technique for the collection of water and environmental samples [1,5,7,10,13,16], including those that directly determined the concentration of antibiotics in water despite the technique being intended for bacterial assessment as opposed to water quality [9,18]. The Mackie and McCartney microbiological technique generally consists of the following guidelines when collecting environmental water samples:

- Collection should be in sterilized containers [9,18], generally made of plastic (high-density polyethylene [10]) or glass [1,5,7].
- Water should not be collected at surface level and instead at a depth of either 20 [18] or 30 [9] centimeters.
- Collection should not include water that has come into direct contact with a hand, a bank, or a wall [9,18].
- Collection in a stream occurs in the direction of the stream's flow [9].
- 200 milliliters of water should be collected at a minimum [1,5,7,9,13,18].
- Samples are meant to be tested within three hours of collection, but if not possible, samples should be kept cold in ice [5,7,10,13], protected from light, and tested within 24 hours maximum [9,18].
- Labels should include a description of the water source and environment and the date and time of collection [9,18].

As environmental conditions can cause antibiotic concentrations to differ and vary, many studies measure certain parameters of water quality, such as pH, temperature, conductivity, and dissolved oxygen to contextualize their findings [1,4,5,7]. Location scouting is often done before sampling takes place in most studies, and sampling sites are chosen based on their proximity to a suspected pollution source [5,7,13,16].

Antibiotic Concentration Studies

Studies dealing with antibiotic concentration utilize some version of liquid chromatography mass spectroscopy (LC-MS) to determine the exact concentration of each type of antibiotic present in a sample [4,7,10,13]. Commonly, samples are taken from a body of water, and those water samples are first processed so that they can be used in LC-MS [4,5,7,10,13]. Sample processing involves some form of acidification and concentration via evaporation, filtration, and extraction [4,7,10,13]. One important aspect of antibiotic concentration studies is that pre-selection of antibiotics to study and detect must occur before any samples are tested, meaning only chosen antimicrobials' concentrations are reported [4,5,7]; generally, antimicrobials are chosen based on past studies and their known stabilities and impacts in the environment [4,5]. From the literature reviewed, several correlations between antibiotic concentration and environmental conditions were reported in addition to raw data regarding concentrations of specific antibiotics. Diwan et al. [4,5] found seasonal variation to occur with antibiotic concentrations with antibiotics appearing at their maximum in fall and at a minimum during the summer in the Kshipra River. At its maximum, sulfamethoxazole was found at a concentration of 2.75 µg/L and throughout all of the seasons, albeit at occasionally very low levels. Norfloxacin and ofloxacin were found only in fall at less than 1 µg/L with some residual beta-lactams also being found during the summer. Meng et al. [13] found antibiotic concentrations to range anywhere from 0 ng/L to 242.1 ng/L in a river in China with sulfonamides, tetracyclines, macrolides, and lincosamides being the most common antibiotic families. In North Carolina, Gray [7] measured antibiotic concentrations ranging from 0 ng/L to 1,227 ng/L with sulfamethoxazole, erythromycin, and danofloxacin being most common. Gray [7] also discovered a seasonal variation in antibiotic concentrations in his study with fall and winter having the highest antibiotic detection rates. Attempting to explain the seasonal variation, Gray [7] hypothesized that the different conditions in each season varied the concentrations, as for example, increased rainfall in spring may have lowered concentrations in that season. Gray and Diwal et al. [4,5,7] also found that pH, temperature, and the time of day of collection affect the

reported antibiotic concentration, as increased adsorption and photodegradation may occur during different conditions.

At face value, the antibiotic concentrations reported appear far too low to have consequential effects and impacts on the environment and overall health, but it is important to note that these are only concentrations of individual types of antibiotics and that the cumulative concentration of these antibiotics will have a damaging effect [7]. However, many of these individual concentrations still remain below the minimum inhibitory concentration (MIC) [4], which refers to the lowest antibiotic concentration that will visibly inhibit bacterial growth [19,20]. Unfortunately, research has shown that concentrations of antimicrobials, pharmaceutical or not, below the MIC can still select for antibiotic resistance in bacterial populations [4,5]. In fact, trace amounts of antimicrobials can select for resistance all the way down to the minimum selective concentration (MSC), which refers to the lowest concentration of antimicrobials that will select for ARBs [20]. The MSC can be anywhere from 1/4 to 1/230 of the MIC [20]. As a result, the low concentrations of antimicrobials found by these studies are actually quite consequential and will lead to some of the aforementioned dire implications of environmental antibiotic pollution.

Antibiotic-Resistant Bacteria Studies

On the other hand, those studies dealing with measuring relative antibiotic presence in the environment by sampling bacteria use some form of the Kirby-Bauer method to test for antibiotic sensitivity [1,4,5,16,23]. Water samples were still taken in these studies, but bacteria were taken from these samples through serial dilutions, filtration, and isolation on selective and differential media [4,5] instead of direct measurements of antibiotic concentrations. However, as with antibiotics being pre-selected in antibiotic concentration studies, the bacteria for study must be pre-selected during the isolation phase of the studies, meaning resistances to antibiotics are only reported for bacterial strains who were chosen for culture. *Escherichia coli* was the most common species of bacteria selected in most studies, which likely was due to its easy identification, association with contamination, and general identity as a 'benchmark' for studies dealing with antibiotic concentrations [4,5,23]. The Kirby-Bauer methodology was then done with select antibiotic discs, such as sulfamethoxazole, tetracycline, erythromycin, and vancomycin [1,4,16,23], against the chosen environmental bacterial strains. Results that demonstrate bacterial resistance to drugs indicate antibiotic pollution in the environment. Bird et al. [1] found bacteria to be most resistant to tetracycline, sulfamethoxazole, and cefoxitin in Louisiana, corresponding to the antibiotics found

in the waters there. Both Diwan et al. and Poonia et al. [4,16] found much of the environmental bacteria in their respective rivers to be resistant to similar antibiotics as those bacteria tested in Bird et al.'s study but also to multiple antibiotics at once with winter and fall having the most resistant bacteria.

Other Tests and General Limitations

While LC-MS for antibiotic concentrations and Kirby-Bauer for ARBs remain the predominant forms of studying environmental antibiotic pollution, other tests do exist or are being developed to continue to study this problem. One such test deals with isolating the DNA of environmental bacterial colonies through centrifugation and then extracting and studying it to search for any ARGs [1,4,5]. Some other methods deal with bacterial morphology, as in one test, engineered bacteria whose antibiotic sensitivities are known produce colored products in environmental samples; those colored products are then studied using spectrometry to determine the sample's antibiotic concentration [14]. Generally used in very low concentrations near the MSC, microfluidics identifies changes in bacterial morphology in the presence of antibiotics for their detection [14]. Aptasensors, oligonucleotides that bind to specific targets, are being developed for antibiotics with different types, such as electrical and fluorescent [14]. Differing from these, which have generally been at the microbial level, the accumulation of antibiotics inside certain animal tissues or plant parts (roots, stems, leaves) can be studied and used to obtain an idea of environmental antibiotic pollution in an area [12].

Despite all of these tests and methods to detect antibiotics in the environment, there still remain many limitations regarding the capability of these tests and methods. One of the major limitations in each of these testing methods is the fact that they are too specific. This means that they are unable to be sensitive to every antimicrobial, including non-pharmaceutical ones, as pre-selection of antibiotics must occur before testing [14]. As a result, antimicrobials, such as heavy metals, that may be present in the water and that may select for some antibiotic resistance in environmental bacteria go unreported and undetected by these studies, most of which do not consider non-pharmaceutical antimicrobials. ARB studies also face these types of limitations, as strains of bacteria to study and antibiotic discs to use have to be pre-selected as well due to problems with culturing bacteria [23], leading to unreported resistances in environmental bacterial strains to certain antibiotics. Ultimately, current methods are not inclusive enough to paint the fullest picture of antibiotic resistance because of how limited their scope is by the act of

predetermination. Some other challenges to environmental antibiotic pollution studies include the high cost in time and money that these require, as extensive, expensive laboratory equipment is required for analysis in most studies [14]; this has led to a desire to create testing methods that could analyze antibiotic presence at sampling sites in the field [14]. In addition, while this is more apparent on a case-by-case basis, certain techniques such as filtration during sample preparation may cause experimental error due to antibiotics being erroneously filtered out during the process [7]. Because of these limitations, antibiotic pollution studies are few and far in between across the world, leading to a dearth of information regarding the extent of the antibiotic pollution problem. In fact, studies that only detect the presence of antimicrobials in the environment and do not provide any information past their detection are currently considered sufficient because of the lack of studies worldwide regarding antibiotic pollution [14].

Kirby-Bauer Methodology

As a final point, because of known limitations in what can be done at the high school level, the Kirby-Bauer methodology has to be used as the main core of any procedure attempting to determine the environmental presence of antibiotics near a potential pollution source. Consequently, research needed to be done on Kirby-Bauer regarding its general procedure, techniques involved, and standards to truly determine its potential for assessing the environmental presence of antibiotics.

History and Overview of Kirby-Bauer

At its core, the Kirby-Bauer disc diffusion susceptibility test determines the degree of efficacy of various antibiotics against certain pathogenic bacteria isolated from diseased patients to aid physicians in determining the best course of action and medicinal therapy against a pathogen [11]. Ultimately, the results obtained from Kirby-Bauer in regards to whether a specific pathogen is either sensitive (susceptible) or resistant against a specific antibiotic allows a physician to determine if it would be effective to prescribe to a patient. The method is named after its authors, W. M. M. Kirby and A. W. Bauer, after they standardized the many different ways in which disc diffusion tests were performed by microbiologists across the world [11]. By having a standard protocol, confusion and unevenness in treatment, diagnoses, and prescriptions would no longer occur.

The Kirby-Bauer disc diffusion test consists of placing several antimicrobial-impregnated 6 millimeter filter paper discs onto a bacteria-inoculated petri dish (or plate). After placing the

discs onto the dish, the plate is then incubated for anywhere from 16-24 hours, after which "zones of inhibition" - areas surrounding each disc where no bacterial growth is found - should be observed on the plate. These zones of inhibition are created by the antimicrobial impregnated inside each paper disc diffusing into the surrounding agar on the plate; diffusion rates are determined by the molecular weight and solubility of the antimicrobial in the agar. The diameter of the zones of inhibition, including the diameter of the discs, are the quantitative data reported from the Kirby-Bauer method; these diameters are then compared against a table listing antibiotics and various ranges for the diameters to be interpreted into a qualitative result, allowing the microbiologist performing the test to determine whether the bacteria on the plate are resistant, intermediately sensitive, or sensitive to the antimicrobials impregnated into each disc. A table of zone diameters exists for each species of bacteria, and these tables are created through extensive *in vivo* experimentation of blood and urine bacterial samples with each antimicrobial [11].

Standards and Procedure

There exists several standards with the Kirby-Bauer method to allow for consistency across test results. Starting with the agar, Meuller-Hinton agar (MHA) is the standard type of agar on which bacteria are inoculated to perform the Kirby-Bauer method [11]. MHA is used because of its predictability, capability to culture most organisms, and how most data has been collected with the agar [11]. Usage of alternate types of agars is discouraged because of the erroneous results they may bring [11], but Daoudi et al. [3] found there to be no difference between nutrient agar (NA) and MHA in terms of the reported qualitative results for a specific strain of *E. coli* with certain types of antibiotics; however, quantitative results differed by at least 4.5 millimeters between NA and MHA. The standard agar depth is 4 millimeters, which is necessary because the diameters of the zones of inhibition are influenced by depth of the agar [11]. Shallower depths will produce erroneously larger zones of inhibition, as antimicrobials within the paper discs diffuse in multiple directions through the agar [11]. The pH of the agar should be anywhere between 7.2 to 7.4 at room temperature [11]. The creation of a 0.5 McFarland standard of inoculum (bacteria to be cultured for testing) is necessary to ensure a standard amount of bacteria is dispensed onto the plate. McFarland standards are a means of communicating the relative density of bacteria inside a broth tube, and the 0.5 McFarland of the inoculum is created through comparison to a 0.5 standard tube in front of Wickerham paper [11]. In regards to disc placement,

they should be placed at least 24 millimeters apart from each other and away from the edges of the plate, there should be no more than 12 discs on 150 millimeter plate and no more than five discs on a 100 millimeter plate, and each disc should be pressed down after placement without disturbing the agar [11]. Zone sizes are measured up to the nearest millimeter and include the diameter of the paper disc itself; interpretation is done through tables from the most recent Clinical and Laboratory Sciences Institute publication [11]. Finally, while not an actual standard, it is recommended that one of *Staphylococcus aureus*, *Escherichia coli*, or *Pseudomonas aeruginosa* are used for instruction with Kirby-Bauer because of the wealth of information known about these species in regards to the method [11]. The general procedure for Kirby-Bauer goes as follows [11]:

1. Dispense MHA into petri dishes to the appropriate depth.
2. Prepare an inoculum tube to a 0.5 McFarland standard with the desired bacterial culture to test; this should be used within 15 minutes of preparation.
3. Dip a swab into the prepared inoculum tube and swab the plate to create a 'lawn of bacteria.' Ensure the swab is not dripping wet before swabbing the plate.
4. Allow the surface of the plate to dry for at least three to five minutes but no more than 15 minutes.
5. Place antimicrobial-impregnated discs onto the surface of the agar one at a time.
6. Replace plate lid, invert plates, and incubate at 33-37°C for anywhere between 16-24 hours.
7. Take the plate out of the incubator and measure zones of inhibition. Interpret results.

Kirby-Bauer with Essential Oils

Because of aforementioned limitations, the Kirby-Bauer method will need to be used to determine the presence of antibiotics in water samples as opposed to how it has traditionally been used to culture environmental strains of bacteria to test against standard antibiotic discs. This is because potentially hazardous strains of bacteria may be cultured if the latter were done. As a result, water from the collection sites will be impregnated into filter discs, meaning non-standard antimicrobial discs will be used in any form of procedure involving Kirby-Bauer. The closest comparison to this type of usage of non-standard antimicrobial discs with Kirby-Bauer are with studies dealing with the efficacy of essential oils as antimicrobials. Some key takeaways from the procedures of studies using Kirby-Bauer with essential oils include how

plates stayed out for 30 minutes after disc placement to allow for diffusion of the essential oils without evaporation in the incubator [17], the usage of a standard comparison disc of either streptomycin [17] or vancomycin [21], the sealing of the plates with parafilm to avoid evaporation [17], and the creation of new standards to interpret quantitative results [17,21].

Personal Research Plan

As previously stated in the literature review, the Kirby-Bauer disc diffusion test will need to be used for any experiment at the high school level (due to safety limitations) to study the environmental presence of antibiotics. However, Kirby-Bauer cannot be used in its traditional sense as discussed in the 'Antibiotic-Resistant Bacteria Studies' section because of the potential for culturing hazardous organisms. Instead, water from any environmental sample will be impregnated into sterilized blank filter discs (the same type as used in regular Kirby-Bauer) and placed onto the plate to determine if antimicrobials are present in that sample's water. However, to the best of my knowledge, such a procedure has not been attempted yet, indicating the novelty and originality of this idea. Ultimately, my research question is, "Can the Kirby-Bauer disc diffusion method be appropriately modified to detect the relative environmental concentration of antimicrobials?". This idea can be split into two parts with Part I being "Can the traditional Kirby-Bauer methodology be modified to be more sensitive to lower concentrations of antibiotics as are typically found in the environment? If so, through what means and to what effect?" and with Part II being "Can a modified version of the Kirby-Bauer methodology detect the presence of and determine the relative concentration of environmental antibiotics?". The hypotheses for these parts are "If both a shallower agar depth and a different agar type are used, then the Kirby-Bauer methodology will be more sensitive to lower concentrations of antibiotics," and "If a version of the Kirby-Bauer procedure modified for low concentrations of antibiotics is used, then the presence of antibiotics in bodies of water nearby waste facilities, livestock farms, and dog parks can be detected," respectively.

Regarding the value and exigence of this idea, as previously mentioned under the 'Other Tests and General Limitations' section, current environmental antibiotic pollution studies are hampered by their specificity and lack of inclusiveness. Antibiotic concentration studies have to predetermine what antibiotics to look for and analyze in their studies, leaving out other potential antimicrobials like heavy metals and other prescription drugs that may not have been selected, and ARB studies with Kirby-Bauer also have to predetermine bacterial strains to study and the

antibiotics to test them against (potentially leaving out other resistances to bacteria). This method of directly testing the water samples disallows the exclusion of any antimicrobial agent present in the environment, as no predetermination of substances would occur, signifying an improvement. Moreover, current studies are too costly, time-consuming, or equipment-intensive, thus limiting the amount of studies currently being conducted on the matter. While this method still necessitates the laboratory environment to be done effectively, Kirby-Bauer is evidently more affordable and accessible than other testing methods, so if the research project proves to be fruitful, this would allow for an alternative means of testing for antibiotic resistance that could be widespread and not limited in its scope.

The materials necessary for this project will be:

- At least 12 1 liter sterile glass containers (if glass containers will not be reused throughout the experiment, then more will be needed)
    - Possibly amber medicine vials
- 200 blank, sterile 6 millimeter filter paper discs
- Antibiotic discs of 15 µg erythromycin
- 60 100 millimeter petri dishes
- K-12 *E. coli* bacteria (chosen because of noted significance in antibiotic pollution studies and ease of use in Kirby-Bauer [4,5,11])
- Either nutrient agar or Mueller-Hinton agar
- Standard laboratory equipment, such as gloves, flasks, incubator, and hot plates

The glass containers and medicine vials will be sourced from recycling, so that is of no cost. The *E. coli*, agar, and standard laboratory equipment will be provided by resources already available from Mrs. Shoemaker's classroom, so that will also be of no cost. Ultimately, the materials I will have to procure myself will be the filter paper discs, erythromycin discs, and petri dishes.

These materials will largely be sourced from Carolina, a classroom science supplier. The blank, sterile filter paper discs come in packs of 50 and range from $13-14 at these stores ($14 x 4 = $56). The erythromycin discs also come in packs of 50 and cost around $20. The 100 millimeter petri dishes come in packs of 30 and cost around $14 each ($7 x 4 = $28). Overall, the cost of this project will be around $100-110.

Before any testing of environmental samples, the Kirby-Bauer methodology will have to undergo some modifications that will have to be experimentally tested. First, as noted while

discussing Kirby-Bauer, changing the agar depth in the plate will cause erroneously different zones of inhibition. Shallower depths cause greater zones of inhibition but also cause greater sensitivity to antibiotics, which means lower concentrations of antibiotics (as would be present in environmental samples) would produce larger, more identifiable zones of inhibition. In a similar fashion, the type of agar can change zone diameters; nutrient agar has been shown to produce larger diameters than Mueller-Hinton agar [3], but this would best be tested in our experimental setting to determine if a switch is necessary. Finally, a method of creating impregnated discs will need to be experimented with, as discs with different amounts of antimicrobial from the standard mass will need to be created by using solutions of different volumes to transfer over the desired lower amounts. Those antimicrobial solutions will be created using rainwater and the erythromycin discs - different concentrations will result by using different volumes of rainwater in each solution. To actually impregnate the discs, around 20 mL of each solution will be transferred into a conical tube with a disc at the bottom and allowed to boil out to transfer the antimicrobial into each disc. While not directly tested for, the effect of boiling on antibiotics will be seen in addition to the efficacy of this method of creating antimicrobial discs.

The prior testing is meant to justify particular decisions in the creation of a novel procedure to finally test environmental samples using Kirby-Bauer. Once the decision is made on all three choices above, environmental samples from landfills or waste facilities, dog parks, and farms will be tested using the previously established Kirby-Bauer procedure with modifications (type of agar, agar depth, and concentration). Controls will be established with known concentrations of antibiotic using the purchased erythromycin discs and rainwater.

Testing will be performed using the typical Kirby-Bauer procedure guidelines with the aforementioned changes to its standards to best fit our needs. Specifically, five trials will be done for each of the experimental proofs of concept and ten trials will be conducted for the testing of each environmental sample.

All raw data for the experimental proofs of concept and the testing of environmental samples will be the diameters of the zones of inhibition measured in millimeters.

For the experimental proofs of concept, the diameters will be analyzed through comparison of the ‘control/standard’ and modified version of the standard. For example, when testing agar depth, if the average diameter for erythromycin discs in 4 mm agar is 15 mm while the average diameter for erythromycin discs in 2 mm agar is 20 mm, then the data I would report

would be somewhere along the lines of "an agar depth of 2 mm increases the sensitivity of bacteria to antibiotics by about a factor of 1.25." The same would occur with the type of agar and the volume of water.

For the environmental samples, the diameters will be analyzed through comparison of established controls with known concentrations and the samples' diameters. Controls will be established with different concentrations of antibiotics, the controls' diameters will be measured and averaged out. This will then be compared with the diameters of the environmental samples to determine the relative concentration of antibiotics in the water sample. For example, if the positive control with the antimicrobial amount of 0.9 μg produces an average diameter size of 10 mm, the negative control produces an average diameter size of 0 mm, and an environmental sample produces average diameter sizes of ~13 mm, then I would report that that environmental sample does contain antimicrobials at a relative concentration of around 0.9 μg/20 mL (or 15 μg/333 mL).

References

1. Bird K, Boopathy R, Rajkumar N, LaFleur G. Water pollution and observation of acquired antibiotic resistance in Bayou Lafourche, a major drinking water source in Southeast Louisiana, USA. Environ Sci Pollut Res Int [Internet]. 2019 Nov [cited 10 Jun 2022]; 26(33):34220-34232. Available from https://doi.org/10.1007/s11356-018-4008-5

2. Castillo-López RI, Gutiérrez-Grijalva EP, Leyva-López N, López-Martínez LX, Heredia JB. Natural alternatives to growth-promoting antibiotics (GPA) in animal production. J Anim Plant Sci [Internet]. 2017 [cited 18 Apr 2022];27(2):349-359. Available from https://www.researchgate.net/profile/Erick-Gutierrez-Grijalva/publication/316671322_Natural_alternatives_to_growth-promoting_antibiotics_GPA_in_animal_production/links/5911e420aca27200fe3d8f40/Natural-alternatives-to-growth-promoting-antibiotics-GPA-in-animal-production.pdf

3. Daoudi Y, Daoudi S, Bennacer S, Saïd A. Effect of culture media on the agar diffusion method for testing antibacterial activity against *Escherichia coli* "HO25". ElWahat Rech Etudes [Internet]. 2017 Dec [cited 11 Jun 2022];10(2):157-165. Available from http://dx.doi.org/10.54246/1548-010-002-006

4. Diwan V, Hanna N, Purohit M, Chandran S, Riggi E, Parashar V, Tamhankar AJ, Lundborg CS. Seasonal variations in water-quality, antibiotic residues, resistant bacteria and antibiotic resistance genes of *Escherichia coli* isolates from water and sediments of the Kshipra River in Central India. Int J Environ Res Public Health [Internet]. 2018 Jun 17 [cited 2 Jun 2022];15(6):1281. Available from https://doi.org/10.3390/ijerph15061281

5. Diwan V, Purohit M, Chandran S, Parashar V, Shah H, Mahadik VK, Lundborg CS, Tamhankar AJ. A three-year follow-up study of antibiotic and metal residues, antibiotic resistance and resistance genes, focusing on Kshipra—a river associated with holy religious mass-bathing in India: protocol paper. Int J Environ Res Public Health [Internet]. 2017 May 2017 [cited 3 Jun 2022];14(6):574. Available from https://doi.org/10.3390/ijerph14060574

6. Finley RL, Collignon P, Larsson DGJ, McEwen SA, Li X, Gaze WH, Reid-Smith R, Timinouni M, Graham DW, Topp E. The scourge of antibiotic resistance: the important role of the environment. Clin Infect Dis [Internet]. 2013 May 30 [cited 15 Jul 2022];57(5); 704-710. Available from https://academic.oup.com/cid/article/57/5/704/311765?login=true

7. Gray AD. The presence, bioavailability, and impact of antibiotics in rural and urban streams of North Carolina [dissertation]. University of North Carolina at Greensboro; 2020. 145 p.

8. Gyawali R, Ibrahim SA. Natural products as antimicrobial agents. Food Control [Internet]. 2014 Jun 8 [cited 17 Apr 2022];46(2014):412-429. Available from http://dx.doi.org/10.1016/j.foodcont.2014.05.047

9. Hooja S, Pal N. Standard operative procedures in microbiology for laboratory technicians [Internet]. Jaipur (IND): SMS Medical College; n.d. [cited 6 Jun 2022]. Available from http://rajswasthya.nic.in/RHSDP%20Training%20Modules/Lab.%20Tech/Microbiology.pdf

10. Huang C, Renew JE, Smeby KL, Pinkston K, Sedlak DL. Assessment of potential antibiotic contaminants in water and preliminary occurrence analysis. J Contemp Water Res Educ [Internet]. 2001 [cited 19 Apr 2022]:120(1). Available from https://opensiuc.lib.siu.edu/jcwre/vol120/iss1/4/

11. Hudzicki J. Kirby-Bauer disk diffusion susceptibility test protocol [Internet]. Washington (DC): American Society for Microbiology; 2009 Dec 8 [cited 11 Jun 2022]. Available from https://asm.org/getattachment/2594ce26-bd44-47f6-8287-0657aa9185ad/Kirby-Bauer-Disk-DiffusionSusceptibility-Test-Protocol-pdf.pdf

12. Mauricio C, Muñoz-Gómez A. Pharmaceutical antibiotics at a significant level in nature: from hospitals, livestock, and plants to soil, water, and sea [Internet]. London (UK): InTechOpen; 2021 May 27 [cited 12 Jul 2022]. Available from https://www.intechopen.com/chapters/76553

13. Meng T, Cheng W, Wang M, Wan T, Cheng M, Zhang CC, Jia ZY. The distribution of antibiotics in water of a river basin in South China. IOP Conf Ser Earth Environ Sci [Internet]. 2017 Aug [cited 3 Jun 2022]:82(1). Available from https://doi.org/10.1088/1755-1315/82/1/012078

14. Parthasarathy R, Monette CE, Bracero S, Saha MS. Methods for field measurement of antibiotic concentrations: limitations and outlook. FEMS Microbiol Ecol [Internet]. 2018 June 5 [cited 11 June 2022];94(8):105. Available from https://academic.oup.com/femsec/article/94/8/fiy105/5033401?login=true

15. Polianciuc SI, Gurzău AE, Kiss B, Ştefan MG, Loghin F. Antibiotics in the environment: causes and consequences. Med Pharm Rep [Internet]. 2020 Jul 22 [cited 13 Jul 2022];93(3):231-240. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7418837/

16. Poonia S, Singh TS, Tsering DC. Antibiotic susceptibility profile of bacteria isolated from natural sources of water from rural areas of East Sikkim. Indian J Community Med [Internet].

2014 Jul-Sep [cited 6 Jun 2022];39(3):156-160. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4134531/

17. Prabuseenivasan S, Jayakumar M, Ignacimuthu S. In vitro antibacterial activity of some plant essential oils. BMC Complement Altern Med [Internet]. 2006 Nov 30 [cited 11 Jun 2022];6. Available from https://doi.org/10.1186/1472-6882-6-39

18. Sample collection & transport [Internet]. Surat (IND): Governmental Medical College, Surat; n.d. [cited 6 Jun 2022]. Available from https://gmcsurat.edu.in/lib/exe/fetch.php?media=pg:microbiology_sample_collection.pdf

19. Seiler C, Berendonk TU. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front Microbiol [Internet]. 14 Dec 2012 [cited 18 Apr 2022]:3. Available from https://www.frontiersin.org/articles/10.3389/fmicb.2012.00399/full

20. Serwecińska L. Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and to public health. Water [Internet]. 25 Nov 2020 [cited 13 Jul 2022];12(12);3313. Available from https://www.mdpi.com/2073-4441/12/12/3313/htm

21. Sharma P, Mack JP, Rojtman A. Ten highly effective essential oils inhibit growth of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA). Int J Pharm Pharm Sci [Internet]. 2013 [cited 11 Jun 2022];5(1). Available from https://doi.org/10.1186/1472-6882-6-39

22. Shea A, McCarthy R, Lindenmayer J. Therapeutic antibiotic use patterns in dogs: observations from a veterinary teaching hospital. J Small Anim Pract [Internet]. 2011 Jun [cited 19 Apr 2022];52(6):310-318. Available from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3106276/

23. Siedlecka A, Piekarska K. The evaluation of the classical Kirby-Bauer antibiogram method for the determination of antibiotic resistant microorganisms in tap water. E3S Web of Conf [Internet]. 2017 [cited 7 Jun 2022]:17. Available from https://doi.org/10.1051/e3sconf/20171700081

24. Ware E. Common antibiotics for dogs and cats [Internet]. Swedesboro (NJ): Wedgewood Pharmacy; n.d. [cited 19 Apr 2022]. Available from https://www.wedgewoodpharmacy.com/blog/posts/common-antibiotics-for-dogs-and-cats.html

Additional References

Bengtsson-Palme J, Kristiansson E, Larsson DG. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol Rev [Internet]. 2018 Jan [cited 21 Feb 2022];42(1);68-80. Available from https://academic.oup.com/femsre/article/42/1/fux053/4563583

Doron S, Gorbach SL. Bacterial infections: overview. Int Encycl Public Health [Internet]. 2008 [cited 17 Jan 2022];273-282. Available from https://doi.org/10.1016/B978-012373960-5.00596-7

Hunting the nightmare bacteria [video, season 2013, episode 14]. 2013 Oct 22. Frontline. WGBH Educational Foundation. [accessed 26 Jan 2022]; 54:46 minutes. https://www.pbs.org/wgbh/frontline/film/hunting-the-nightmare-bacteria/

The trouble with antibiotics [video, season 2014, episode 1]. 2014 Oct 14. Frontline. WGBH Educational Foundation [accessed 31 Jan 2022]; 53:40 minutes. https://www.pbs.org/wgbh/frontline/film/trouble-with-antibiotics/

Weinstein MP, Lewis II JS, Bobenchik AM, Campeau S, Cullen SK, Galas MF, Gold H, Humphries RM, Kim Jr TJ, Limbago B, et al. Performance standards for antimicrobial susceptibility testing [Internet]. Wayne (PA): Clinical and Laboratory Standards Institute; 2020 Jan [cited 11 Jun 2022]. Available from https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf

Yeatts E. Antibiotic resistance and bacteriophages. Virginia Beach (VA): MSA Explore; 2020 Dec [cited 25 Jan 2022]. Available from https://sites.google.com/vbschools.com/explore--day6/home