

## The Impacts of Multidrug Resistant Bacteria and Sewage Purification Limiting Antibiotic Spread in Habitats (SPLASH)

### Antibiotics and The Threat of Resistance

According to the United States Center for Disease Control (CDC), antibiotics are medicines that have been developed with the intention to fight certain bacterial infections (“Antibiotic Use Questions and Answers.”). The use of antibiotics to fight against bacterial infections began in 1928 with Alexander Fleming’s discovery of penicillin as a way to fight infections. In the 1940s, penicillin became readily available for the general public. Although incredibly effective, it would ultimately lead to overuse and the eventual rise to antibiotic resistance among bacteria (“Antibiotic Resistance”). From the late 1920s/30s and onwards, the scientific community has introduced various antibiotics (i.e. tetracycline in the 1940s, erythromycin in the 1950s, and linezolid in the 2000s), all of which have been observed in relation to resistant bacteria as seen in Figure 1 (Pazda et al. 2019). Initially, the problem of antibiotic resistance was primarily restricted to healthcare settings such as hospitals, but as resistance increased, the problem expanded into community settings (Schaefer et al. 2015). Even now, the problem of antibiotic resistance is disregarded and worked around through the designation of new antibiotics, which only acts to increase the chances for resistance to occur across more strains of antibiotics.

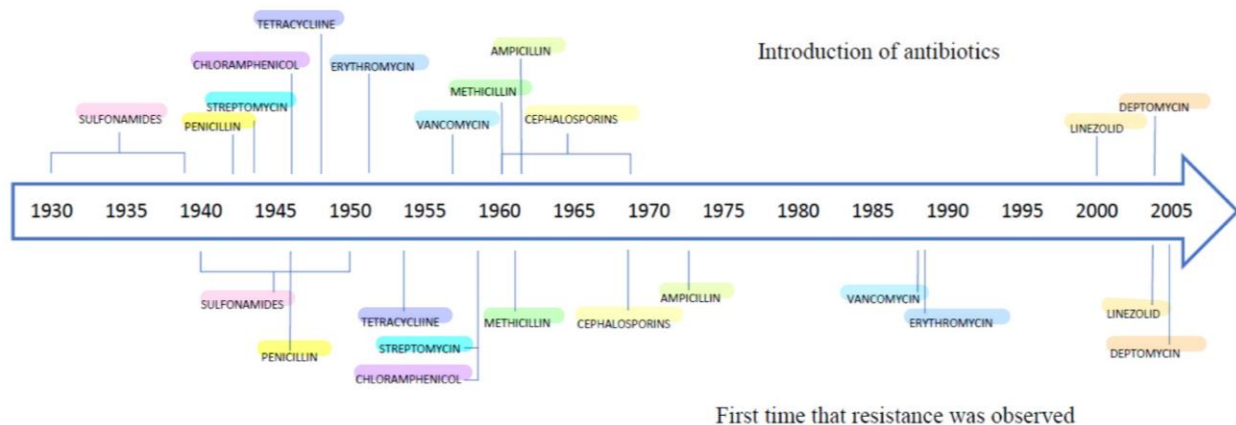


Figure 1; Taken from Pazda et al. 2019.

Antibiotic resistance is the ability of bacteria (or other organisms) to fight off the very antibiotics designed to combat them (“About Antibiotic Resistance”). The World Health Organization (WHO) states that antibiotic resistance is a global problem which effectively registers the corresponding use of antibiotics ineffective, resulting in increased fatalities and expenditures in the medical field (longer hospital stays, increased costs) (“Antibiotic Resistance”). The CDC cites antibiotic resistance as the “greatest public health challenge of our

time,” reporting a staggering 2.8 million people in the United States alone succumbing to an antibiotic resistant infection per year (“Antibiotic/Antimicrobial Resistance”). This resistance can occur in different ways, one of those being the introduction of antibiotic compounds into areas with naturally occurring bacteria. The bacteria which are able to survive in the presence of antibiotics in the natural environment will inevitably be the most fit to survive. Once established, the populations of these bacteria will grow exponentially within the environment due to lack of competition. Bacteria can also transfer ARGs, or antibiotic resistant genes, between each other, propagating the resistance to a greater population (Amarasiri et al. 2019).

Antibiotic resistant bacteria (ARBs) and ARGs have been increasing in prevalence as well as concentrations. ARBs are the specific bacteria that have developed resistance to antibiotics; i.e. methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a result of resistance to various types of antibiotics that the staph bacteria has acquired (“MRSA Infection,” Mayo Clinic). The overall prevalence of antibiotic resistance does not only cause concern on national and global levels, but also within the local level. Evidence of the effects of antibiotic resistance can be seen locally in Florida from a variety of cases, including: The *Tallahassee Democrat* reported a football coach acquiring MRSA after visiting St. Teresa beach, and the *Environmental News Service* reported an environmental documentary filmmaker shooting at Fort Myers beach and also acquiring MRSA (Miller 2019; Castle 2004).

Antibiotic resistance poses a major liability to Florida's population as well as its tourism, agriculture and many other industries the state relies on. We focused our solution to initiate the reduction of antibiotics into the environment, in an effort to reduce overall levels of resistance seen in Florida's population and its environment.

### Looking at Data from Florida

The number of antibiotic resistant bacteria has been increasing seemingly in relation with the increasing use of antibiotics since their introduction in 1928. For example, in a report on antibiotic resistance from the CDC, they presented a 15% increase between 2010 to 2017 in erythromycin resistance shown in Group A *Streptococcus* bacterium as shown in Figure 2

(“Erythromycin- Resistant Group A *Streptococcus*.”). Group A

*Streptococcus* is a common bacterium, with up to 24,000 cases reported annually in the United States, including infections like pneumonia and Necrotizing fasciitis. The most common treatment for this is a prescription of the antibiotic erythromycin (Group A *Streptococcal* (GAS) Disease). This alarming trend caught our attention when we discovered it was mirrored in dolphins in the environment. Adam Schaefer, an epidemiologist at Florida Atlantic University in

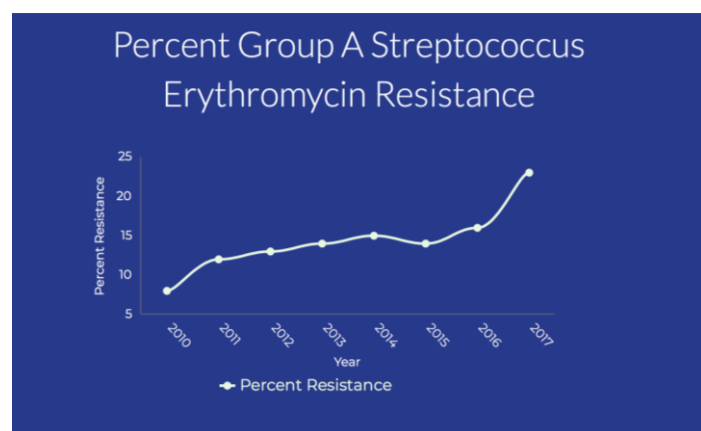
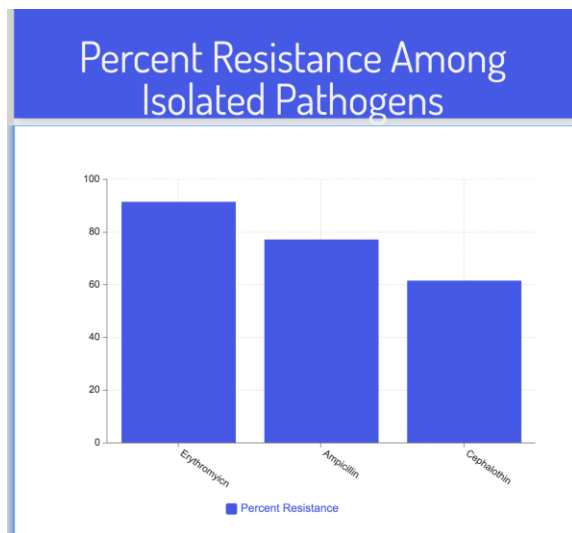


Figure 2



*Figure 3*

Boca Raton, collected tissue samples from dolphins in the Indian River Lagoon in Florida, over a 12-year period (Schaefer et al. 2015). The Indian River Lagoon is a complex system of grouped lagoons and waterways which stretches down 40% of Florida's east coast and is home to one of the most biodiverse populations found in the state (Indian River Lagoon). Schaefer found that 88.2% of the isolated bacterial pathogens were resistant to at least one antibiotic, and within every pathogen, resistance to at least three antibiotics increased over time. He also found resistance to be highest in erythromycin, ampicillin, and cephalothin, as seen in Figure 3.

This data supports that antibiotics are being polluted into the environment from some source (i.e. agriculture, aquaculture, wastewater treatment plants, etc.). Given that dolphins do not consume or come into contact with antibiotics, it is apparent that the increasing use of antibiotics is contributing to their increasing prevalence within Florida's ecosystem. Although the concentrations of antibiotics being released may not be considered toxic to the human body alone, they are still able to promote resistance.

### Sources of Antibiotic Resistance and Our Decision

Antibiotic pollution is a problem driven by many independent sources. Agriculture, extensive human prescription, and proper/improper disposal can all release amounts of antibiotics into the environment. As previously stated, this allows for the alteration of an environment in which resistance is then favored. When researching and reviewing the numerous contributing factors, we focused on the relative impact of the source, as well as our ability to efficiently deal with the source across the entire region of Florida. We focused our efforts in researching three main routes of antibiotic resistance into the environment: aquaculture, agriculture, and wastewater treatment plants (WWTPs). Figure 4 shows the various channels that antibiotics can enter and circulate within the environment.

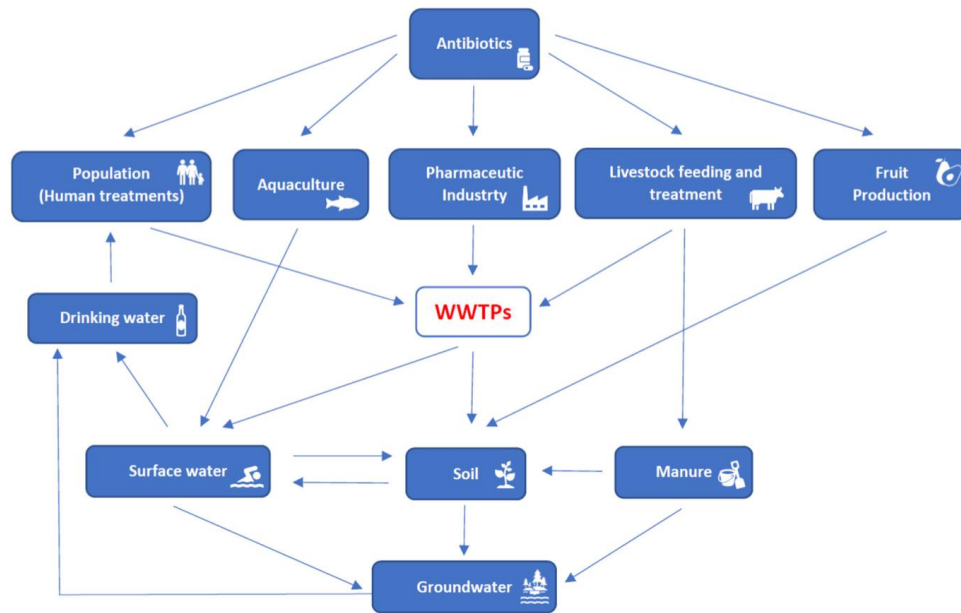


Figure 4; Taken from Pazda et al. 2019.

Aquaculture, or the process of growing aquatic plants and animals for food, uses large amounts of antibiotics, oftentimes releasing these compounds directly into the surrounding ecosystems (Done et al. 2015). Although posing a threat to the specific ecosystems, we identified other sources which proved to be larger in impact than aquaculture, including agriculture and wastewater treatment plants. Florida's agriculture industry consumes millions of pounds of antibiotics a year for use in our staple industries, including beef and orange production (Dall 2019).

Cattle require multiple antibiotics to stay healthy, for the purpose of stimulating growth, and are used in excess in many areas (He et al 2020). Orange groves require multiple treatments per year to maintain healthy groves and fight against bacterial infection (Dall 2019). Orange groves, as seen in the 2019 Florida State University iGEM team who addressed the issue of citrus greening, are often treated regardless of infection due to the prevalence of the issue. This treatment contributes to the rise of antibiotic resistance. Overall, agriculture is a top contributor to the current problem Florida faces concerning antibiotics, however, the multiple ways and means of use within the agricultural entity, offers multiple concerns for an agriculture related solution. It would require individualized plans for each instance of pollution across the state and would face more push back due to necessary funding and requirements for installation and maintenance. After evaluating the various tracks of antibiotic resistance, we were led to the path of WWTPs.

Antibiotics are not always entirely digested by the human body, resulting in residual amounts in the excrement that leaves the body, and ultimately an accumulation at wastewater treatment facilities. In our research, we came across studies which depicted certain concentrations of antibiotics, even after the completion of the treatment process, are released along with the effluent (water leaving the WWTPs) (Batt 2007). To perpetuate this problem,

Florida is a leader in wastewater reclamation, in which we take the treated effluent from these plants, and use it for irrigation purposes in numerous residential and recreational areas across the state (“Florida’s Reuse Activities”). Reclaimed water gathered from water sprinklers in Miami was tested for antibiotics, in which they found 127 nanograms/L as the median concentration of erythromycin in each sample, one of the highest occurring concentrations among six other antibiotics (Panditi 2013). This leads us to believe that the practice of using reclaimed water is not only capable but is actively contributing to the spread of these antibiotic compounds throughout the state. This offers the chance that antibiotic compounds are being spread throughout Florida inside populated areas without regard for the implications it could cause.

From a solution perspective, this route offered us an ideal, closed system that is common across the state, which allowed us the best chance for an efficient and widely applicable solution for Florida. From this information we chose to conduct further research into WWTPs to understand their contribution to a higher degree, as well as to identify other factors within this route which needed to be addressed.

Given the systematic nature of wastewater treatment plants and the heightened opportunity to implement an effective solution at the congregation of all wastewater, we chose to focus our efforts on wastewater treatment plants, to stop the emission of these antibiotic compounds. A study out of Miami, Florida showed the ineffectiveness of the current wastewater treatment in targeting antibiotics (Leitz and Meyer 2004). As seen below in Figure 5 (incoming water influent) and Figure 6 (outgoing water effluent), although the amount decreased from the influent (water coming into the plant) to effluent, there were still antibiotics of measurable concentrations left in the water.

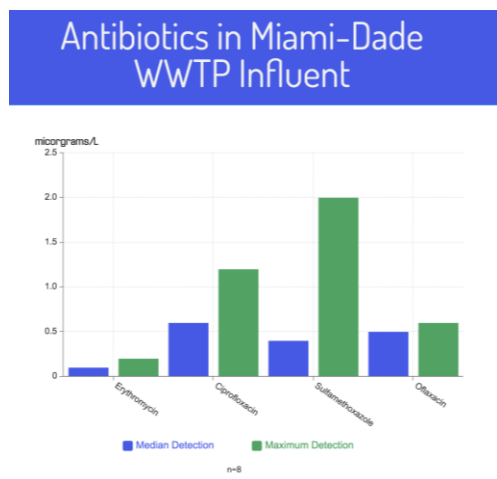


Figure 5

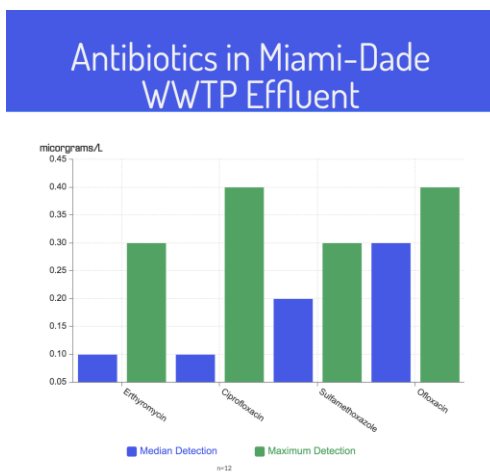


Figure 6

### Investigation of Wastewater Treatment Regulations

Identifying the problem of antibiotic resistance in WWTPs led us to the question of the types of testing and monitoring that were being done for antibiotics, ARBs, and ARGs in the

water. Research studies from various parts of the United States showed presence of one or more of the aforementioned substances in the water. We searched for parties responsible for tracking WWTPs to investigate how these antibiotics, ARBs, or ARGs were being monitored, and what was being done to help mitigate the problem. On a national level, the Environmental Protection Agency (EPA) is in charge of water quality, while the Florida Department of Environmental Protection (FDEP) regulates the water quality of the state. Our team experienced unprecedented obstacles in contacting experts and officials, due to the effects of the COVID-19 pandemic. Despite major efforts to generate a large pool of information, the pandemic and its effects prevented



*A preview of a WWTP; taken from the tour of Mike Kelley's WWTP*

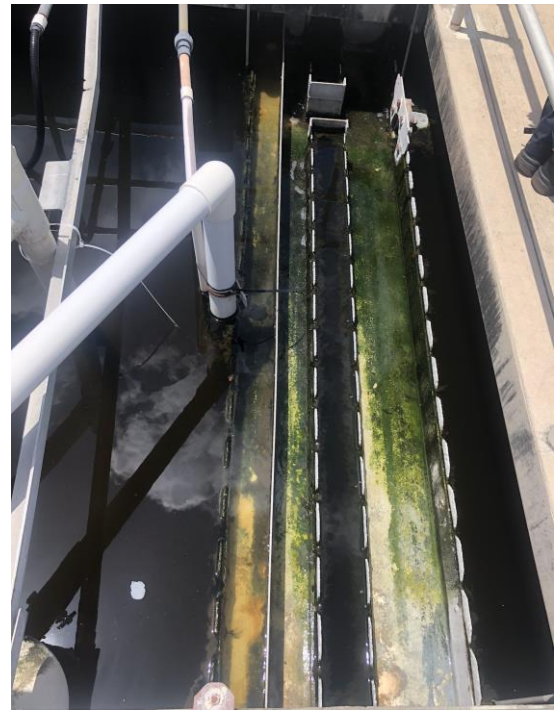
us from achieving our desired level of expert and official resources. Even after getting in contact FDEP officials, we faced difficulty receiving clear direction on if any department of the organization oversees the regulation of antibiotics in WWTPs. However, we did have success in contacting a few officials from the FDEP. Our following email correspondence led us to David D. Whiting, the Deputy Division Director over the Laboratory and Water Quality Standards Programs for the FDEP. He was able to provide some information on questions regarding antibiotic, ARB, and ARG monitoring being conducted. The primary insight that we gained from this correspondence was that the FDEP is not currently testing WWTP effluents for antibiotics of any kind. Any detection of antibiotics in the effluent would be characterized as a generic prohibition.

Another expert we reached out to was Dr. Youneung Tang, an engineering professor here at Florida State University, who has experience in wastewater treatment plants as well a degree within the field of water quality management. In a Zoom interview with Dr. Tang, he confirmed that neither the EPA, nor FDEP monitor levels of antibiotic concentrations in the water. He explained that testing the water included common parameters with previously established guidelines (i.e. Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), concentrations of certain organic matter, etc.). He did however state that antibiotics could be classified as an emerging contaminant, part of a group of materials under the Pharmaceuticals and Personal Care Products (PPCPs) which could pose a potential threat to the environment, however, more research would be required before mandated testing could be implemented (Wang 2016).



## Integrated Human Practices: The Development of Our Design

Upon making the decision to tackle the problem of antibiotic resistance in WWTPs, our team had the difficult task of brainstorming solutions to our chosen problem. Initially, our first iterations to a solution included a version with an algae-based solution as well as a bacteria-based solution, both of which would help to eliminate antibiotics and/or ARGs at a certain stage in the WWTP process. With COVID-19 restrictions in place, the team was able to send one member on a socially distant preliminary tour with Mike Kelley, owner of the South Shores Water Treatment Plant. She observed algae growth on the top layers of the water. Upon this discovery, Mike Kelley mentioned that he let the algae grow because he believed it would ultimately lead to cleaner water. Our design team later created an algae-based solution that was well-received by the whole team. However, a follow-up Zoom meeting with Mike Kelley revealed that as a WWTP owner he preferred a bacteria-based solution, on the basis that the use of bacteria in WWTPs is not a new concept and would be well-received.



*Algae in water from Mike Kelley's WWTP*



*Our initial meeting with Mike Kelley*

Additionally, while he did allow some algae to grow in his plant, he had received pushback from inspectors because of it. His advice and expertise therefore led the team to a different route than the initial algae-based solution. We then pivoted to a bacteria-based solution with the stakeholders (WWTP operators, owners, and workers) in mind. Upon choosing the bacteria-based solution, we had to select the specific bacteria to base our project on. Ultimately, we decided that *E. Coli* would best satisfy our needs, due to its extensive history and correlating research, as well as its previous use by the UCAS 2016 iGEM project which aimed to degrade the antibiotic tetracycline.

Another important choice that was largely influenced by experts in the field of wastewater treatment was the decision to target antibiotics or antibiotic resistant genes. According to various research studies, antibiotics and ARGs were both found to be present in the water (Griffen et.al

2020). However, our team was undecided if pursuit of the antibiotics or the genes would be more beneficial when opting for the most effective solution. Dr. Youneng Tang's continued correspondence and expertise helped guide us with this decision. Dr. Tang advised the team that creating a solution that attacked ARGs would be difficult to design. ARGs exist mostly inside the bacteria until the bacteria have been degraded, after which they then are free-floating genes. Dr. Tang qualified that a solution using bacteria to attack genes would be a novel approach, yet lacking in literature (i.e. specific concentrations of free-floating genes vs. genes inside the bacteria) to help support the solution. A bacteria-based solution to attack the antibiotics would therefore be a more feasible approach and had previous literature to support the solution.

Our final choice to pursue a bacterial solution was the result of various sources of input which offered multiple reasons for the course. Kelley and Dr. Tang both stated that it would be more feasible to implement the bacteria solution, due to the fact that the use bacteria as a means of treatment in the WWTP for degradation of other compounds is already practiced. Also, the algae solution was believed by them to be too inefficient, not only because the algae would require sunlight to function in the manner we wished it to, but also because the algae also does not filter antibiotics well in fast-moving water, making it unsuitable for most stages in wastewater treatment plant.

Next for our design, we had to choose which antibiotic/antibiotic family to target. Based on our initial inspiration for the project, Adam Schaefer's 2015 publication on dolphins, we discovered that the antibiotic erythromycin was cited as the greatest resistance among the bacteria isolates taken from the dolphin samples (Schaefer et al. 2015). Additionally, the continued presence of erythromycin in the numerous studies we analyzed allowed us to feel secure in our decision (Leitz and Meyer 2004). Erythromycin is used to treat various infections, such as respiratory tract infections and STDs (in particular, syphilis) ("Erythromycin: MedlinePlus Drug Information"). However, erythromycin is not the only antibiotic that our design targets; our product was designed to be able to target the macrolide antibiotic family. The macrolide antibiotic family includes erythromycin, clarithromycin, azithromycin, fidaxomicin and telithromycin ("Macrolide Antibiotics"). This makes our bacteria functional towards multiple different antibiotics, and therefore more effective. Our genetically engineered E. Coli produces the enzymes EreA and EreB to target and break down erythromycin as well as all other macrolides by hydrolyzing the lactone ring.

Finally, we considered the environmental, moral, and social consequences of the problem we chose as well as for the solution we designed. Our overall mission in undertaking this project, was to decrease antibiotic resistance and therefore mitigate the spread/rise in the environment. We engineered bacteria that breaks down macrolide antibiotics in order to fight antibiotic resistance. The possibility of this engineered bacteria escaping the WWTP and contributing to the very problem the team had chosen to help solve was an issue that needed to be addressed within the design. To account for this, we developed a toxin/antitoxin (TA) kill switch. The engineered bacteria produce a toxin that can destroy the bacteria as well as the anti-toxin. If the bacteria are in the WWTP, they can produce the toxin and antitoxin which are harmless together



and would not damage the engineered bacteria. If, however, the engineered bacteria escaped the WWTP and were introduced to the environment, the production of the anti-toxin ceases and only the toxin is produced, forcing the bacteria to degrade.

### Considerations for Our Final Design

There were pros and cons to the team's agreement to go forth with a genetically engineered bacteria solution. One positive for this solution is the ease of possible implementation. Being that there are already existing companies which sell natural bacteria to WWTPs, such as Aquafix, for purposes such as filtering and cleaning the wastewater, we expect less opposition in the implementation of our bacteria solution. Natural bacteria are integrated into the WWTPs by simply adding them into the wastewater and allowing them to do their desired job. While our bacteria are genetically engineered, they would still closely conform and follow the pre-existing standards of bacterial integration. Another positive to our bacteria solution is its versatility. If our team had chosen to pursue the algae solution, the number of possible WWTPs in which we could implement our solution in would be greatly reduced, due to the requirement of a bioreactor to be present in the WWTP for proper function. With the genetically engineered bacteria solution, the scope of possible implementation in WWTPs increases significantly, consequently increasing our project's ability to affect the most benefit.

Despite remaining superior to other options, the bacteria solution is not flawless. Before the final decision, the team was concerned about what implications our project could have on the public's perception of WWTPs. In recent years, the general public has caused major pushback regarding the implementation of genetically modified organisms (GMOs) in various sectors. Florida State University's 2019 iGEM team experienced this pushback from farmers when they suggested a GMO for their citrus greening problem. Since some WWTP's effluent is reused for drinking water or for irrigation systems, it is possible that our design could receive significant pushback from the public as well.

Furthermore, development of a TA kill switch with the engineered bacteria resulted in consequences that needed to be addressed. The TA kill switch works by converting methane into methanol using methane monooxygenase and then from methanol to formaldehyde using methanol dehydrogenase. The ParE2 uses a constitutive promoter, so it is not dependent on the environment, while the ParD2 uses a formaldehyde inducible promoter. In the WWTP, the formaldehyde will induce the antitoxin generator which will produce ParD2 (antitoxin), the ParD2 will then bind to ParE2 (toxin) which will create a harmless molecule. However, if our engineered bacteria does escape from the wastewater treatment plant, it will not have any methane to convert into formaldehyde, and thus will stop producing the antitoxin, leaving the toxin unbounded, and resulting in the bacteria's death. Since our system uses methane to convert methanol and formaldehyde, we were concerned that it could leave some methanol and formaldehyde in the effluent, which can be a health hazard. Methanol is flammable and formaldehyde is toxic to the human body. However, further discussion with Dr. Tang informed us that the methanol and formaldehyde by-products would be broken down within the WWTP

(by other bacteria or through various stages of water processing) and were also not tested/regulated for by the EPA/FDEP.

All considerations made were in accordance with our goal to prioritize the needs of the WWTP operators. Kelley emphasized cost (funding, or lack thereof, from government sources), ease of implementation (additional equipment to the plant), and a solution that had general support from various treatment plants. We aimed to create a design that addressed these needs. We also initially intended to design a solution that targeted a broad spectrum of antibiotics and their families. Ideally, a solution that targeted all the antibiotics present in WWTPs and reduced concentrations to the highest degree would have been our ideal goal. Since creating bacteria that produce multiple enzymes meant to attack various antibiotics would only hinder the bacteria's efficiency, we needed to find an alternative solution which still achieved our goal of addressing the largest possible spread of antibiotic pollution. This was satisfied by a team decision which settled on attacking one family of antibiotics instead of attacking all antibiotics. Our goal of maximum removal proved to be unrealistic, however, the team was successfully able to identify and design a solution which maintained the largest effects in addressing the issue of antibiotic resistance.

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