Evaluation of the Environmental Prevalence and Toxicity of Sulfonamides and Their Metabolites

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I pledge my word of honor that I have abided by the Washington College

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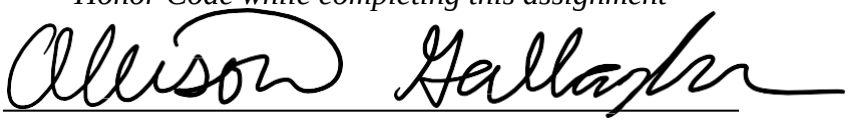

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Accepted in partial fulfillment of the requirements

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Dr. Leslie Sherman, *Thesis Advisor*

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Abstract

Antibiotics are one of the most used drugs worldwide. Sulfonamides (SNs) were the first class of antibiotics discovered, and remain among the top used antibiotics, as they are commonly used in humans, agriculture, and aquaculture. Due to the heavy use of not just antibiotics but SNs specifically, many SNs are found in the environment. Sulfamethoxazole (SMX) is one of the most prevalent in the environment out of all the sulfonamides. SMX is heavily metabolized by the human body, and thus many different metabolites are excreted into the environment. Some of these metabolites are inert, yet some are still active against bacteria, or can easily be converted back to the active SMX compound through either wastewater treatment, or microorganisms living within the human digestive tract. Once present in the environment, these antibiotics and their metabolites can cause many issues such as antibiotic resistance (ABR) and disruptions in aquatic organisms' endocrine and reproductive systems. These contaminants also lead to environmental pollution, which can disrupt entire ecosystems. ABR possess a great threat to the current ease of treatability of bacterial infections. This is a global issue that is rapidly growing. Many programs have been put in place to combat the ABR pandemic. However, the rate of antibiotic discovery needs to surpass the rate of bacterial evolution if the human race is to make any progress against this issue.

Table of Contents

Chapter 1: An Introduction to Antibiotics

The History of Antibiotics

Antibiotics are drugs that are commonly prescribed worldwide due to their wide array of different uses. These drugs are frequently employed in humans to prevent the growth of unwanted microorganisms.[1] Nearly 200,000 tons of antibiotics are used every year and this number is only growing bigger.[2] Between the years 2000 and 2010, antibiotic usage increased by about 36%. Approximately three-fourths of this increase came from the countries of Brazil, Russia, India, China, and South Africa alone.[3] Antibiotics are also heavily used throughout the agricultural industry as therapeutics and growth promoters.[4] Throughout 2020, over 160,000 tons of antibioticswere given to farm animals worldwide.[5] This number is projected to be 200,000 tons by 2030. [5] In2010, the projection for 2030 was 105,000 tons, showing that this number is increasing more rapidly than originally anticipated.[6]

Many believe that Alexander Fleming's discovery of penicillin was the first antibiotic released to the public. However, there were several other drugs that were used for treatment before penicillin.[7] Although the effects of penicillin were discovered in 1929, it took Fleming nearly 15 years along with the help of Ernst Chain to be able to isolate penicillin from the mold that produced it so that it could be incorporated into a drug.[7] Clinical trials for penicillin did not begin until 1943. Meanwhile, one of the first antibiotics widely used was a sulfonamide named Prontosil (Figure 1) which was released in 1935.

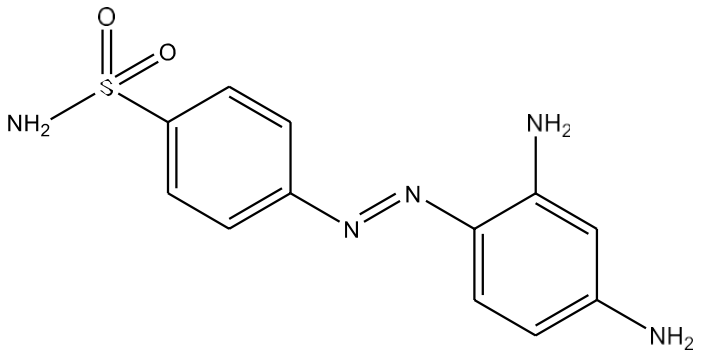

Figure 1. The structure of Prontosil, the first sulfonamide.

Gerhard Domagk was the first to show that Prontosil was inactive against *Streptococcus pyogenes* in vitro, however it became active against these bacteria in vivo.[7] This showed that Prontosil required some sort of metabolism before it was effective. This drug is part of a large group of antibiotics that are still commonly used today called sulfonamides (SNs). Sulfonamides became a crucial tool to save soldiers throughout World War II, and after the war became the first antibacterial medication to be used on a large scale to treat many infections.[7] Although Prontosil has been replaced with more effective treatments, many of these sulfonamides are still used today.

Following the discovery of Prontosil, the late 1930s through the 1950s became a time of rapid antibiotic discovery. This period has been coined the "Golden-Age" of antibiotics. The post-World War II era brought the discovery of many new classes of antibiotics leading up to the 1970s. From the year 1944 to 1972, human life expectancy increased by over 8 years.[7] Many scientists have credited a large portion of this sharp increase to the development and widescale use of antibiotics.[7]

By the 1980s many researchers started to look at how to increase the effectiveness of the antibiotics currently on the market rather than continuously discovering new ones. This shift

largely had to do with the onset of antibiotic resistance.[7] Fleming had originally warned the world about the dangers of antibiotic resistance when accepting his Nobel Prize in 1945, and the problem has continued to grow ever since.[8] His speech included the following words of caution: "the time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant".[9]Antibiotic resistance poses a massive problem in the treatment of bacterial infections. When bacteria become resistant to the drugs that are typically used to treat them, getting rid of these infections becomes increasingly difficult. Although it was rare in the 1980s when the issue first surfaced, it is a very common issue today.[9]

Throughout the 80s and up until 1992, the awareness of antibiotic resistance and the issues it was causing continued to grow. The main issue caused by increased antibiotic resistance is that this can lead to diseases that are continuously harder to treat with antibiotics.[2] As it became increasingly obvious that something had to be done about this issue, several different reform groups began to materialize with the goal of increasing awareness and decreasing antibiotic resistance. Steps to decrease antibiotic resistance include educating the public on the proper use of antibiotics, health care professionals only prescribing these drugs when necessary, and farmers only using antibiotics to control infection in livestock rather than to stimulate growth.[10] However, these groups, such as the Alliance for the Prudent Use of Antibiotics, were largely unsuccessful until this issue became fully globalized and politicized in 1992.

By 2001 the Senate had produced a task force that developed a plan entitled "Public Health Action Plan to Combat Antimicrobial Resistance". This plan consisted of 84 action items including increased surveillance of the issue, increasing research and funding in the field to develop new antibiotics that the bacteria were not resistant to, and decreasing antibiotic prescriptions.[11] There

have been several new antibiotics introduced within the past several years, however the pace of antibiotic discovery is not keeping up with the rapid growth of antibacterial resistance.[10] There is a concerning lack of economic incentive within the antibiotic research community. Due to this lack of funding, many large pharmaceutical companies have begun to shut down their antibiotic departments, and thus the academic community is expected to take over. However, this community is also struggling with a lack of funding and are therefore unable to keep up with the need for antibiotic discovery.[12]

Antibiotics Throughout the Environment

Despite these actions being taken, antibiotics can still be found throughout the environment, which heavily contributes to antibiotic resistance. These antibiotics can be found in concentrations that are toxic to the biota present. Some waste water treatment plants have mechanisms to remove antibiotics, however the concentration of antibiotics found in the environment suggests that the treatment plants are not as effective as hoped.[13] Because of this, the highest concentrations of antibiotic resistant bacteria can be found in and around waste water treatment plants.[1] Concentrations of antibiotics that are typically found in the environment may not be toxic to humans, but can be extremely detrimental to wildlife.[3] This is due to the fact that these pharmaceuticals are designed to be readily absorbed by the body and cross many biological membranes. Pharmaceutical compounds are often designed with Lipinski's rule of five in mind. Lipinski's rule of five includes four rules that increase the chances of the molecules being membrane soluble, and thus absorbed by the body. These rules include: the molecular weight is less than 500 Da, the lipophilicity of the molecule (logP) is less than 5, there are less than 5 hydrogen bond donors, and less than 10 hydrogen bond acceptors.[14] Since most drugs are designed with these parameters in mind, they are absorbed at a high rate by organisms in a contaminated environment.[3]

Additionally, due to disposal of unused medications, landfills tend to have high concentrations of antibiotics. For example, Figure 2 shows the top 25 pharmaceutical and personal care products (PCPPs) reported in landfill leachates around the globe from 1993 to 2018.[15] Nearly half of these are antibiotics, more than any other category. The three most common antibiotics in the landfill are sulfamethoxazole, sulfamethazine, and sulfadiazine.[15] These three drugs are all a part of the antibiotic class of sulfonamides, which will be the focus of this paper.

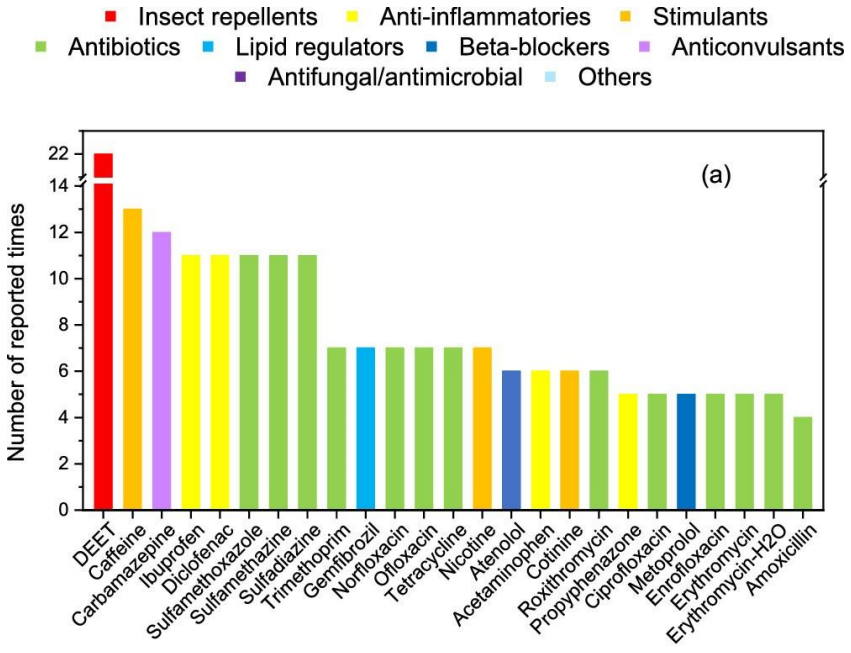

Figure 2. The number of times each of the 25 most frequently environmentally present pharmaceutical and personal care products (PPCPs) were reported in landfills.[15]

Chapter 2: Structure and Function of Sulfonamides

The Uses of Sulfonamides

Sulfonamides can be used to treat a wide array of bacterial infections. The most common of these infections include urinary tract infections, pneumonia, and bacterial meningitis. Sulfonamides are also commonly used to control seizures and other medical ailments.[16] These sulfa drugs can also be used topically rather than orally to treat ear and eye infections along with burns and vaginitis. One major drawback of sulfonamides is their potentially serious side effects. Taking too many sulfa drugs can cause kidney damage or the development of hepatitis, amenia, or other blood disorders.[16] Since these drugs were among the first developed, most remain relativelysimple to produce, and therefore affordable. Their low price point makes them a common treatment in many developing countries.[16]

Sulfonamides are also heavily used in veterinary medicine. In China alone, over 6,000 tons of antibiotics are used annually in agriculture.[17] Sulfonamides are among the most used antibioticsin this field. Globally, sulfa drugs used in veterinary medicine account for 2.3% of the total numberof antibiotics used yearly. These drugs are often used as feed additives to improve growth.[17] This is again due to their low cost and effectiveness against common bacterial infections.[18]

While sulfonamides alone are not widely used in humans anymore due to high bacterial resistance, a combination drug sold under the brand name of Bactrim contains a mixture of sulfamethoxazole and trimethoprim. This antibiotic is one of the most commonly prescribed drugs used to treat urinary tract infections (UTIs).[19] The sulfamethoxazole in Bactrim works because it is structurally similar to para-aminobenzoic acid (PABA). PABA typically binds to dihydropteroate synthase to convert PABA into folic acid within the bacteria. Sulfamethoxazole competes with PABA for the binding site on this enzyme, and thus folic acid production is greatly

decreased, and bacterial growth slowed.[20]

Additionally, sulfamethoxazole (SMX) is one of the most used sulfonamides in veterinary medicine, in both agriculture and aquaculture (Figure 3) and thus sulfa drugs are still of high concern due to their promotion of antibiotic resistance and toxicity to other organisms. Out of all the sulfonamides, SMX is the most resistant to biodegradation in the environment. This drug was found to have a half-life of up to 72 days in water.[13] Any compound having a half-life of over 2 months in water is considered a persistent organic pollutant (POP), making this drug of high concern. Since the half-life is so long, this drug can continue to accumulate in the environment at a faster rate than it is being biodegraded.

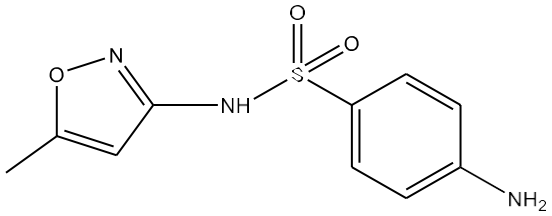

Figure 3. The structure of sulfamethoxazole (3-sulfanilamido-5-methyl-oxazole).

The Chemical Properties of Sulfamethoxazole

The biodegradation of SMX can be affected by the environment's microbial activity, light exposure, degree of oxygenation, pH, and the concentration of other drugs present in the environment.[13] Higher temperatures have also been shown to increase biodegradation, but mostly due to an increase in biodiversity and micro-organism activity at these higher temperatures.[13] One study computed the minimum environmental half-lives of SMX and its metabolites during both aquatic summer and winter conditions. Both half-lives are shorter than the half-life of SMX in water, mentioned above, of 72 days.[13] During the summer at 47° N latitude, the half-lives were calculated to be anywhere from 1.3 hours to 14 days. Contrastingly, these numbers were

calculated to be 6 hours to 63 days on a typical winter day.[21] Under acidic conditions, SMX and most of its metabolites undergo photolysis approximately 30 times faster than in a neutral environment.[21] These acidic conditions most likely speed up the electrophilic substitution mechanism of the biodegradation.[13] This process will not occur under sterile conditions which suggests that it is not a hydrolysis reaction.[13] Not only is sulfamethoxazole biodegrading in the environment, but this parent compound is metabolized in the body, and several metabolites are also excreted into the environment.

When ingested, on average, 14% of sulfamethoxazole is excreted in the original form, the rest being metabolized by the body.[13] On the whole, 90% of antibiotics are excreted in the drug's original form.[2] This shows that SMX is much more heavily metabolized in the body than the typical antibiotic. About 90% of SMX and its metabolites are eliminated through renal excretion.[22] There are five known major metabolites of the drug, as shown in figure 4.

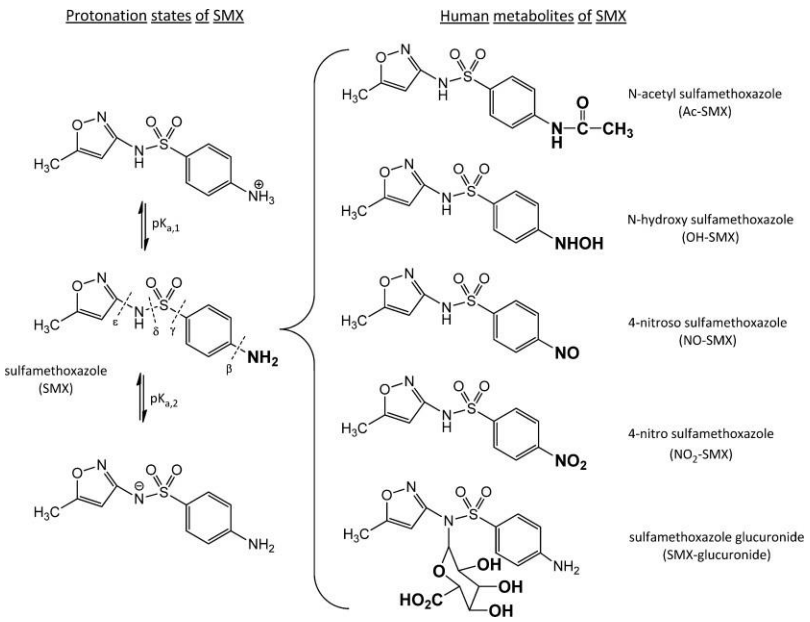

Figure 4. Major known metabolites of sulfamethoxazole. The left panel also shows major cleavage points on the SMX molecule.[21]

Most of this metabolism takes place in the liver and is mediated by cytochrome P-450.[22] The most common of these metabolites is N-acetyl sulfamethoxazole (Ac-SMX), accounting for about 50% of the metabolite products. During wastewater treatment, this compound can be transformed back into the original SMX molecule. Sulfamethoxazole glucuronide (SMX-glucuronide) accounts for about 9% of the product, and N-hydroxy sulfamethoxazole (OH-SMX) accounts for 2.2%. OH-SMX has also been shown to be reduced back to the parent drug by microorganisms living in both the human and pig livers through an enzyme system involving cytochrome b_5, nicotinamide adenine dinucleotide-cytochrome b_5 reductase, and CYP2D.[23] The least common metabolites of SMX are 4-nitro sulfamethoxazole (NO_2-SMX) and 4-nitroso sulfamethoxazole (NO-SMX. The later of the two is even more toxic than SMX itself.[21] NO-SMX can be transformed back into SMX in the presence of an electron donor such as environmental organic matter or chloride.[21] OH-SMX can also be converted quite easily to NO-SMX under both neutral and basic environmental conditions.[21]

One study looked at the effects of pH conditions on excretion of SMX. Changing the pH within the body had several different effects on the absorption, metabolism, and excretion of SMX which can be seen in figure 5.[22] The half-life of elimination was longer (11 hours) under acidic urine conditions compared to a half-life ($T_{1/2}$) of only 9 hours under alkaline conditions.[22] Additionally, the SMX was absorbed faster under basic urine conditions than acidic. Basic conditions also caused a greater percentage of the parent compound to be excreted. Under urine conditions of pH between 7.5-8.5, 36% of the original parent compound was excreted at SMX, whereas under acidic urine conditions between pH 5.5-6.0, only 9% of the parent compound was excreted as SMX.[22]

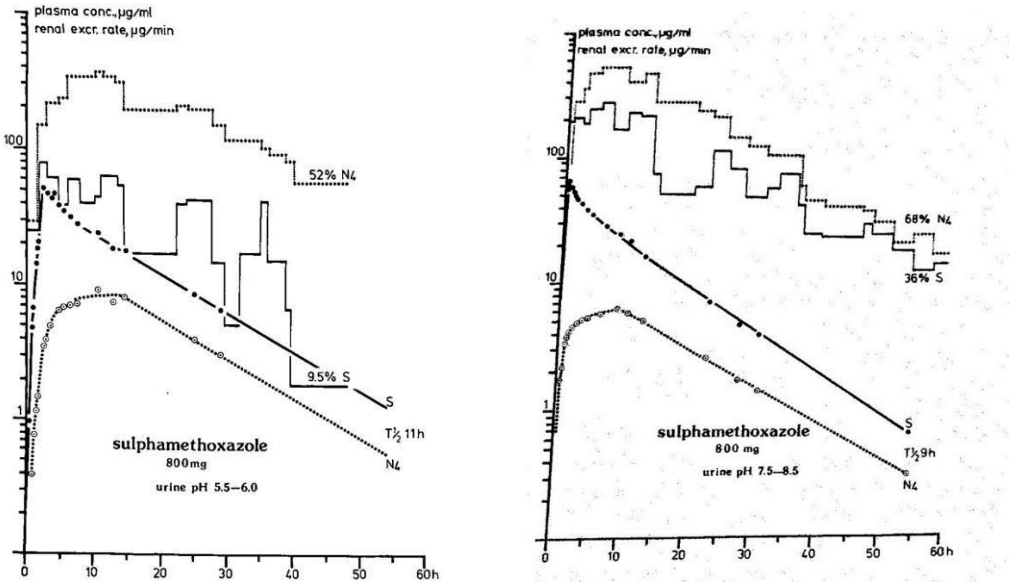

Figure 5. The curved lines in each graph represent the plasma concentration (y-axis) over time (x-axis) of sulfamethoxazole (S-solid lines) and its main metabolite, N-acetyl sulfamethoxazole (N4-dotted lines) in µg/mL. The jagged lines show the renal excretion rates (y-axis) over time (x-axis) for both SMX and Ac-SMX. The left graph shows these parameters under acidic conditions and the right graph represents basic conditions.[22]

The degradation of SMX and these major metabolites can produce many different products. Much of this degradation occurs as photolysis within the environment. Scientists have shown that the primary mechanism of SMX photolysis is cleavage of the molecule at several different points (Figure 4, left panel). However, the major mechanism of SMX photolysis, with a conversion of 20%, is δ-cleavage to produce sulfanilic acid, shown in figure 6.[21]

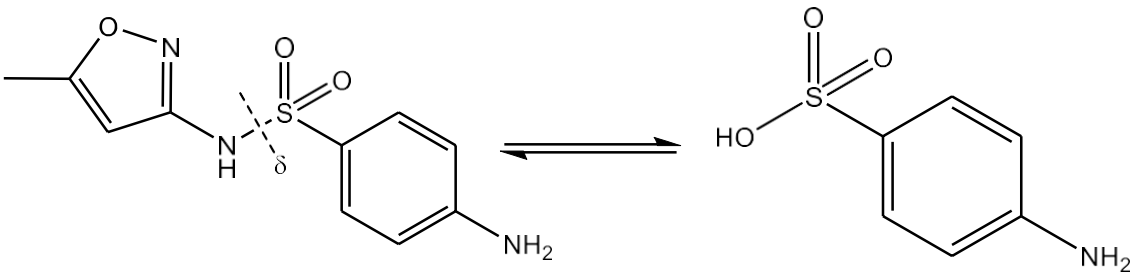

Figure 6. δ-cleavage of sulfamethoxazole during photolysis to create sulfanilic acid.

Cleavage reactions tend to be the predominant reaction in the photolysis of SMX and Ac-SMX. However, in NO_2-SMX and NO-SMX, the nitro group is very resistant, and therefore these metabolites undergo forms of substituent modifications.[21] All major photolytic products of SMX and its major metabolites are given in figure 7.

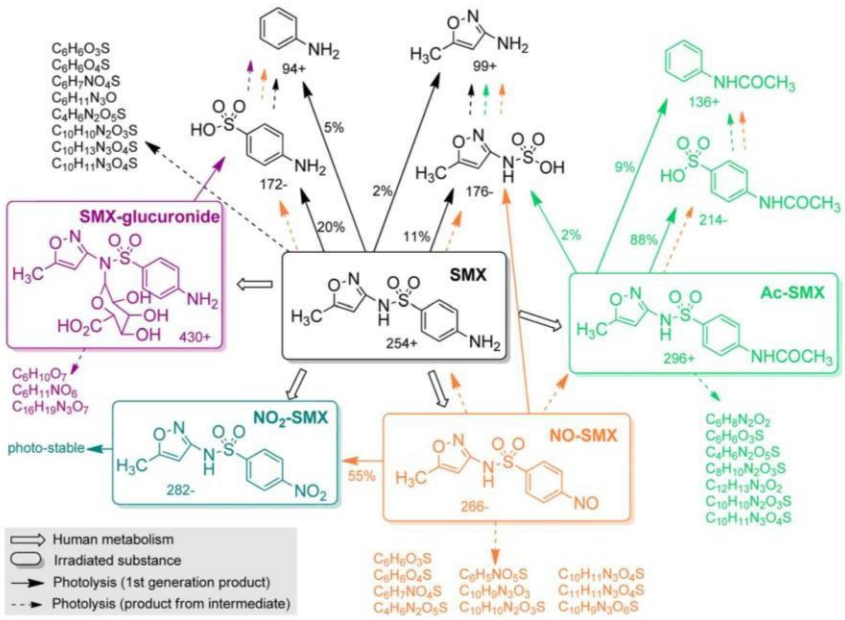

Figure 7. Photolysis products of SMX and its human metabolites. Each metabolite of SMX is represented in a different color. OH-SMX is not shown because in basic and neutral environments, it is very quickly converted to NO-SMX in the presence of oxygen. [21]

There have been three proposed mechanisms of SMX metabolism in the microalgae species *Scenedesmus obliquus*. In the major pathway, the C-N bond is cleaved, similar but not identical to the δ-cleavage of SMX seen in photolysis represented in figure 6. This intermediate, benzenesulfonamide, can then either be deaminated or methylated. Methylation creates ortho-toluenesulfonamide which is then hydroxylated to produce the final metabolite. Both benzenesulfonamide and ortho-toluensulfonamide have been found in waste, ground, and surface waters.[24] In an alternate pathway, SMX can be deaminated to form deamino-SMX. A third pathway yields 4-nitro-SMX through nitrosation of the primary amine in SMX.[24] These pathways are summarized in figure 8. Several other studies have shown the presence of these metabolic intermediates in the environment, suggesting that these microalgae have a significant influence on the breakdown of SMX present in the environment.[24]

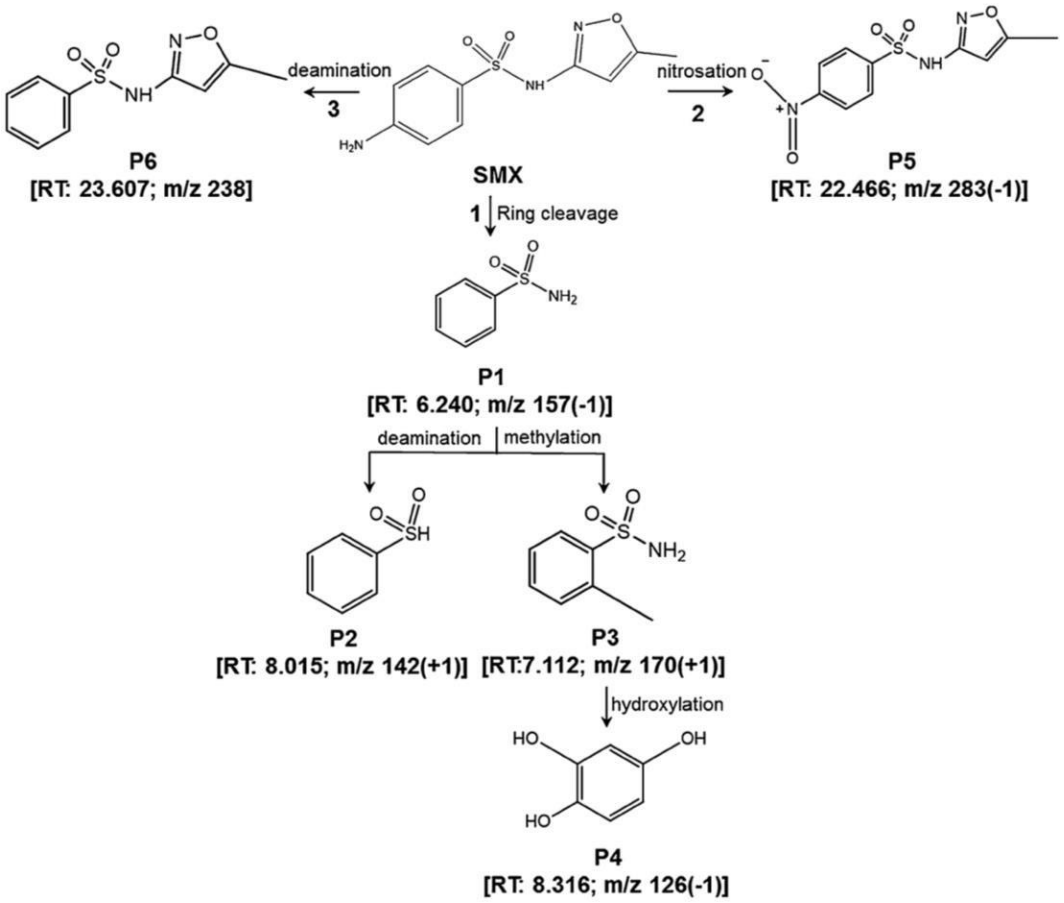

Figure 8. The three proposed metabolic pathways of sulfamethoxazole in *Scenedesmus obliquus*.[24]

Not only are humans ingesting this drug knowingly, but crops can also accumulate sulfonamides in their edible tissues, posing another route of exposure to these drugs. Corn, cucumbers, tomatoes, carrots, and lettuce are all very widely eaten crops that have been shown to accumulate sulfonamides. Sulfonamides are more commonly taken up by plants than any other antibiotic.[25] Since these antibiotics are largely metabolized by the plants, exposure of antibiotics through consumption of crops has largely been ignored due to low concentrations of the parent compound. Figure 9 represents the many different metabolites of SMX in the *Arabidopsis thaliana*, a plant often used to model plant behaviors.[25] However, these metabolites can be converted back

to the parent compound easily during the human digestion of the eaten crop in the low-pH environment of the gastric and intestinal portions of the digestive tract.[25] This has been shown to occur within the first 30 to 60 minutes that these compounds are present in an in vitro Rumen Stimulation System.[25]

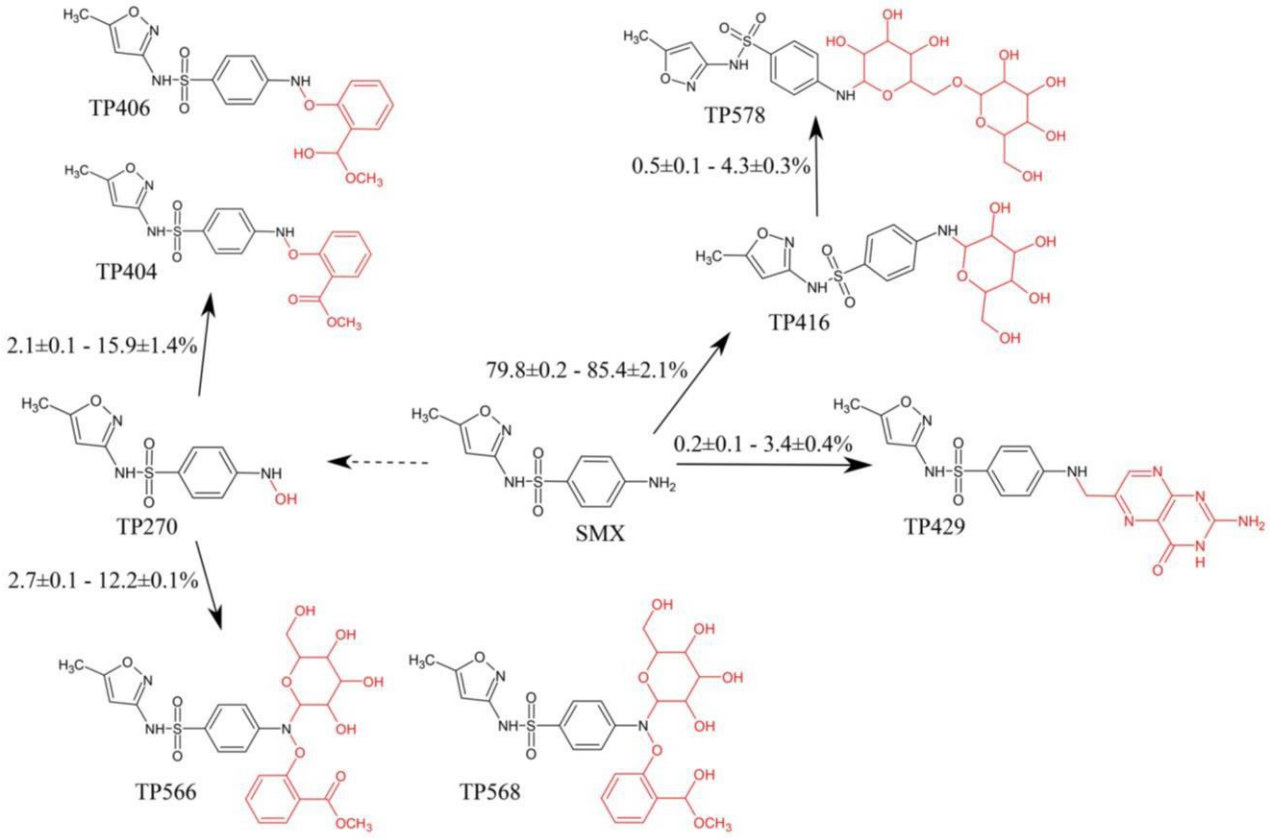

Figure 9. Metabolites of SMX in *Arabidopsis thaliana.*[25]

The Chemical Properties of Sulfadiazine

Sulfadiazine is also a very commonly used sulfonamide, especially in animal agriculture. Agriculture can be yet another major route of antibiotic exposure to humans and, therefore, it is important to look at how animals metabolize these drugs. Exposure through animals can come through both their excretion of antibiotics into the environment, and through humanconsumption of animal products. Figure 10 shows the two major metabolites of sulfadiazine

produced in pigs. The main metabolite is N-acetyl sulfadiazine, a compound that is no longer active against bacteria. However, there is evidence that once in the environment, microorganisms can cleave the added acetyl group to transform this compound back to the parent drug, thus becoming active once again. This retransformation has been shown to occur during manure storage, as well.[26] Traces of an alternate compound, 4-hydroxysulfadiazine, was also found as a metabolite of sulfadiazine in pigs. This compound does have microbial activity. These metabolites were found in both excreted urine and fecal matter of pigs, making them ever more difficult to contain.[4]

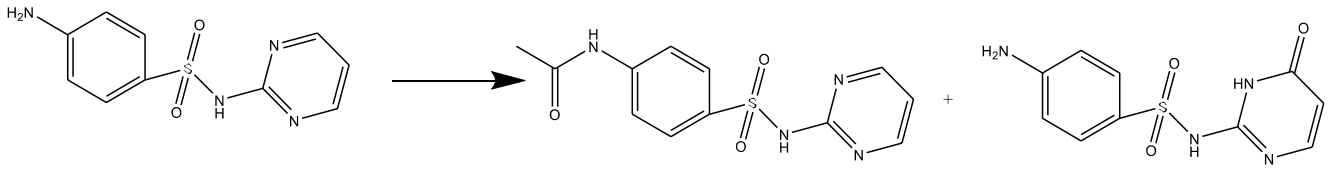

Figure 10. Sulfadiazine is metabolized into N-acetyl sulfadiazine and 4-hydroxysulfadiazine in pigs.[4]

Overall, many of these metabolites, whether they come from humans, plants, or animals can be active against microbes in their current form, or easily transformed back into the parent drug which possess strong antimicrobial characteristics. Once excreted, these compounds are released into the environment and remain there until they are biodegraded, undergo photolysis, or are taken up by aquatic or terrestrial organisms. The presence of so many antimicrobial agents in the environment is one of the leading causes for antibiotic resistance in bacteria.[2] Actions have been taken to prescribe fewer antibiotics. However, the prescription of antibiotics is not the only mechanism through which bacteria are exposed to these drugs. Therefore, it is important that researchers not only study the impact antibiotics have on humans, but also their impact in the environment. Although antibiotic resistance is the major concern of antibiotics in the environment, these drugs can cause a myriad of other issues.

Chapter 3: The Concentrations and Effects of Sulfonamides in the Environment

Presence of Antibiotics in the Environment

The presence of antibiotics in the environment causes many adverse effects. One of the reasons why this issue is so prevalent is that there are many ways for pharmaceuticals to enter the environment. The more ways in which these drugs enter ecosystems, the harder they become to regulate. Many of the inputs into the environment come from waste-water treatment plants (WWTPs). Not only are the parent drugs and their metabolites present in sewage due to human excretion, but expired or unused drug are often improperly disposed of through the act of being flushed down the toilet.[2,3] The highest concentrations of antibiotic resistant bacteria discovered throughout the environment were found to be at WWTPs.[1] As previously discussed, antibiotics are used heavily in agriculture and aquaculture, and animal excretion of the parent drug and its metabolites is another way pharmaceuticals are introduced into the environment.[3] Up to 58.5 mg of sulfonamides are used to produce 1 kg of meat.[13] Additionally, antibiotics are often used to control bacterial infections in plants either through direct injection or soil drenching.[2] Soil drenching is a process that directly introduces antibiotics into the environment, with the farmer having little control over how far these drugs leach into the surrounding soil. The manure used to fertilize crops can also be contaminated with pharmaceuticals that were given to the animal through the feed, and the metabolites of these drugs.[2] Concentrations of sulfonamides in manure can reach up to 400 mg kg^{-1}.[1] Crops that are exposed to pharmaceuticals often bioaccumulate these compounds, as shown through the study with *A. thaliana* mentioned above, and can then expose humans to even higher concentrations than what can be found in the environment.[2] Moreover, areas where these antibiotics are produced can contain high environmental concentrations due to improper disposal of waste products.[3]

Many studies have shown that antibiotics are the most concentrated class of pharmaceuticals within the environment. One such study looked at the concentrations of several different classes of pharmaceuticals in the plasma and bile of fish.[3] These numbers are different than the concentrations found in the environment due to bioaccumulation, but they allow for comparisons. The most concentrated drug was found to be lincomycin, often used to treat bacterial infections in humans who are allergic to penicillin. The concentration of lincomycin within the fish was measured to be 567 ng mL^{-1}.[3] Sulfonamides were also detected in this study. Sulfamethazine was found at a concentration of 144 ng mL^{-1}.[3] Comparing these concentrations to the next most concentrated class of drugs shows just how concentrated antibiotics can be within the environment. Nonsteroidal anti-inflammatory drugs (NSAIDs) were the next most concentrated class with a concentration within the fish plasma of only 16.5 ng mL^{-1}.[3] Out of all fish that were found to have a detectable concentration of pharmaceuticals in their body, 35% of the time this pharmaceutical was an antibiotic.[3] An alternate study showed that sulfamethazine had the highest concentration out of all antibiotics within invertebrates with a measured concentration of 430 ng mL^{-1}.[3] Concentrations of sulfamethoxazole have been measured in freshwater as well. Since these measurements were taken straight from the water, rather than an organism, they are lower due to the lack of bioaccumulation, but still detectable. Sulfamethoxazole concentrations in freshwater can range from 259.60ng L^{-1} to 385.00 ng L^{-1}.[6] This proves that not only are fish exposed to these drugs through aquaculture, but also in their everyday natural environments.

The Effects of Antibiotic Exposure from the Environment

Overexposure to pharmaceuticals in the environment has been shown to cause a myriad of problems in organisms. The most predominant issues include but are not limited to changes in behavior, and disruption of the endocrine and reproductive systems.[2] When the reproductive

system of an organism is not functioning properly, this could threaten the survival of the species.[2] However, these pharmaceuticals typically do not exist alone in the environment. One study showed that an average of 26 different pharmaceuticals were found in sewage treatment plants across the United States.[27] This proves that it is also important to study the possible synergistic effects of mixtures of pharmaceuticals, yet the effects of many of these drug cocktails are unknown.[27] Unknowingly ingesting two or more different drugs through contaminated food or water could have potentially harmful effects due to drug to drug interactions.[2] A cocktail that has been studied is the mixture of acetaminophen, carbamazepine, gemfibrozil, and venlafaxine. This mixture consisting of common classes of antibiotics at environmentally relevant concentrations was shown to have significant impacts on cumulative embryo production in zebrafish.[1] This shows the synergistic effects of mixtures of antibiotics. However, these drugs can cause adverse effects on their own as well.

Whether or not these effects are imposed on humans directly or indirectly, they could eventually affect humans, nonetheless. For example, in concentrations less than 0.1 mg L^{-1}, antibiotics have been shown to slow algae growth immensely. This leads to a decrease in the number of algae present, and since algae is such a crucial component in the food web within aquatic environments this can affect the entire ecosystem resulting in seafood shortages for humans.[2] Antibiotics used to treat bacterial diseases in aquaculture can lead to decreased growth, body malformation, microbiota dysfunction, and immune suppression in fish. They can also induce oxidative stress, affect antioxidant capacity, and trigger DNA damage.[28] Additionally, high aquatic concentrations of antibiotics have been proven to lead to fish population disturbances and endangered species extinction.[28] These concentrations can bioaccumulate within the fish or other human crops, and unknowingly ingesting these antibiotics can lead to

increased antibacterial resistance within the bacteria present inside the human body.[2] Typically, this would not be an issue until an opportunistic prokaryote surfaces. These organisms are always present within the body but can only cause problems when the host's health declines.[29] If these bacteria are antibiotic resistant, the disease states they cause are much more difficult and potentially more expensive to treat. For many bacterial species, it does not take much exposure time to develop antibiotic resistance due to their extremely fast reproduction rate.

Chapter 4: Sulfonamide Toxicity

Sulfonamide Toxicity in Bacterial Species

Since sulfonamides are designed to kill bacteria within the human body, it is also important to know how toxic these drugs are towards bacteria within the environment. In one study, Adameck et al. assessed the development of antibiotic resistance of several sulfonamides (SNs) in bacteria. These experiments found that after 18 hours of exposure to 3.9 mg L^{-1} of SNs significant growth inhibition of micro-organisms was observed. When increased to 15.6 mg L^{-1}, the inhibition reached 42%. After 48 hours, the inhibition dropped below 20% for both concentrations (Figure 11). In fact, the growth inhibition after 48 hours was less than after only 18 hours for every concentration of SNs studied in both species of bacteria. These results strongly suggest that the organisms had undergone an adaptation to become resistant to the toxic effects of the sulfonamides.[13]

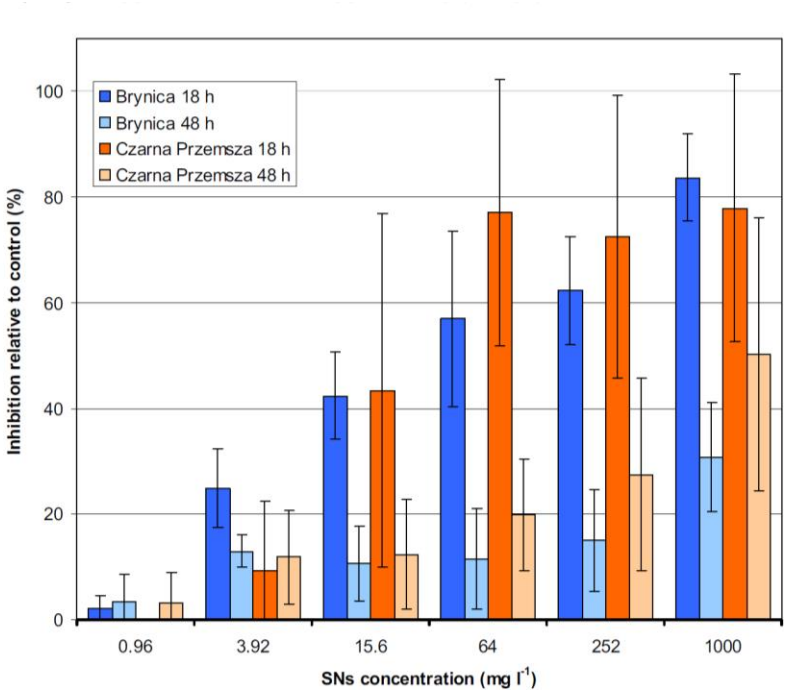

Figure 11. The growth inhibition of sulfonamides in two different species of bacteria over 18 hours compared to 48 hours.[13]

Sulfamethoxazole and Sulfamethazine Toxicity in Microalgae

Xiong et al, conducted a separate study that looked at the toxicity of SMX along with another common sulfonamide, sulfamethazine (SMZ) in a species of microalgae named *Scenedesmus obliquus*. This study also observed the effects of these two drugs are combined, as they are often found this way in the environment.[24] These scientists observed that in concentrations between 0.075-0.2 mg L^{-1} SMX significantly inhibited *S. obliquus* growth anywhere from 25.7-83.0% after 96 hours when compared to the control.[24] These drugs inhibit growth by causing damage to the organisms' cells. SMX can impair cell structures, cause an overproduction of reactive oxygen species, disintegrate cell membranes, degrade nuclear material, inhibit metabolic activities such as photosynthesis, and affect the energy production within the chloroplasts and mitochondria.[24]

In the above study, the concentration at which the effects of the drug are halfway between no effects (baseline) and the maximum effect possible (EC_{50}) was calculated after 96 hours of exposure. The EC_{50} of SMZ was calculated to be 1.23 mg L^{-1}, SMX to be 0.12 mg L^{-1}, and the mixture of the two to be 0.89 mg L^{-1}.[24] An EC_{50} below 1 mg L^{-1} is said to be very toxic to aquatic organisms while anything between 1 and 10 mg L^{-1} is said to be toxic. These classifications mean that SMX, and the mixture of the two drugs are both considered very toxic, and SMZ on its own it still considered toxic.[24] The risk quotient (RQ) shows how dangerous the compounds are ecologically. This is calculated by dividing the measured environmental concentration by the predicted no-effect concentration of the drug.[24] If the RQ is greater than 1, the compound possesses a severe risk to the environment. The RQ of SMZ in wastewater is 187.7, SMX in wastewater is 111.2, and antibiotic mixture RQs can reach up to 273.5, proving that at high concentrations, these antibiotics can be severely hazardous.[24]

Sulfamethoxazole Toxicity in Fish Species

A separate study conducted by Limbu et al. saw similar effects of SMX in a fish species, *Oreochromis niloticus*, that Xiong et al. had found in their species of algae. In this study, fish were exposed to two separate concentration levels of a mixture of SMX and oxytetracycline (OTC). The first level was the low environmental concentration (LEC) of SMX/OTC which represents the legal limit of antibiotics allowed to be present in the environment. Fish were exposed to 260 ng L^{-1} of SMX and 420 ng L^{-1} of OTC in a water bath to stimulate the exposure route of LECs. The other route of exposure was the legal aquaculture dose (LAD) of SMX/OTC mixture, conducted by feeding each fish 100mg/kg body weight/day of SMX and 80mg/kg body weight/day of OTC.[28]

Overall, this study found that both concentrations of SMX/OTC had adverse effects on fish growth, lipid body composition, and nutrient digestibility.[28] Expression of the proteins that regulate the tight junctions in *O. niloticus* intestine, which allow for the passage of larger molecules and xenobiotics into the body, significantly decreased.[28] The microbiota of the intestines was also largely affected. The exposure to SMX/OTC at both concentrations also impaired the liver's detoxification mechanism, increased oxidative stress, and caused DNA damage.[28] The immune response in the fish exposed decreased while inflammation increased.[28] This occurs because the expression of macrophage migration inhibitory factor (MIF) is upregulated in the liver. This cytokine stimulates inflammatory pathways by binding to a transmembrane protein called CD74 as well as upregulating other pro-inflammatory cytokines.[28,30] Increased expression of MIF can also lead to cancer through binding to p53, an important tumor suppressor and preventing cellular apoptosis of mutated cells.[31] This study also found that exposure to both LECs and LADs of SMX/OTC affected the metabolism of glucose and lipids within the fish.[28] Lastly, this study looked at the human risk of eating fish exposed to these levels of concentrations. It was found that

only fish exposed to LADs posed any threat to human health, especially in children.[28] This is of concern because these concentrations are legally allowed to be used in aquaculture and, therefore, humans can very easily consume a fish fillet with this concentration of antibiotics present. A summary of these findings can be found in figure 12. As the image shows, the impacts of exposure to SMX are vast and affect many different crucial functions within an organism.

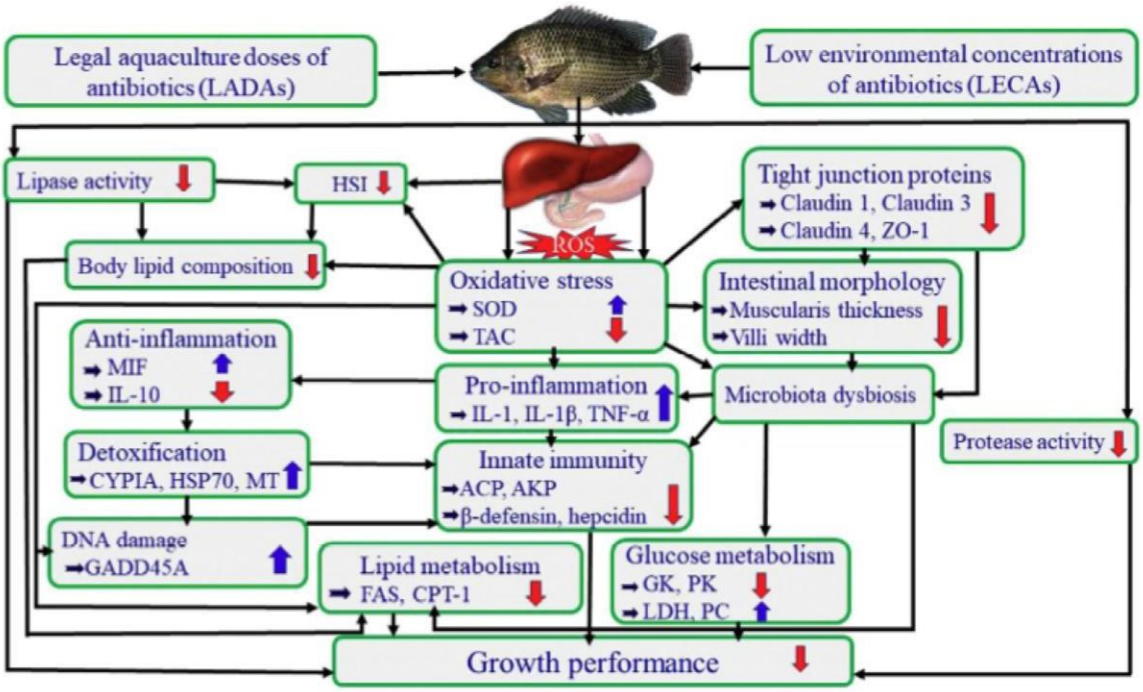

Figure 12. The effects of exposure of *O. niloticus* to legal aquaculture doses of antibiotics (LADAs) and low environmental concentrations of antibiotics (LECAs).[28]

Chapter 5: Actions to Prevent Antibiotic Resistance

Programs to Prevent Antibiotic Resistance

The major lack of funding within the antibiotic research community is one of the major reasons that humans are struggling to keep up with the growing rate of antibiotic resistance. In 1990, there were 8 large pharmaceutical companies working on antibiotic development, however, in 2020 this number has fallen to 4.[32] Many companies have withdrawn from this field due to a lack of profit. However, there have been several global initiatives established to attempt to increase the funding within this field. The major initiatives include the Innovative Medicine Initiative's (IMI) "New Drugs for Bad Bugs" program, the Global Antibiotics Research and Development Partnership (GARDP), the Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator (CARB-X), and Novo Holdings' "REPAIR Impact Fund".[12]

Many of these programs have similar goals with different approaches. The current goal of GARDP is to make infection treatable for everyone, everywhere.[33] The primary focus of this program lies on sexually transmitted infections, sepsis in infants, and infections in hospitalized children and adults.[33] CARB-X is a program focused on preclinical discovery and development as well as Phase 1 clinical trials of newly discovered antibiotics.[32] Additionally, another obstacle is the fact that access to antibacterial assays and expertise is limited---most services at the moment require a fee. The Community for Open Antimicrobials Drug Discovery (CO-ADD) initiative in Australia attempts to fix this issue.[12] This initiative allows scientists access to free antimicrobial screening, and since its beginnings in 2015 has received over 300,000 samples from 47 different countries.[12]

The IMI was first developed in 2008 to help close the nearly 20-year gap between bacterial evolution and the development of new antibacterial strategies.[34] Since this program's beginnings, it has launched several programs to combat this issue, including the "New Drugs for Bad Bugs"

(ND4BB) program. Launched in May of 2012, this program uses 7 cross-linked research topics to assist in discovering and developing new and effective antimicrobial treatments (Figure 13).[34] The IMI has put several other programs in place as well. The TRANSLOCATION program, launched in January 2013, aims to assist researchers in the discovery and optimization of Gram-negative bacterial infection treatments through the sharing of information on the mechanisms through which drugs enter and exit Gram-negative bacteria.[34] The European Gram-negative Antibacterial Engine (ENABLE) also helps with discovery of new treatments against resistant Gram-negative bacteria. The program does this by assisting drug discovery projects through the discovery phase via collaboration.[34] The IMI has many other programs underway and many others still under development.

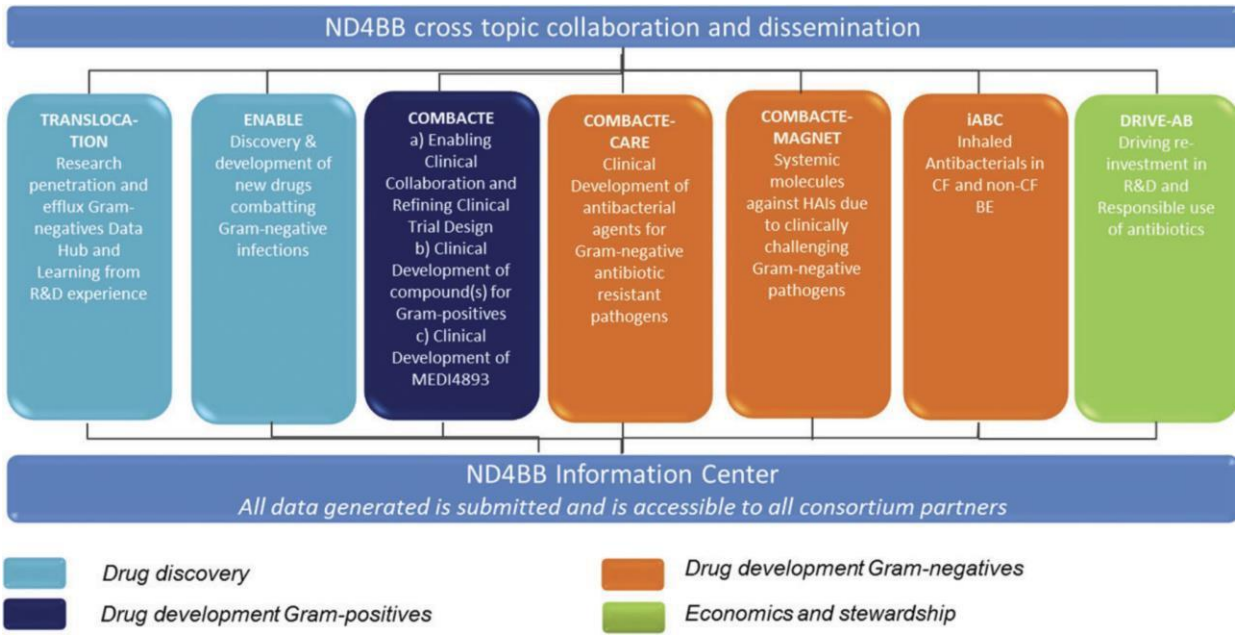

Figure 13. The "New Drugs For Bad Bugs" (ND4BB's) 7 research topics.[34] (BE=bronchiectasis, CF=cystifibrosis)

In the United States the Food and Drug Administration (FDA) passed an act entitled the Generating Antibiotics Incentives Now Act (GAIN) that allowed for certain antibacterial or antifungal drugs that aim to treat serious, life-threatening infections to be considered "Qualified Infectious Disease Products" (QIDPs). As a QIDP, a drug receives priority review status along with the possibility for a fast-track designation, allowing the drug to be on the market up to five years early than if it were to go through the typical drug review process.[35] These programs are promising, however more participation globally needs to occur for them to work effectively.

Not only is it important to prevent the spread of antibiotic resistance, but it is also important toprevent these antibiotics from entering the environment in the first place. Although it is difficultto find actual regulations placing limits on the concentrations of drugs that are allowed to be released into the environment, there is a program that the United States Environmental Protection Agency has put in place to help combat this issue. This program is entitled the Pollution Prevention Act (P2 Act) and was passed by Congress in 1990.[36] This act focuses on preventing pollution before it occurs. This act has three main points. The first is to prevent pollution whenever possible. Ifprevention is not possible, then the contaminant should be safely recycled. When recycling is not possible, the pollution should be treated in an environmentally safe manner.Finally, if none of the above are feasible, then the contaminant can be disposed of or released in an environmentally safe manner as a last resort.[36] While this act does show some progress, the fact that more detailed guidelines were impossible to readily locate is alarming. Prevention of pharmaceutical pollution is the number one way to stop the adverse effects these drugs create in the environment, and, thus, this information needs to be better publicized.

Conclusions

Overall, antibiotics are present in the environment and causing detrimental effects. Although the focus of this paper, sulfonamides are not the only antibiotics present in the environment in noticeable concentrations. As seen in figure 2, there are many other kinds of antibiotics, and several other classes of drugs seen throughout our environments.[15] This wide array of antibiotics raises an even larger concern about antibiotic resistant bacteria. The more antibiotics the bacteria are exposed to within their own environment, the more desensitized they will become to additional antibiotic drugs. This exposure, if lethal to the bacteria, can also create large shifts in the biodiversity of the foundational bacteria within an ecosystem. This is a direct concern for humans, as soon, our bacterial infections will not be able to be treated by any of our current antibiotics. The presence of a wide array of drugs in the environment poses more of a threat to the organisms living directly in the environment. While it is extremely important to keep the ecosystems of the world alive and healthy, this is less of a direct concern for human beings. However, that does not mean it should be ignored.

Antibiotics in the environment pose a significant problem for humans, yet there are very few studies that reveal these direct effects. Most of the studies mentioned in this paper document the effects that sulfonamides have on exposed micro bacteria or indicator plant species. However, little is known about what can happen when a human being is exposed to these drugs accidentally rather than ingesting an antibiotic purposefully. More data should be collected to study the actual concentrations humans are exposed to indirectly. These antibiotics can leak into the waters that we swim in, be fed to the animals we eat, or bioaccumulate in the plants we consume, but over the course of a year, or a lifetime, how much exposure occurs?

Even without this data, many realize this is a problem that needs to be fixed sooner rather than later. Indeed, steps to decrease the concentration of these antibiotics in the environment need to be taken before it is too late. These environmental concentrations of antibiotics are causing rapidly growing antibacterial resistance among other issues. Antibiotic resistance is not reversable, and therefore it must be slowed as much as possible. More public education on the issue and less prescribing of antibiotics could be potential immediate steps toward a solution. However, humans need to stay ahead of the bacterial evolution and must continue to research and develop the drugs to help resolve this issue. There are numerous programs that have been put in place to promote the research and development of newantibiotics to get ahead of the resistant bacteria. However, when trying to find regulations to prevent the release of drugs into the environment in the first place, it was extremelydifficult to find anything concrete. The EPA needs to set more absolute guidelines to inform the public about the cleanliness of their environments. If these regulations do exist, they need to be more accessible and publicized more effectively. Overall, the actions that have been taken to date are heading in the right direction. However, more needs to be done to ensure that humankind stays ahead of antibiotic resistant bacteria.

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