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Microbial Degradation of Sulfonamide Antibiotics

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MICROBIAL DEGRADATION OF SULFONAMIDE ANTIBIOTICS

by

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MICROBIAL DEGRADATION OF SULFONAMIDE ANTIBIOTICS

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Certain microbes can transform antibiotics in the environment. However, little is known about the identity of these microbes and their antibiotic biotransformation processes. The objectives of this study were to (1) isolate bacterial strains capable of transforming antibiotics, (2) determine the biotransformation kinetics of antibiotics, (3) characterize the effects of background carbons on the biotransformation kinetics, and (4) identify biotransformation products under various environmental conditions. Sulfadiazine (SDZ) was used as the model antibiotic in this study due to its frequent occurrence in livestock wastes. Surface soil from a cattle feedlot was collected to enrich potential SDZ degrading bacteria. A mixed culture was obtained after several cycles of enrichment in a mineral solution containing 10 mg/L SDZ as the sole carbon and energy source. Despite repeated efforts, no single SDZ degrading strain could be isolated from the mixed culture. 16S rRNA gene sequence analysis showed that the culture consisted primarily of two major bacterial species, *Brevibacterium epidermidis* and *Castellaniella denitrificans*. The degradation kinetics of SDZ by the mixed culture could be described using a mirrored logistic function, with a biotransformation rate measured to be at 4.86 $mg \cdot L^{-1} \cdot d^{-1}$. Three types of background carbons were tested: diluted R2A medium, glucose, and humic acid. The mixed culture had the fastest and slowest SDZ biotransformation rates when diluted R2A and humic aicd were used as the background carbon, respectively, at concentrations equivalent to SDZ on a carbon basis. The mixed culture could also degrade other sulfonamide compounds such as sulfamethazine and

sulfamerazine, at transformation rates slower than that of SDZ, but could not degrade sulfathiazole. Using liquid chromatography tandem mass spectrometry, we identified 2-aminopyrimidine (2-AP) as a major biotransformation product of SDZ in the absence and presence of the background carbons tested. Another biotransformation product detected was confirmed to not be 4-aminobenzenesulfonate, the remaining structure after the cleavage of 2-AP from SDZ. This work presents a comprehensive study of microbial biotransformation of SDZ under various environmental conditions.

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CHAPTER 1: INTRODUCTION

1.1 General background information and literature review

As the global demand for meat increases, the agricultural community has gradually turned to antibiotic usage as both a preventative measure against sickness and a growth promotor during the livestock production process. Currently, it is estimated that up to 80% of antibiotic usage in the United States is derived from agricultural usage alone (Haller et al., 2002). Similarly, up to 90% of the antibiotics directly administered to livestock are excreted as either the parent compound or one of its metabolites (Larcher and Yargeau, 2012). The introduction of pharmaceuticals into soil, surface water, and groundwater can cause the dispersal and persistence of antibiotic resistance properties in surrounding microbial communities.

It has been proven that sulfonamides can persist in the environment for many months after initial introduction into the environment (Garcia-Galan et al., 2008) via the application of manure to agricultural fields (Sukul et al., 2006). All specific antibiotics in the sulfonamide class possess a similar general structure (see Figure 1.1) containing the presence of an aniline structure and an amide group, connected by bonds to a sulfonyl group. One sulfonamide is differentiated from another by the presence of a unique functional group (denoted as "R" in Figure 1.1) connected to the molecule's amide group. Marked similarities in structure between antibiotics of the same class allow for reasonable comparisons to be made within the class, and could be a potential reason why the degradation patterns of one sulfonamide can be rationally applied to another without the need for initial testing (Ingerslev and Halling-Sorensen, 2000).

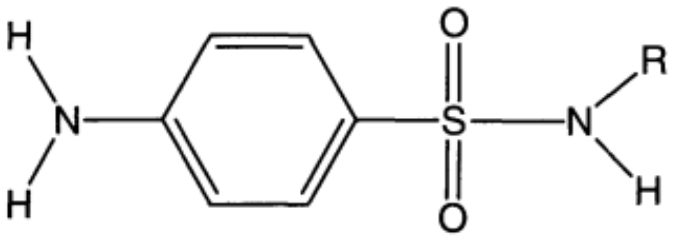


Figure 1.1. General skeletal structure of a sulfonamide molecule, different sulfonamide compounds have different functional group R (Sukul et al., 2006).

The use of microbial communities to degrade sulfonamides in wastewater treatment applications is hindered by the lack of knowledge pertaining to the behavior of sulfonamide-degrading cultures in the presence of both labile and recalcitrant nutrients. There are limited cases in which background nutrients have been proven to influence the microbial degradation of sulfonamides and other contaminants throughout the literature. Namely, one study concluded that when sulfamethoxazole was introduced into a system containing a mixed culture, the compound was only utilized as a carbon and/or nitrogen source after the depletion of acetate and ammonium nitrogen (Drillia et al., 2005), suggesting that sulfonamides are not a preferred substrate for resistant organisms. Similarly, Zhang *et al*. also discovered that a novel single-species culture able to degrade sulfadoxine could not do so without the addition of tryptone, suggesting a more extreme case of co-metabolism as a necessary condition for the use of sulfonamides as energy sources (Zhang et al., 2012a). In either case, the lack of ability/reluctance of organisms to utilize sulfonamides as a sole carbon source proves the profound effect background substances can have on the ability of a biological removal system to function.

Sulfadiazine (SDZ) is one sulfonamide that is often used as a test subject throughout the literature. Several studies have pioneered the determination of metabolites

and pathways associated with SDZ degradation. In one study, a species responsible for the partial mineralization of SDZ in previously manured soils was identified as *Microbacterium lacus* Strain SDZm4 (Tappe et al., 2013), citing it as a potentially important member of bacterial community responsible for degrading SDZ in nature. Through use of LC/MS technology, these researchers also verified the work of Topp *et al*., who found that degradation of sulfamethazine by bacteria originating from the same genus produced a stable pyrimidine product which was produced proportionally to SDZ degradation (Topp et al., 2013). In the hopes of discovering other metabolites, Ricken *et al*. employed *ipso*-hydroxylation to fragment intermediates formed from a sulfamethoxazole parent compound (Ricken et al., 2013). A lack of accumulation of polar metabolites after fragmentation serves to bolster the pyrimidine structure as the only stable product of sulfonamide degradation, a sentiment echoed by Larcher *et al*. in a summarization of current literature on the subject (Larcher and Yargeau, 2012). In keeping with such work, this study aims to lessen the knowledge gap pertaining to microbial degradation of sulfonamides by employing SDZ (and other lesser sulfonamides) as the main research subject of this study.

1.2 Objectives

The objectives of this study were to (1) isolate bacterial strains capable of transforming antibiotics, (2) determine the biotransformation kinetics of antibiotics, (3) characterize the effects of background carbons on the biotransformation kinetics, and (4) identify biotransformation products under various environmental conditions.

In order to accomplish these objectives, multiple degradation trials were run to experimentally determine the extent two multi-species cultures (one naturally produced in nature and one artificially created) could degrade SDZ in several conditions. The most successful "mixed" culture was also tested on similar sulfonamides to determine possible degradation pathways associated with the degradation process. Once degradation rates were established in all cases, LC/MS (liquid chromatography/mass spectrometry) and HPLC (high performance liquid chromatography) technology were used to both qualitatively and quantitatively examine the extent of degradation as well as the products resulting from degradation. In the case of LC/MS, C-14 labelled SDZ was added to samples to aid in the identification of degradation products.

1.3 Introduction to main study components

All bacterial cultures used in this research originated from soil taken from a cattle feedlot antimicrobial agents were used in livestock. In order to obtain a working culture, bacteria were enriched from this raw soil sample using a growth medium containing SDZ, allowing only bacteria with resistance properties to persist and grow in the mixture. The resulting "mixed culture" (made up of both known and unknown species) also yielded several isolates that were extracted using 16S RNA sequencing.

Of the culturable genera found in the original soil sample, *Brevibacterium* and *Castellaniella* were proven to be by far the most abundant. Similarly, two species belonging to these genera: *Castellaniella sp.* and *Brevibacterium epidermidis*, were chosen for pure culture creation. Bacteria belonging to the *Castellaniella* genus are gram negative, whereas the *Brevibacterium* genus contains only gram positive species. After

the exhaustion of possible scenarios attempting to use pure cultures to degrade SDZ, the mixed culture was subsequently used for all remaining degradation experiments.

The degradation of SDZ by mixed culture proved more successful than that of previous pure culture experiments. In order to provide degradation results with applicability to a realistic environment three conditions were chosen, each providing the culture a substrate source other than SDZ. Similarly, each degradation trial was run multiple times for the purpose of statistical analysis. Depending on the predictability of the degradation pattern exhibited by each background carbon the number of additional trials run differed, with each condition being tested a minimum of three times.

After examining degradation trends, tests were conducted in order to determine the versatility of the mixed culture as well as the region on the sulfonamide molecule which is most suseptible to microbial attack during degradation. Several sulfonamides posessing varying degrees of structural similarity to the SDZ molecule were evaluated: sulfamethazine (SMT), sulfamerazine (SMR), and sulfathiazole (STZ). While the literature has established that 2-aminopyrimidine (henceforth referred to as "2AP") is reasonably the only known product of SDZ degradation (Larcher and Yargeau, 2012), it was essential to confirm the importance of the presence of this exact structure to the degradation process by the mixed culture. Differences in sulfonamide degradation rates resulting from altering the struture of the non-aniline ring in a sulfonamide compound allow the bacteria dismemberment location on the structure to be identified.

Guided by information obtained from the previously outlined degradation experiments, an attempt was also made to identify previously undiscovered SDZ

degradation products and quantify known and expected products. A liquid chromatography/mass spectrometry (LC/MS) analysis was conducted on an Agilent Triple Quad machine equipped with a radioactivity detector (Agilent Technologies, Santa Clara, CA) to qualitatively identify degradation products and track the radioactive signal emitted by the radio-labelled SDZ parent compound. The addition of a radiolabeled signature to the parent compound allowed for the ability to track products originating from the aniline ring.

Additionally, an analysis was performed on a Waters 2695 Alliance High Performance Liquid Chromatography System (HPLC) (Waters, Milford, MA) for a dual purpose. This allowed for a quantitative determination to be made regarding the amount of potential degradation products (e.g., 2AP and sulfanilic acid, or SA) produced under multiple background carbon conditions. Additionally, it also allowed for a direct comparison in SDZ concentration detection between HPLC and UV-Vis methods.

1.4 Thesis organization

The first chapter of this thesis explains the motivation for the research that was conducted, including previously published conclusions pertaining to sulfonamide antibiotics in the environment, as well as the shortcomings associated with these published findings. The objectives of this study are also elaborated on, as well as the steps taken to achieve said objectives. All major components of the study are further introduced. In the second chapter, the materials and methods needed for the completion of all experiments are introduced. Chapter 3 details the results of said experiments, as well as discussion of pertinent results. Lastly, chapter 4 details the main conclusions

drawn over the course of this study. Similarly, potential pathways for future research are explored based on these findings. Two appendices are present after this chapter detailing solution recipes.

CHAPTER 2: MATERIALS AND METHODS

2.1 SDZ degradation by artificial co-culture

2.1.1 Pure culture plate counts

After the creation of two pure culture stocks for *Castellaniella sp.* and *Brevibacterium epidermidis,* growth curves for the two species were constructed. For each species curve, five evenly spaced time points over a 25 hour period were determined using plate counts. Agar plates were created, using R2A growth medium (see Appendix A) as a base, in order to allow for colony formation throughout the sampling process.

At the start of the growth curve experiment, 100mL of R2A medium was supplied to two 250mL flasks. In each flask, 100μL of a single thawed bacterial species sample was inoculated into the flask and immediately sampled, thus marking the "time 0" point of the analysis. At each time point, multiple dilution ratios were performed in order to effectively capture the single best representation of bacterial growth in the reaction flask at the time point. A phosphate buffer solution (henceforth referred to as "PBS", see Appendix A) was used as the background dilution liquid for all serial dilutions.

At each time point, the necessary amount of 2mL vials were filled with 900μL of autoclaved PBS; similarly, 1mL of liquid was removed from the reaction flask and placed in a separate 2mL vial. To begin each serial dilution, 100μL of bacterial slurry was removed from the 1mL store and added to the first vial containing PBS. After vortexing the suspension, 100μL of liquid was removed from this vial and added to the next vial containing PBS. This process was repeated until the desired dilution ratio was achieved.

A 100μL volume of the final diluted mixture was then deposited and spread evenly onto an agar plate. Throughout the duration of the growth experiment, 250mL flasks were incubated at 30°C on a shaker rotating at 120rpm. The reaction flask opening was covered with a cotton stopper and aluminum foil to ensure an aerobic growth environment with a minimal risk of airborne contaminants entering the flask. Agar plates were stagnantly incubated at 30°C for 4 days before colonies were counted.

2.1.2 Growth curve construction and co-culture creation

Raw plate count values were used to construct a straight-scale growth curve by dividing the colony forming unit (or "CFU") by the relative volume of the solution. Equation 2.1 was used to compute CFU/mL values for each time point. In this case, *C* refers to the number of colonies present on the plate, while 0.1mL is the volume of sample taken from the original reaction flask. *DF* is the dilution factor for each time point; one dilution factor was chosen for each time point of several serial dilution options. These values were then plotted against the time points at which the measurements were taken (see Figure 2.1). Through the creation of this figure, the optimum time for the harvesting of both species was determined and used in the creation of an artificial co-culture. The total necessary cultivation time for the *Brevibacterium epidermidis* culture was determined to be 20 hours, whereas the *Castellaniella sp.* culture was grown for 18 hours before harvesting.

$$\frac{C}{0.1mL} \cdot DF = CFU/mL \qquad (2.1)$$

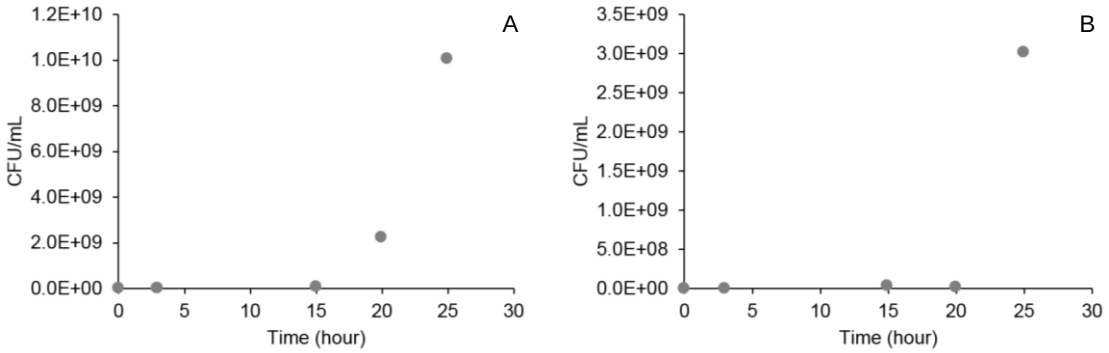


Figure 2.1. Growth curves for *Castellaniella sp*. (A) & *Brevibacterium epidermidis* (B)

To create the artificial co-culture, 100μL of each thawed bacteria culture were grown in separate flasks containing 100mL of R2A medium. Flasks were incubated at 30 degrees Celsius on a shaker rotating at 120rpm for the necessary time outlined by the growth curve results. After the incubation period, 50mL of each bacterial slurry was put into two separate 50mL sterile Genemate vials (Bioexpress LLC, Kaysville, UT) and centrifuged at 10,000rpm for ten minutes. Following initial centrifuging, bacteria were washed two additional times in PBS solution at the same speed. Following final washing, 5mL of PBS solution was added to each vial and vortexed to create two working bacteria stocks.

2.1.3 Co-culture degradation experiment procedure

After both cultures were created, three vials were prepared serving as the abiotic control, SDZ Only, and SDZ+R2A cases. In each vial, 10mL of macronutrient and 10μL of each micronutrient (Tappe et al., 2013) were added (see Appendix A), along with 10μL of a 10mg/mL SDZ stock solution (see Appendix A). In the SDZ Only and

SDZ+R2A vials, 50μL of each previously made bacterial culture were added. Additionally, in the SDZ+R2A vial, 100μL of R2A growth medium was added. Volume discrepancies between vials were corrected using PBS buffer solution. Each vial received a cotton stopper covered with aluminum foil and was placed on a shaker rotating at 120rpm within an incubator set at 30°C for 7 days.

Daily sampling was conducted in a biosafety cabinet, in which 300μL of solution from each experimental condition was collected and centrifuged at a speed of 14,000 rpm for 5 minutes. The supernatant was measured for absorbance at 260nm (Jen et al., 1998) on a DR2000 Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). This wavelength was also confirmed as the optimal wavelength for measuring SDZ absorbance on the spectrophotometer (see Figure 2.2).

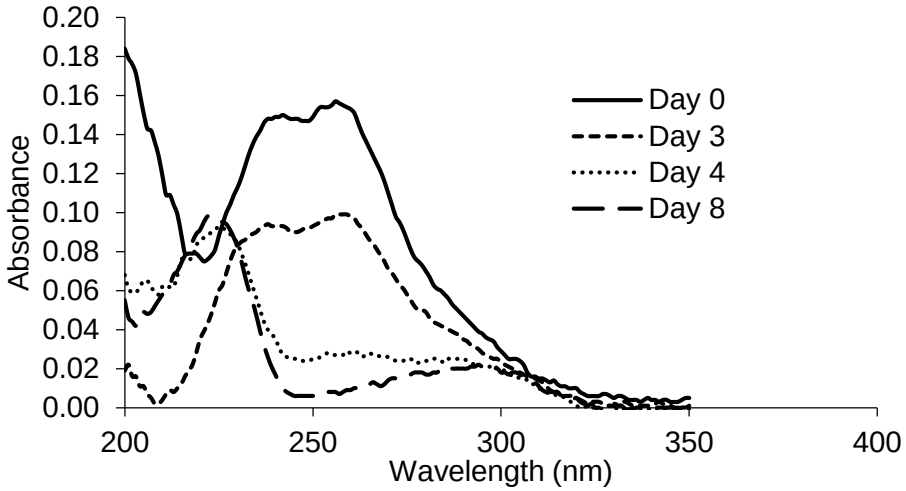


Figure 2.2. UV spectra showing decrease of SDZ parent compound at the absorbance signature wavelength of 260 nm.

Raw absorbance values were then converted to a final SDZ concentration using an experimentally predetermined absorbance vs. concentration curve (see Figure 2.3), which

was also used to perform similar concentration transformation calculations for subsequent mixed culture experiments.

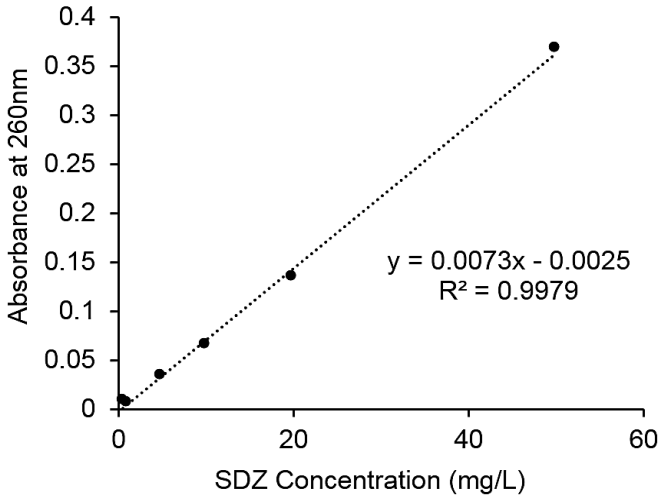


Figure 2.3. Absorbance vs. concentration relationship for control case

For the three remaining background carbon conditions used throughout the duration of the study, test solutions were created by adding the necessary volume of medium to Nanopure water to attain a total volume of 10mL of test solution (therefore producing the same concentrations present in the final degradation vials). A 1.5μL droplet of test solution was then measured at 260nm wavelength. The absorbance value obtained for the concentration in question was then subtracted from the absorbance reading obtained from the sample bearing the same background carbon concentration. Comparatively, glucose and R2A skewed absorbance readings far less than that of humic acid. Ultimately, no correction was made for glucose degradation replicates (absorbance values for glucose were negligible compared to that of SDZ absorbance). To determine the final concentration of SDZ in a sample, absorbance values were first corrected if

necessary (using the relationships present in Figure 2.4), then absorbance values were plugged into the "y" value of the trend SDZ line to solve for concentration (previously shown in Figure 2.3).

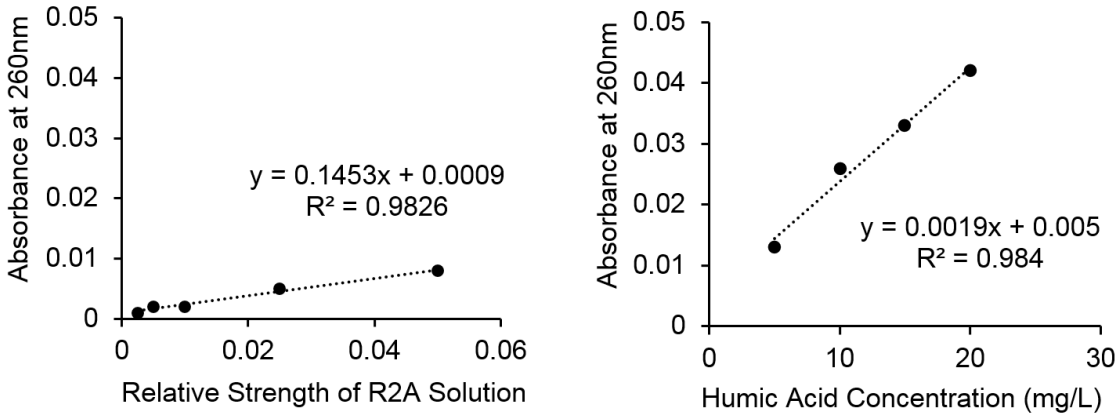


Figure 2.4. Absorbance vs. concentration relationships for R2A growth medium and humic acid additives

2.2 SDZ degradation by mixed culture

2.2.1 Background carbon selection

In order to examine the effect of background carbon media on degradation profiles, four trials were done without any additional substrate provided. In this case, SDZ stock solution was added to reaction vials to achieve a final SDZ concentration of 10mg/L. In trials where background carbons were present, this concentration of SDZ was maintained in order to effectively compare results.

The addition of glucose as a background carbon source serves several purposes. As a simple carbohydrate, glucose is a rudimentary form of sugar which can be directly and easily synthesized through the Embden-Meyerhof pathway within a bacterial cell (Madigan et al., 2009). Glucose is a common additive when studying the effects of

sulfonamides on a microbial community, as the "effect of sulfonamides is very likely linked to substrate addition to promote microbial growth" (Hammesfahr et al., 2008). Similarly, the addition of glucose to a sample can provide the necessary nutrients to induce microbial respiration (Thiele-Bruhn and Beck, 2005) and ensure cell activity. However, the simplicity of the glucose molecule has potentially limiting consequences, as this substrate may make antibiotics an unattractive substrate option by comparison when the complexity of both molecules are considered.

Conversely, R2A growth medium is a rich source of multiple complex substrates that was also used as an alternative carbon source in this experiment. Due to the fact that R2A is also used during the incubation and growth step of the experiment, it is known that the mixed culture is receptive to the mixture and able to use the ingredients as substrates. Due to the complex nature of some of the medium ingredients, the exact carbon content of the R2A medium is unknown. While relative strength is a suitable alternative in order to measure the qualitative benefit the medium has for degradation capabilities of the bacteria, it is unlikely that a broader application of R2A medium as a degradation promoter can be applied on a larger scale than batch laboratory tests.

Humic acid was used as a final background carbon alternative, due to the fact that "humic substances are the most common forms of organic carbon in the natural environment" (Islam et al., 2005). Similarly, the introduction of humic acid serves to create a situation mimicking the natural organic matter (NOM) present in wastewater; noting that the literature cites that artificial wastewater can be simulated with a concentration of humic acid up to 30 mg/L in final solution (Zhang et al., 2012b). Humic

acid has been proven an essential contributor to either the inhibition or stimulation of microbial growth, depending on the bacteria that are present in the environment (Tikhonov et al., 2010). Additionally, because the exact microbial composition of the mixed culture is unknown, the effects of humic acid addition could lead to clues as to the species present. To better investigate the effects of the quality of background carbon present, several final concentrations (or strengths) of each carbon were analyzed (shown in Table 2.1).

Table 2.1. Background carbon concentrations and corresponding COD values

Carbon type	Concentrations/Strengths Used	COD Value (mg/L)
Glucose	5 mg/L	7.93
	10 mg/L	12.80
	20 mg/L	22.53
	50 mg/L	51.73
	100 mg/L	100.40
R2A Growth Medium	1/400	11.42
	1/200	17.13
	1/100	28.57
	1/40	62.86
	1/20	120.02
Humic Acid	5 mg/L	7.27
	10 mg/L	13.41
	15 mg/L	19.56
	20 mg/L	25.71

2.2.2 Mixed culture degradation experiment procedure

To create the bacterial suspension used for the analysis, 500μL of the mixed culture was added to 50mL R2A medium containing 50mg/L of SDZ (see Appendix A for procedure for creation of SDZ stock). The culture, covered with a cotton stopper and foil, was incubated at 30ºC on a shaker at 120rmp for 12 hours. The culture was then

harvested and diluted with autoclaved Nanopure water until the optical density of the culture reached 1 at 600nm wavelength. The dilution and measuring step was done before washing to ensure that bacterial population had multiplied to a suitable level for analysis before it was removed from the R2A growth medium. Next, 50mL of this diluted bacteria stock was washed three times in a centrifuge operating at 10,000rpm, using PBS buffer as the suspension medium during the washing process. After the final wash, cells were re-suspended in 5mL PBS to create a working bacteria stock.

Degradation experiments were conducted under four conditions: SDZ only, SDZ+R2A, SDZ+glucose, and SDZ+humic acid. After the addition of 100μL of working bacteria stock, 10mL of macronutrient and 10μL of each micronutrient (Tappe et al., 2013) were added to each degradation vial (see Appendix A). Similarly, the desired background carbon (glucose, humic acid, or R2A medium) was also added along with 10μL of the SDZ stock solution (see Appendix A) to obtain a final SDZ concentration of 10mg/L. For each background carbon, different final concentrations were used, according to Table 2.1. Finished vials were then incubated at 30 °C at 120rpm. Although a cap was used to prevent potential contamination or unnecessary evaporation during incubation, the amount of liquid in the vial compared to the total vial volume suggests that an aerobic environment was maintained throughout the degradation process. This claim is bolstered by the fact that vials were opened in a sterile environment once a day for sampling, allowing fresh air to reenter the vial.

Daily sampling was conducted in a biosafety cabinet, in which 200μL solution from each experimental condition was collected and centrifuged at a speed of 14,000 rpm

for 5 minutes. The supernatant was measured for absorbance at 260nm (Jen et al., 1998) on a DR2000 Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Absorbance values were then corrected for background caused by substrate carbon. Further, corrected absorbance values were then converted to concentration using the appropriate predetermined absorbance vs. concentration curve.

2.2.3 COD test for background carbon conditions

COD tests for each background carbon concentration were performed to relate the COD contained in each sample to the solution concentration (see Figure 2.5). In the case of R2A, COD was related to the strength of the additive compared to the original solution due to the fact that the exact concentration of nutrients is unknown. Once test concentration values were selected, the value was plugged into the "x" value of the appropriate trend line equation to determine the COD value for a sample containing that amount of background carbon. COD tests were done according to Standard Methods protocol (1999) using pre-made low-range COD reaction vials obtained from Hach (Loveland, CO).

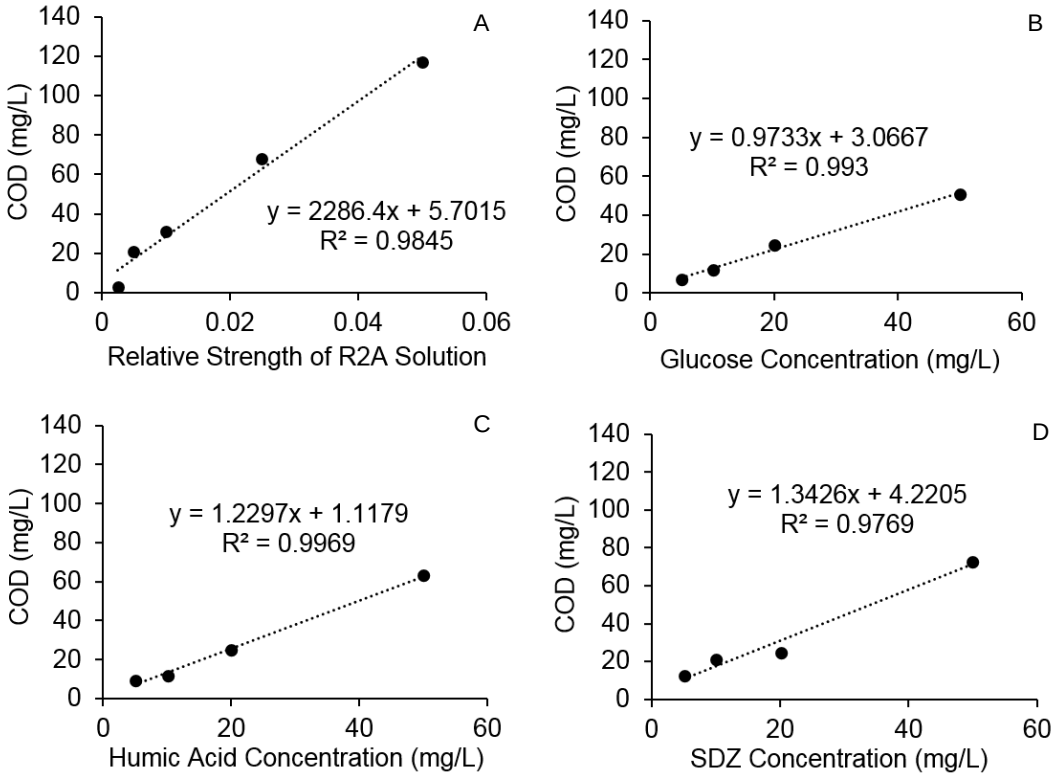


Figure 2.5. COD vs. concentration relationships for R2A growth medium (A), glucose (B), humic acid (C), and the no background control case (D)

2.2.4 Software modelling of degradation rates

A logistical model (Equation 2.2) was adopted to describe the degradation kinetics of SDZ (Tappe et al., 2013). The initial concentration of SDZ in a sample (in mg/L) corresponds the C_o variable, while the final concentration of SDZ in a sample (in mg/L) is denoted by C_∞. The degradation rate constant r has final units of day^{-1}, while time t is also measured in days. In order to obtain these values, experimental data were used to fit a model of Equation 3.1 in MATLAB (MathWorks Inc.). Once predicted C_o, C_∞, and r values were obtained from the program, these constants were plugged into Equation 2.2 to create a predicted degradation curve, which was plotted along with the

actual corrected concentration values to obtain a final degradation curve and rate for the sample, as shown in Figure 2.6.

$$C(t) = C_O - \frac{C_O}{1+\left({C_O}/{C_\infty}-1\right)e^{-rt}} \qquad (2.2)$$

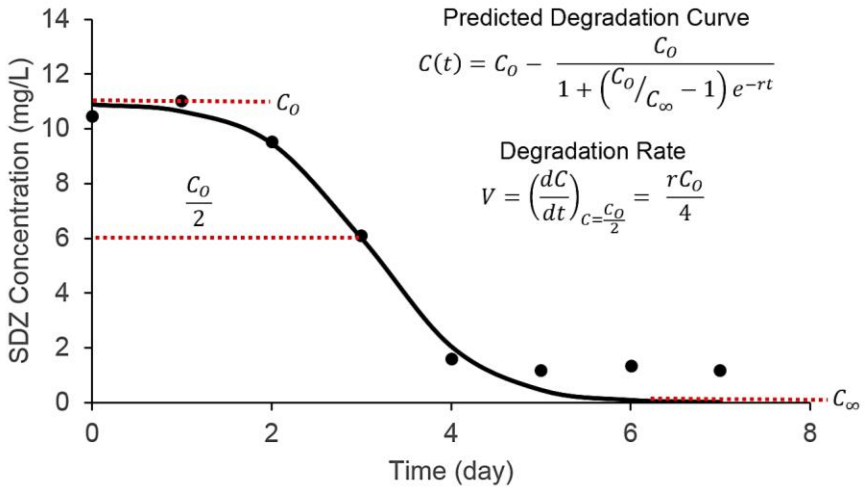


Figure 2.6. Determination of SDZ degradation rates in MATLAB

These constants was also used to determine a degradation rate (with units of mg/(L·day), given by Equation 2.3, in which all constants have the same meaning as outlined above.

$$V = \frac{rC_O}{4} \qquad (2.3)$$

2.3 SMR, SMT, & STZ degradation by mixed culture

2.3.1 Mixed culture degradation experiment procedure

To create the bacterial suspension used for the analysis, 500μL of glycerol stock of the mixed culture was added to 50mL R2A medium containing a final concentration of 50mg/L of the necessary sulfonamide stock (see Appendix A). The culture, covered with foil, was incubated at 30ºC on a shaker at 120 rpm for 12 hours. The culture was then harvested and diluted with autoclaved Nanopure water until the optical density of the culture reached 1 at 600 nm wavelength (Jen et al., 1998). Next, 50mL of this diluted bacteria stock was washed three times in a centrifuge operating at 10,000 rpm, using PBS buffer as the suspension medium during the washing process. After the final wash, cells were re-suspended in 5mL PBS to create a working bacteria stock.

In order to effectively compare sulfonamide degradation rates to that of the principal research subject, no background carbons (other than the sulfonamide molecule) were provided to the mixed culture. After the addition of 100μL of working bacteria stock, 10mL of macronutrient and 10μL of each micronutrient (Tappe et al., 2013) were added to each degradation vial (see Appendix A), along with 10μL of the necessary 10mg/mL sulfonamide stock solution (see Appendix A).

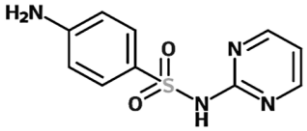
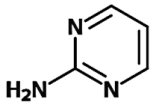
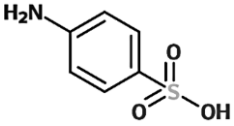
2.4 SDZ degradation products

2.4.1 Preparation of standard solutions for HPLC analysis

In order to properly encompass SDZ, 2AP, and SA concentrations to be measured degradation process, several volume increments of these three analytes were used in the creation of standard solutions (see Appendix B), each at a final volume of 10mL. Sulfamethazine was used as an internal standard due to its similarity in structure to the

SDZ molecule, while SA and 2AP show a molecular structure that is clearly derived from the parent compound (as shown in Table 2.2).

Table 2.2. Structural comparison between three analytes chosen for HPLC analysis

SDZ	2AP	SA
		

Analyte and internal standard responses were then used to create standard curves (see Figure 2.7) from which final analyte concentrations in the samples were determined. In order to create standard stock solutions, previously made SDZ solution (see Appendix A) was diluted by adding 100μL of 10mg/mL SDZ solution to 9.9mL of Nanopure water to create a 0.1mg/mL stock solution. Similarly, a 0.1mg/mL 2AP stock was created by adding 0.05g of 2AP powder (Aldrich, St. Louis, MO) to 5mL of Nanopure water, and subsequently adding 100μL of this concentrated solution to 9.9mL of Nanopure water. Lastly, an internal standard stock was created by adding 0.025g of SMT powder to 10mL of HPLC grade methanol. After powder was fully dissolved, 100uL of this solution to 9.9mL of methanol to create a 25mg/L working SMT stock to be added to both standards and samples. All solutions were filtered using a sterile syringe and 0.2μm pore size filter before use. To run standards, 200μL of the analyte standard stock for a given concentration was added to a 300μL LC vial insert, along with 50μL of internal standard (resulting in a final concentration of 5mg/L, or 1250ng, of SMT in each standard increment).

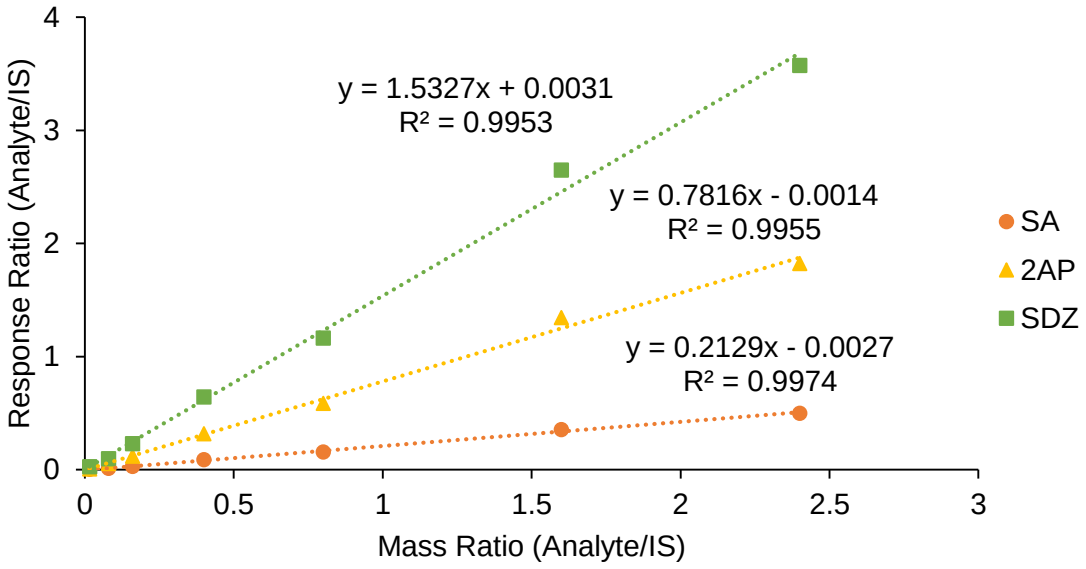


Figure 2.7. Standard curves for HPLC analysis. Optimum wavelengths at which analytes and internal standard (IS) were analyzed at were 267nm (for SDZ and SMT), 301nm (for 2AP), and 249nm (for SA)

2.4.2 Preparation of HPLC samples

Due to the wide variety of background carbon concentrations used in this study one concentration of each background carbon was analyzed for degradation products: 10mg/L humic acid, 10mg/L glucose, 1/400 strength R2A solution, as well as no background carbon control. These four cases were chosen due to their proximity to each other in terms of COD abundance. This allowed microbes in each case access to a similar beginning quantity of carbon substrate.

All samples were prepared using the previously outlined procedure for mixed culture samples. A total volume of 400μL of sample was obtained each day of the analysis before UV-Vis measurements were taken. This liquid was then filtered using a sterile syringe and 0.2μm pore size filter to remove any bacterial constituents from the

solution. The removal of any microbes stopped further degradation from occurring, preserving the amounts of both parent and degradation product(s) at each time point. These samples were then refrigerated until further analysis using HPLC with DAD. Before use, 200μL of this filtrate was added to a 300μL LC vial insert, along with 50μL of internal standard stock (resulting in a final concentration of 5mg/L, or 1250ng, of SMT in each sample). A mobile phase of 0.1% formic acid was used as the first solvent, while HPLC grade methanol was used as a second solvent.

2.4.3 Preparation of LC/MS samples (non-radiolabeled)

All samples containing no C-14 labelled SDZ were prepared using the procedure outlined for mixed culture samples. A total volume of 400μL of sample was obtained each day of the analysis before UV-Vis measurements were taken. This liquid was then filtered using a sterile syringe and 0.2μm pore size filter to remove any bacterial constituents from the solution. These samples were then refrigerated until further analysis using LC/MS technology. Before use, 200μL of this filtrate was added to a 300μL LC vial insert, along with 50μL of internal standard stock (resulting in a final concentration of 5mg/L, or 1250ng, of SMT in each sample).

2.4.4 Preparation of LC/MS samples (radiolabeled)

The base solution for all radioactive samples originated with the same steps taken in section 2.4.3. Radiolabeled SDZ was purchased from American Radiolabeled Chemicals (St. Louis, MO) with C-14 on the aniline ring of the molecule. A final mass of 15370ng of C-14 labeled SDZ was combined with 10 mg/L non-labelled SDZ, resulting

in final radioactivity of 649.3 dpm/μL in each sample vial at the start of the degradation trial. A mobile phase of 0.1% formic acid was used as solvent one, while HPLC grade methanol was used as a second solvent. A linear gradient was produced using these two solvents; sample and background gradient were run through a HyPURITY C18 column (Thermo Fisher Scientific, Waltham, MA).

CHAPTER 3: RESULTS AND DISCUSSION

3.1 SDZ degradation by artificial co-culture

No degradation by artificial co-culture was observed (see Figure 3.1), regardless of amendment with R2A as an additional substrate. Due to the fact that the mixed culture derived from the same source was able to degrade SDZ, several conclusions regarding the co-culture can be made. Firstly, these results are echoed by that of Accinelli *et al*., who suggested that sulfonamide degradation is best achieved through the use of an entire microbial community, not a single bacterial species (Accinelli et al., 2007).

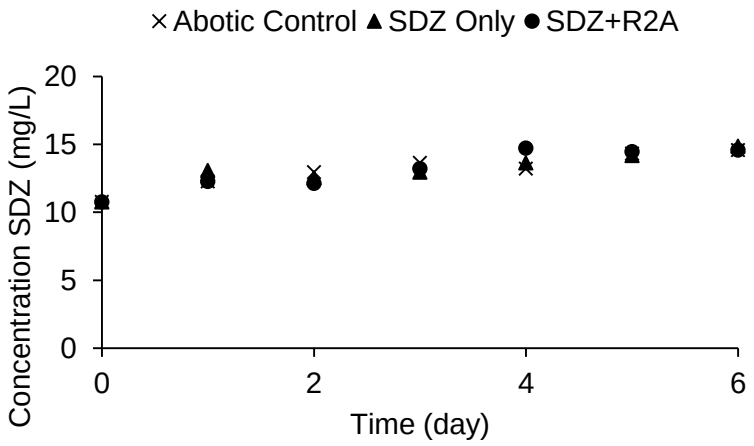


Figure 3.1. Degradation of SDZ by artificial co-culture made up *Brevibacterium epidermidis* and *Castellaniella sp*.

Similarly, readily cultivable species in the mixture are not necessarily representative of the entire population in the culture; while these species can tolerate SDZ, they may not be able to use it as a substrate to the same degree as other culture constituents. When compared to the results of a study by Tappe *et al*., it should be noted that *Microbacterium lacus* was the main species responsible for the biodegradation of

SDZ in that study (Tappe et al., 2013). The fact that this genus was not found in our mixed culture samples, coupled with the fact that culturable genera in our sample were also incapable of degrading SDZ, leads to the conclusion that another unidentified species is responsible for the biodegradation of SDZ in this mixed culture. Furthermore, the *Brevibacterium epidermidis* and *Castellaniella sp*. species in question may have acquired resistance properties through the process of horizontal gene transfer as previously shown by Zhang *et al*., a study in which cattle manure containing high levels of SDZ parent compound and other antibiotics was able to confer resistance properties when applied to soil (Zhang et al., 2013). Known isolates are not always an accurate representation of the capabilities of the entire microbial community, proving that current gene databanks may not contain the necessary information to identify all bacteria capable of degrading sulfonamides.

3.2 SDZ degradation by mixed culture

Background carbon condition influenced the shape of the degradation curves (Figure 3.2), and all degradation patterns could be well described by the logistic model. Without any background carbon, the mixed culture exhibited a 2-day lag time before substantial degradation occurred. Compared to the situation without any background carbon, the mixed culture exhibited a shorter lag time in the presence of diluted R2A medium (~1 day), and comparable lag time in the presence glucose and humic acid.

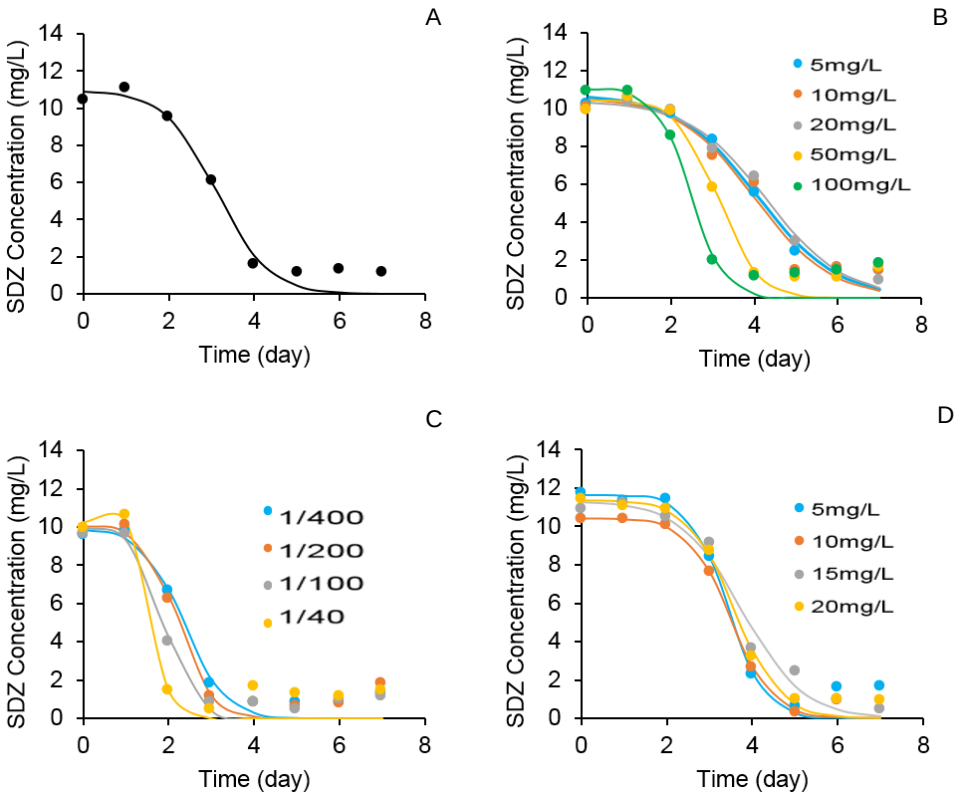


Figure 3.2. Representative SDZ degradation patterns in the presence of no background carbon (A), glucose (B), R2A growth medium (C), and humic acid (D)

Additionally, when the glucose concentration was increased, the degradation pattern the bacteria exhibit is similar to that shown by bacteria given no additional carbon source. While humic acid and R2A carbon sources vary in lag time observed, both types of background carbon show a sharp immediate decrease in SDZ concentration after the lag time is completed. The concentration and presence of the background carbon appeared to have an impact (either positive or negative) on the degradation rate in some cases ($p < 0.05$) (Figure 3.3). These results are also shown in Table 3.1.

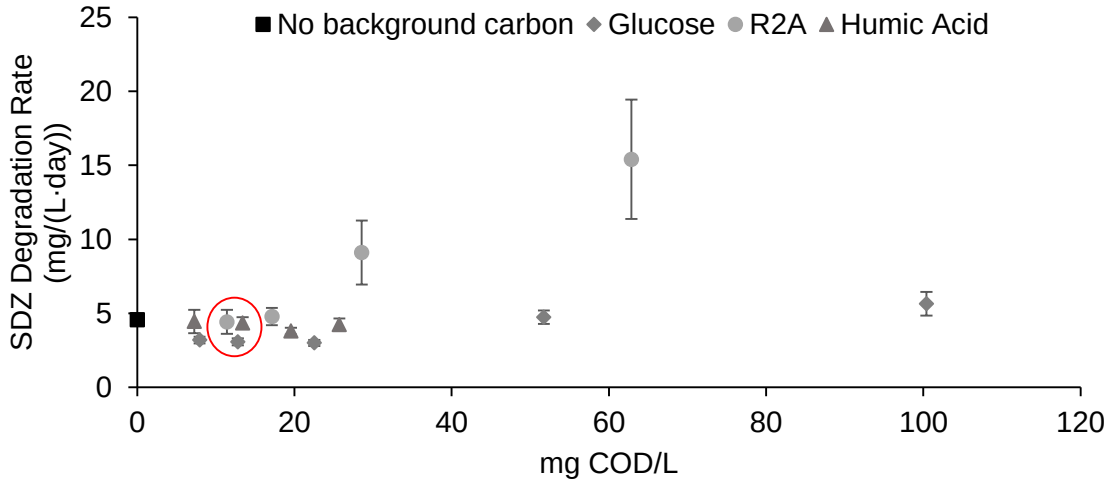


Figure 3.3. Effect of background carbon abundance on SDZ degradation rates by mixed culture. Degradation rates displayed are the mean degradation rates of at least 3 separately conducted trials. Error bars correspond to the standard error associated with the trials conducted for that condition. Circled points correspond to the conditions used for HPLC and LC/MS analysis.

Table 3.1. Mean degradation rates, standard errors, and standard deviations for all experiment conditions

Sample	Mean Degradation Rate mg/(L·day)	Standard Error	Standard Deviation	Considered Statistically Significant when Compared to Control
No background carbon	4.55	0.39	0.78	
Glucose 5mg/L	3.20	0.23	0.39	✓
Glucose 10mg/L	3.07	0.23	0.40	✓
Glucose 20mg/L	3.00	0.21	0.36	✓
Glucose 50mg/L	4.73	0.45	0.78	
Glucose 100mg/L	5.64	0.80	1.38	
R2A 1/400 strength	4.42	0.81	1.81	
R2A 1/200 strength	4.77	0.58	1.29	
R2A 1/100 strength	9.11	2.16	4.82	
R2A 1/40 strength	15.40	4.03	9.02	✓
Humic Acid 5mg/L	4.45	0.77	1.57	
Humic Acid 10mg/L	4.34	0.40	0.80	
Humic Acid 15mg/L	3.80	0.22	0.44	
Humic Acid 20mg/L	4.23	0.43	0.86	

R2A 1/40 strength was the only background nutrient condition that led to a higher SDZ degradation rate than the no background carbon condition. R2A medium is an undefined medium. An undefined medium has complex ingredients, such as yeast extract, which consist of a mixture of many, many chemical species in unknown proportions. Our observation was corroborated by another study, in which an undefined growth medium initially increased the ultimate mineralization of radiolabeled phenanthrene (Carmichael and Pfaender, 1997). Similarly, Boonchan *et al*. also demonstrated that utilizing a PAH contaminant as a sole microbial carbon source is not as effective when compared to amendment with undefined media (Boonchan et al., 2000). In either case, it is clear that compounds possessing a complicated aromatic ring structure (such as sulfonamides) are not easily degraded in a barren environment. The results of this analysis also indicate the concentration of the undefined medium in the background needs to be sufficiently high to have a significant impact on the SDZ degradation rate, as SDZ degradation rates only significantly increased at the highest relative strength of R2A medium tested.

Conversely, recorded bacterial reactions to the addition of humic acid in the literature show less consensus, yet yield important clues as to the response of our mixed culture. In a study conducted by Lee *et al.*, the biodegradation of 17β-estradiol was hindered as humic acid concentrations increased, while sorption of the compound subsequently increased with the humic acid additions (Lee et al., 2011). The effects of sorption in our study can be reasonably eliminated because SDZ has a low K_{oc} value and is polar in nature. This is evident when comparing the sorption coefficients of SDZ and 17β-estradiol in the literature; determined values for 17β-estradiol were reported to be up

to three magnitudes higher than SDZ (Holbrook et al., 2004, Sukul et al., 2008). Coupled with the degradation trends and production of degradation products witnessed in this analysis, it can be inferred that the mixed culture is predominantly responsible for the observed decrease in SDZ concentration. However, the a study conducted by Bialk *et al*. has also claimed that humic acid itself could lead to a lack of bioavailability of sulfonamides, due to its ability to "chemically incorporate" sulfonamide compounds into its own structure (Bialk et al., 2005). While this could impact possible degradation products, we conclude that the presence of humic acid in solution does not have a significant impact on the degradation process itself in. Similarly, the statistically negligible differences in SDZ degradation rates where humic acid was added prove that the abundance of natural organic matter (NOM) in a system has little effect on microbial degradation of sulfonamides.

The addition of labile nutrients in a system has potentially negative consequences on the speed of potential biodegradation of a contaminant. In one study, ultimate estrogen removal was decreased as glucose concentrations increased in the presence of microbes derived from activated sludge additives. When compared to a case where no glucose was added to the reactor, estradiol degradation rates were over five times slower as glucose concentrations were increased to 50 mg/L (Li et al., 2008). In this study, it was likely that glucose was quickly utilized as the preferred substrate by the mixed culture, slowing down the utilization of SDZ.

3.3 SMR, SMT, & STZ degradation by mixed culture

The mixed culture exhibited different degradation capabilities for different sulfonamide compounds; while it could not degrade STZ, it was able to degrade SMT and SMR albeit at lower degradation rates (Figure 3.4). With the addition of one methyl functional group to the previously bare non-aniline aromatic ring of the molecule, the overall degradation rate of SMR by the culture decreased by nearly half. A similar trend is seen with sulfamethazine, suggesting that the addition of more than one functional group to the same ring does little to change the degradation rate if a first functional group is already present. Instead, the second functional group could have a greater impact on the production of possible degradation products.

The lack of degradation of STZ is therefore most likely due to the presence of the cyclo-pentane ring structure present in this molecule, producing a change in electron density when compared to that of the pyrimidine-like structure (due to the elements present in the ring). In a study examining the photo-degradation of SDZ and STZ compounds, this trend was also demonstrated, showing that the electron density of the non-aniline ring structure on a sulfonamide might also be linked to the extent of biodegradation in the environment (Batista and Nogueira, 2012). These discrepancies clearly prohibit the bacteria culture from attacking and utilizing the molecule for substrate. Differences in sulfonamide degradation rates resulting from altering the structure of the non-aniline ring in a sulfonamide compound further allowed the bacteria dismemberment location on structure to be identified as the bond between the non-aniline ring and the amide group.

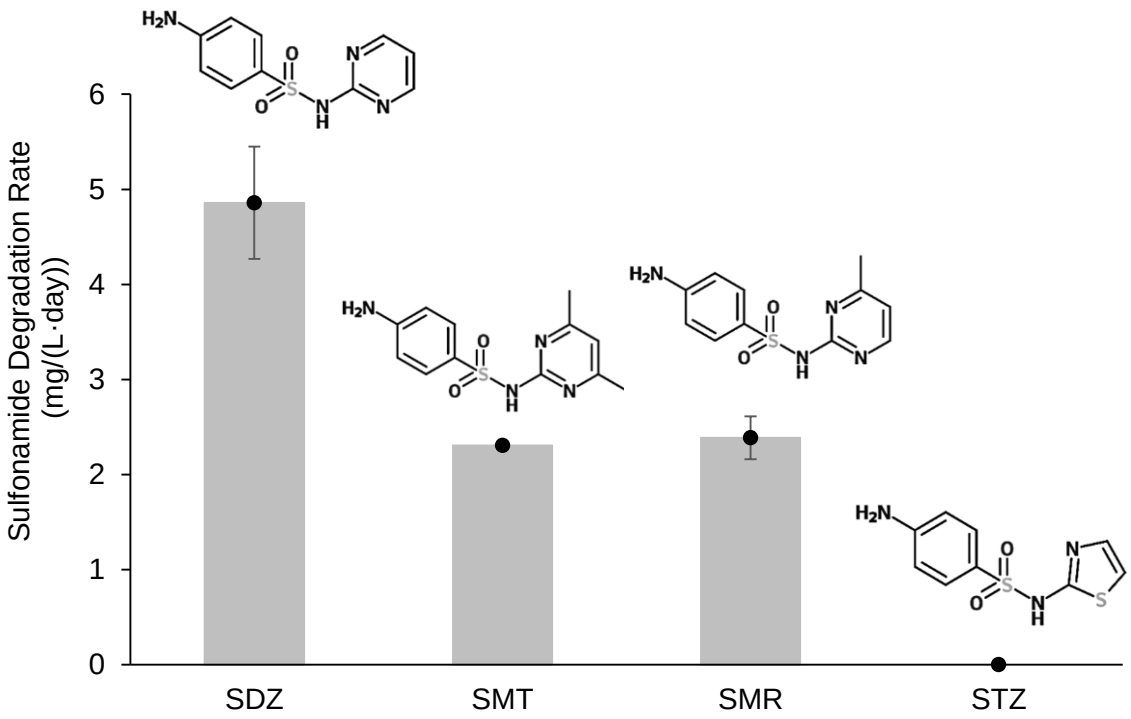


Figure 3.4. Sulfonamide degradation rates via mixed culture. Error bars correspond to the standard deviation of the results of 3 independent trials.

These results are consistent with those of Perez et al., who suggested that the ability of a single bacterial culture to degrade multiple sulfonamides (including STZ) is due to the fact that enzymes present in sulfonamide degrading bacteria work on the entire sulfonamide class, not just a single sulfonamide (Pérez, Eichhorn et al. 2005). These findings suggest that this logic only holds when the non-aniline ring present on the parent compound is of similar structure to that of an aminopyrimidine molecule; if this is not the case, enzymes may be rendered ineffective or not produced at all.

3.4 SDZ degradation products

3.4.1 Qualitative analysis of degradation products

In order to accurately portray the degradation products present in the presence of each background carbon, chromatograms were examined from day 7 samples to identify prevalent masses. Background carbons clearly had an effect on the degradation products associated with the degradation of the parent compound, as several degradation products of unknown structure emerged (see Figure 3.5). These products were produced in addition to the 2AP molecule (not shown), which is a previously identified product of SDZ degradation (Tappe et al., 2013). All solutions, regardless of background carbon type and presence, also produced two metabolites with mass-to-charge ratios of 195 and 115. In addition to these metabolites, solutions containing humic acid also produced a compound with a mass-to-charge ratio of 300. The solution containing SDZ as the only bacteria substrate produced an additional metabolites with a mass-to-charge ratio of 240, also at high levels comparatively. The presence of the 195 and 115 metabolites in all background carbon scenarios implies the possibility that these compounds are indeed metabolized products that the mixed culture produces in order to function that are not related to SDZ degradation. No realistic depictions of possible structures resulting from SDZ degradation for the 240 and 300 mass compounds were able to be determined.

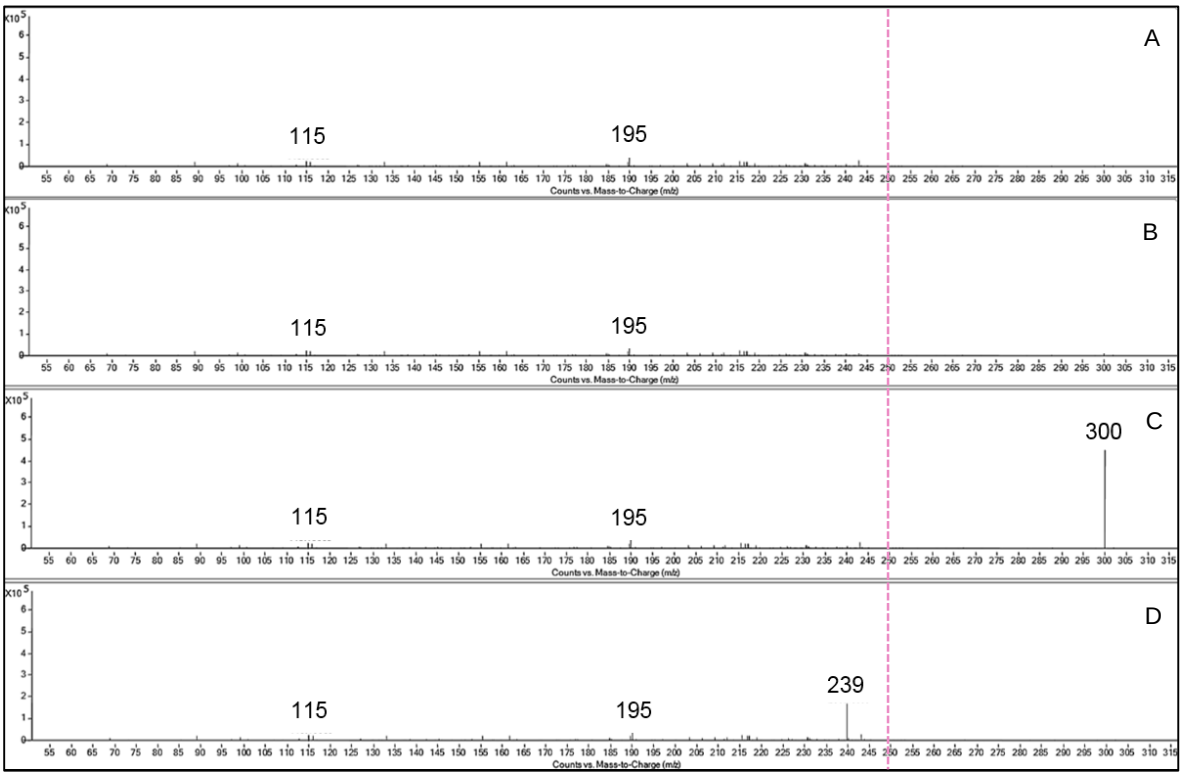


Figure 3.5. Qualitative mass/charge results of SDZ degradation for R2A growth medium (A), glucose (B), humic acid (C), and the no background control case (D). The dashed line indicates where the parent compound falls on the m/z scale.

3.4.2 Qualitative analysis of radioactivity detector output

In order to illustrate the radioactivity present on day zero of the experiment in all samples, the no background carbon control case was used as baseline to which radioactive signals arising from later points could be compared. Throughout the degradation process, the radioactive signature present in solution did not decrease (as shown in Figure 3.6), although liquid chromatography derived chromatograms verified that the parent compound had fully degraded by the end of the trial. With the addition of C-14 labelled SDZ, it was expected that radiolabeled degradation products would be produced. However, the stable high radioactive signal detected throughout the 7 day trial for all background carbons

indicates a scenario in which the labelled SDZ degradation product is inherently not able to be ionized and quantified using LC/MS ESI technology.

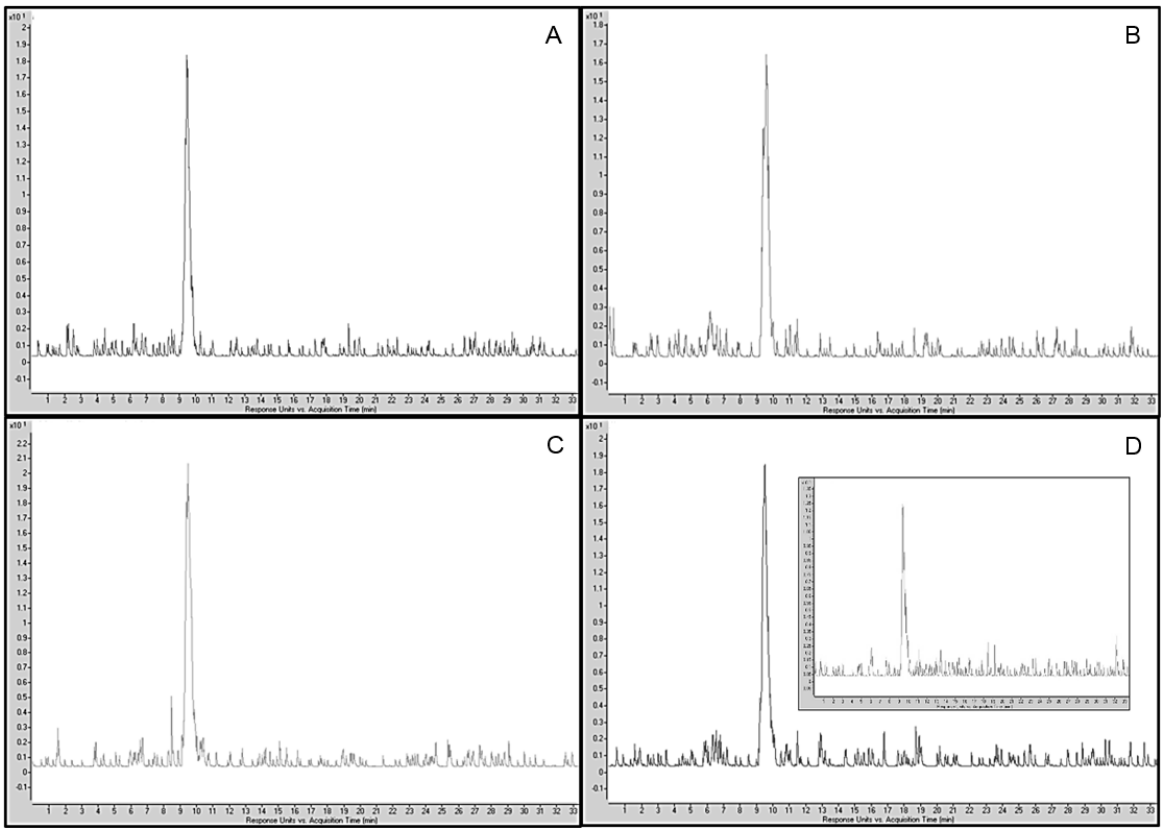


Figure 3.6. Lack of change in radiolabeled signature produced between background carbons between day 7 and day 0 (the insert) for glucose (A), R2A growth medium (B), humic acid (C), and the no background carbon control case (D). Axes represent response (Y) vs acquisition time (min) (X).

3.4.3 Quantitative analysis of SDZ degradation and 2AP production

Analyte responses for all samples were outputted in the form of peak area after analysis on the HPLC machine. Using these raw areas for both the analytes and internal standard, "response ratios" were determined for each analyte by dividing a given analyte peak area by the internal standard peak area for a given time point. These response ratios

were transformed to mass values using the equation given by the linear relationship for the appropriate standard curve. This value was then transformed to a milligram value for each analyte (keeping in mind that the internal standard was at a constant mass of 1250ng in all samples). Using the sample volume of 0.0002L, these analyte values were ultimately transformed to mg/L concentration values at each time point.

Using these concentration values, a degradation rate was determined for the control case, R2A, glucose, and humic acid using Equation 3.2 and its corresponding MATLAB code. Respectively, the rates were found to be 4.68, 5.60, 2.55, and 3.91. It should be noted that these HPLC-derived degradation rates fall within the standard error limits of the corresponding mean degradation rates for these conditions determined through UV-Vis analysis. Such a comparison has not been previously noted in the literature, and proves the feasibility of either technique to accurately measure microbial degradation of SDZ. This could prove to be a valuable consideration when determining the economic feasibility of a project.

The final mass of 2AP in each sample on day 7 was also determined in order to calculate the total yield of the metabolite for each condition. Once these values were obtained, a theoretical 2AP yield was determined using the appropriate SDZ concentration on day 0 of the analysis as maximum value for 2AP production. In this case, the one-to-one molar relationship of 2AP and SDZ was used, along with the molar masses of each compound, to derive the maximum amount of 2AP that could be produced from the initial amount of SDZ in the sample. Using the previously determined

ultimate amount of 2AP produced by day 7, the percent yield of 2AP for each condition was calculated by dividing the actual 2AP yield by the theoretical 2AP yield.

From this Equation, the total percentage of 2AP recovered from the no background carbon control case, R2A, glucose, and humic acid conditions were all above 90%. These yields are a promising indication of the effectiveness of the mixed culture. Due to the molar ratio of the parent compound to the 2AP metabolite, it is reasonable to conclude that the culture is degrading nearly all the parent compound by the end of the trial period, regardless of background carbon presence. It should also be noted that these results mimic the general trend exemplified by Tappe *et al.*, who first discovered the proportionate relationship between SDZ and its 2AP metabolite (Tappe et al., 2013). As evidenced by Figure 3.7, when molarity is used as an alternative to concentration as a means to measure SDZ degradation and subsequent 2AP production, the compounds behave proportionately to one another, further allowing for a comparative evaluation of SDZ degradation between conditions. When such results are applied to a wastewater treatment application, it is clear that the rate of degradation will be the limiting factor in the application of this mixed culture. Although nearly all the parent compound is degraded by day seven regardless of additive, the hindrance or support the additive lends to the system throughout the trial could still render some conditions unfavorable. If quick and complete degradation of SDZ is desired, an undefined medium or NOM additive would be the best suited solution.

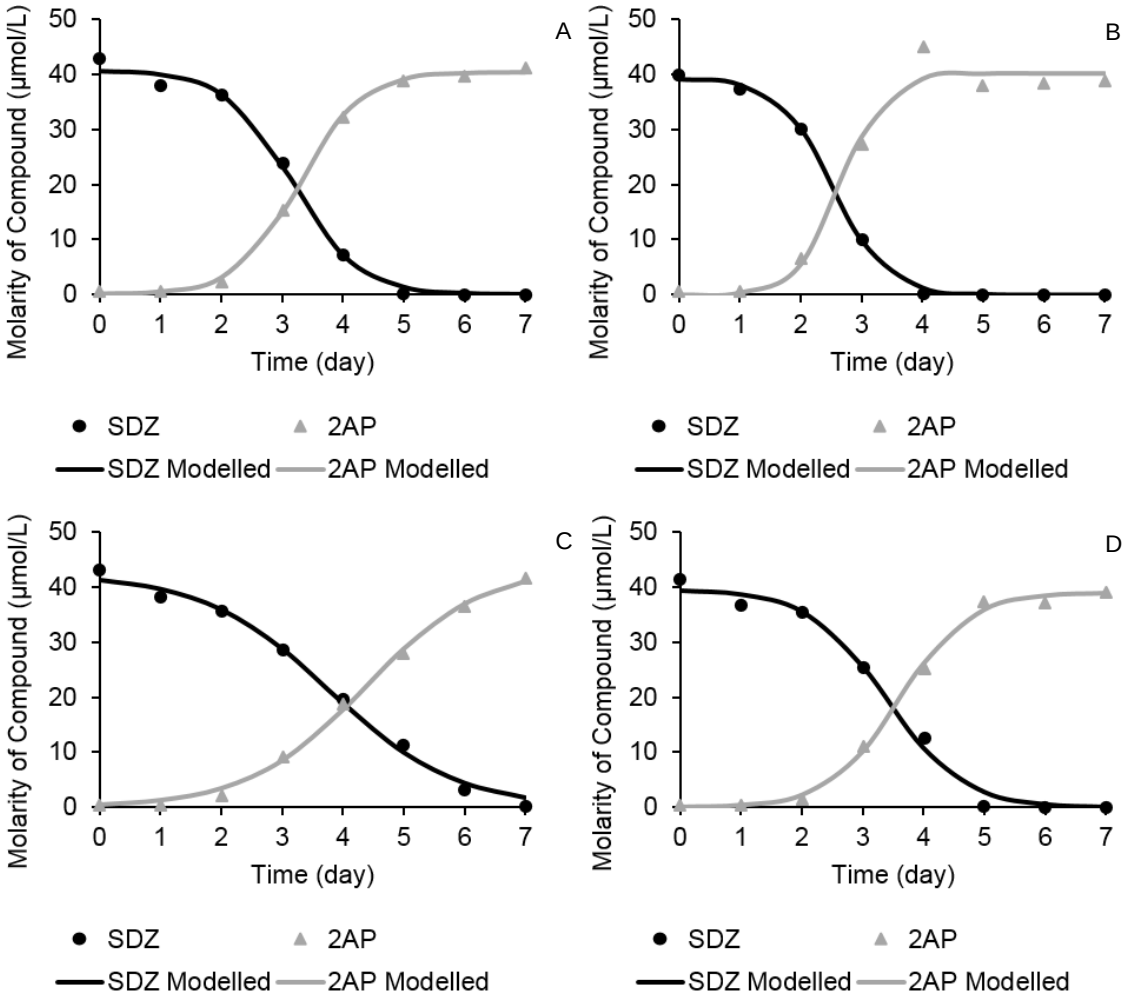


Figure 3.7. Proportionate SDZ degradation and 2AP production in terms of molarity for the no background control case (A), R2A growth medium (B), glucose (C), and humic acid (D)

CHAPTER 4: CONCLUSIONS AND FUTURE WORK

4.1 Study conclusions

After extensive review of the data collected, several conclusions were drawn:

- The use of pure bacterial cultures to degrade SDZ and other like contaminants is not likely to result in degradation in a natural environment. Pure isolates derived from the mixed culture used in this series of experiments were not able to degrade the contaminant, even though resistance properties were present.
- This mixed culture exhibited an affinity for recalcitrant substrates over labile substrates as a main carbon source. Recalcitrant carbon additives reduced lag times and/or hastened the degradation of the SDZ contaminant, proving the viability of the mixed culture in a natural environment where complex substrates are often present in greater amounts than labile ones. While the mixed culture is able to utilize SDZ as a substrate, it is not the preferred substrate.
- The versatility of the mixed culture was proven to an extent by its ability to degrade sulfonamide contaminants with similar structure to that of SDZ. While this shows promise for the mixed culture's ability to degrade multiple sulfonamides at once, the structure of the sulfonamides present must be taken into account. The presence of an aminopyrimidine structure on the non-aniline ring of the molecule is essential for degradation to occur by way of this mixed culture.
- The fact that 2AP recovery rates exceeded 90% in all conditions proves that the ultimate effectiveness of the mixed culture (regardless of its surrounding

environment) is substantial. Such high recovery rates correspond to the inactivation of over 90% of the parent compound in solution. Degradation patterns exemplifying this trend were further verified using HPLC analysis, which was verified as comparable to that of UV-Vis technology when measuring SDZ degradation rates and exemplifying degradation trends.

4.2 Suggestions for future research

Based on the findings of this research, several further steps can be taken to expand upon the conclusions drawn here:

- Degradation testing done with this mixed culture should be performed at multiple concentrations of SDZ (both above and below 10mg/L final concentration in solution). This would allow for an approximate determination of the concentration of SDZ necessary to inhibit bacterial function and lessen or prevent parent compound degradation. Based on previously published literature, it is possible that realistic concentrations of SDZ often found in the environment could be degraded in a quicker period than 7 days by this mixed culture.
- Recently, Tappe et al. has suggested the existence of a "*Terrabacter*-like bacterium, denoted strain 2APm3" that can fully mineralize the 2-AP compound that arises from SDZ degradation (Tappe et al., 2015). Losses of 2-AP in this research could suggest partial mineralization of the compound by 2APm3 present in the mixed culture. Genomic techniques should be applied to determine if 2APm3 is present in the culture, and to what extent.

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Appendix A. Solution Recipes

All recipes listed below correspond to a 1L final volume unless otherwise stated.

Table A1. R2A Growth Medium Recipe

Chemical	Amount (gram)
Proteose Peptone	0.5
Casamino Acid	0.5
Dextrose	0.5
Soluable Starch	0.5
Dipotassium Phosphate	0.3
Magnesuim Sulfate	0.5
Sodium Pyruvate	0.3

Table A2. PBS Washing Solution Recipe

Chemical	Amount (gram)
Sodium Chloride	8
Potassium Chloride	0.2
Disodium Phosphate	1.44
Monopotassium Phosphate	0.24

Table A3. Macronutrient Solution

Chemical	Amount (gram)
Disodium Phosphate	0.7268
Monopotassium Phosphate	0.3522
Magnesium Sulfate Heptahydrate	0.05
Ammonium Chloride	0.306

Table A4. Micronutrient Solution A

Chemical	Amount (gram)
Ethylenediaminetetraacetic acid (EDTA)	0.015
Zinc Sulfate Heptahydrate	0.0045
Ferrous Sulfate	0.003
Manganese (II) Chloride	0.001
Boric Acid	0.001
Sodium Molybdate	0.0004
Copper (II) Sulfate	0.0003
Cobalt (II) Chloride	0.0003
Potassium Iodide	0.0001

Table A5. Micronutrient Solution B

Chemical	Amount (gram)
Calcium Chloride Dihydrate	0.0045

Table A6. Micronutrient Solution C
Mass for 50mL final volume

Chemical	Amount (gram)
Biotin	0.005

Table A7. Micronutrient Solution D
Mass for 50mL final volume

Chemical	Amount (gram)
Thiamin	0.002

Protocol for creation of 10mg/mL SDZ stock solution (total volume 5mL)

- Measure 0.05g SDZ powder (Alfa Aesar, Ward Hill, MA)
- Carefully add powder to a 15mL sterile Genemate tube, wash excess powder remaining on the measuring dish into the tube (using a total of 1.5mL of Nanopure water)
- Make a 0.1M sodium hydroxide solution by adding 0.04g sodium hydroxide to 10mL of Nanopure water (autoclave and cool before further use)
- Add autoclaved sodium hydroxide to SDZ slurry in 20uL increments, vortexing the mixture after each addition; continue until the SDZ is fully dissolved
- Add additional Nanopure water as needed to bring the final volume of the solution to 5mL
- Using 0.2μm filter and sterile syringe, filter the solution into a new 15mL vial to create a sterile solution
- Wrap vial in aluminum foil and refrigerate between uses

NOTE: This procedure was used for the creation of all sulfonamide stock solutions (including the experiments outlined in Chapter 4), base powders for other sulfonamides studied were obtained from the following sources:

- Sulfamethazine: Aldrich (St. Louis, MO)
- Sulfamerazine: Aldrich (St. Louis, MO)
- Sulfathiazole: MP Biomedicals (Santa Ana, CA)

Appendix B. Standard Recipes

Table B1. Standard stock solution increments (for HPLC)

Amount of 0.1mg/mL SDZ solution added	Amount of 0.1mg/mL 2AP solution added	Amount of 0.1mg/mL SA solution added	Final mass of each analyte in standard solutions	Final concentration of each analyte in standard solutions
1.5mL	1.5mL	1.5mL	3000ng	15mg/L
1mL	1mL	1mL	2000ng	10mg/L
0.5mL	0.5mL	0.5mL	1000ng	5mg/L
0.25mL	0.25mL	0.25mL	500ng	2.5mg/L
100μL	100μL	100μL	200ng	1mg/L
50μL	50μL	50μL	100ng	0.5mg/L
10μL	10μL	10μL	20ng	0.1mg/L

Table B2. Standard stock solution increments (for LC/MS)

Amount of 0.1mg/mL SDZ solution added	Amount of 0.1mg/mL 2AP solution added	Final mass of each analyte in standard solutions	Final concentration of each analyte in standard solutions
1.5mL	1.5mL	3000ng	15mg/L
1mL	1mL	2000ng	10mg/L
0.5mL	0.5mL	1000ng	5mg/L
0.25mL	0.25mL	500ng	2.5mg/L
100μL	100μL	200ng	1mg/L
50μL	50μL	100ng	0.5mg/L
10μL	10μL	20ng	0.1mg/L