Are aquatic snails reservoirs and vectors of microbes bearing antibiotic resistant genes?

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Abstract

The role of Stagnicola elodes, a freshwater snail, as a reservoir and vector for transporting antibiotic resistant genes (ARGs) was explored under laboratory conditions. We hypothesized that ARG-bearing microorganisms would become part of the snail gut microbiome allowing ARGs to be spread from their initial point of origin should snails be transported or the input of pharmaceuticals cease. We exposed snails in jars for 14 days wherein they grazed on biofilms that contained microbes resistant to the antibiotic sulfamethoxazole (SMX). Snails were then transferred to fresh media with no SMX for either a 7-day hibernation or depuration period. SMX-related ARGs were quantified in the 14-day treatment and 7-day depuration and hibernation systems. On day 14, treated jars had 6.6% of SMX resistant genes in the water phase, and the 7-day hibernation and depuration vessels had an average of 3.9% and 1.4%, respectively, relative to the total water-borne microbial community. Methods of extracting snail samples for SMX-related ARGs were unsuccessful. This was attributed to the snails' having extensive mucus sugars that interfere with extraction. Our findings suggest that ARGs could be transferred to new environments from snails excreting gut flora in their feces, warranting further investigation.

Keywords: antibiotics, resistance, sulfamethoxazole, snails

1. Introduction

Antibiotics are a class of drugs used to prevent and treat bacterial infections, to inhibit bacterial growth, and to promote growth in animals raised for human consumption (Government of Canada, 2014). Microbial resistance to antibiotics can occur naturally, but the misuse (e.g., not completing a prescribed dose) and overuse (e.g., over-prescribing) of antibiotics in people and animals exacerbates the problem (Government of Canada, 2014). Overtime, antibiotic resistance genes (ARGs) continually become present in the gene pool of the bacterial population as a useful feature for survival (Marti et al. 2014). Currently, ARGs are considered a global threat to human and ecological health and are of particular concern in systems where sewage effluents can contaminate surface waters with resistant microbes. In addition, antibiotics are only partially metabolized and are then excreted with urine and feces, either to sewage treatment plants or directly into water or soil contaminating the natural environment (Martinez, 2009). Of particular concern are wastewater lagoons used by small municipalities when full wastewater treatment plants are impractical or not feasible. In Manitoba, Canada, an estimated 350 small communities (population <10,000) rely on these types of wastewater lagoons (Manitoba Land Initiative, 2004). There is evidence for both pharmaceutical releases from these systems as well as ARG formation. For example, in the rural community of Grand Marais, Manitoba, Canada,

wastewater is treated through a passive sewage lagoon prior to passage through a treatment wetland, following release into surface waters. Anderson et al. (2013), found that there was no significant reduction of ARGs with the treatment process used in Grand Marais indicating that ARGs are being released into local surface waters from lagoon effluent. Other wastewater treatment processes in rural municipalities have also observed ARGs in sewage effluents. For example, the wastewater facility in Dunnottar, Manitoba, Canada comprises a primary and secondary lagoon system and a full-scale sub-surface passive filtration system. Anderson et al. (2015) reported that approximately 99 and 58% of sulfonamide and tetracycline resistant bacteria, respectively, were effectively removed from the lagoon treatment system. However, prior to release a pilot-scale filter appeared to both reduce resistant bacteria (58%) in wastewater and harbour them in subsequent biofilms, where relative abundances of sulfonamide and tetracycline genes were observed to be greatest (Anderson et al. 2015). The formation of biofilms in the filter appear to provide an optimal condition for development of ARGs in these dense microbial communities, leading to a greater proportion of bacteria carrying ARGs in outflow. Snails in wastewater lagoons spend the majority of their life cycle in the wastewater exposed to antibiotics and ARGs. Some snails, such as *Stagnicola elodes*, a common freshwater species, demonstrates tolerance to pollution (Corderio et al. 2015) and could thrive in such conditions. Biofilms, a major food source for snails, are aggregations of bacteria that live in a highly structured and organized community and can develop antibiotic resistance (Marti et al. 2014). Snails that consume biofilms that contain ARGs then have the potential to develop ARGs within their own gut. The hibernation of adult snails provides a plausible mechanism for ARGs to overwinter within the gut if ARGs are assimilated within the gut flora. Cheatum (1934) analyzed snail hibernation patterns in the family Lymnaeidae and found that if water freezes rapidly snails become frozen within the ice. If ice over happens slowly, snails will continue activities until the entire mass of water is frozen solid, resulting in snails becoming embedded in the ice or migrating from cooler littoral areas to burry themselves into sediments to overwinter. They observed that when thawed, snails were found to be alive, and observed mortalities were attributed to old age or parasitism, not the freezing (Cheatum, 1934).

The objective of this study was to characterize the role of the snail *Stagnicola elodes* as a vector and reservoir of ARGs on the Manitoba landscape through a laboratory study. Specifically, we attempted to address three questions; 1) do ARGs become part of the snail gut microflora, 2) if ARGs do become part of the gut microflora, do they persist if ARGs are removed from the environment, and 3) can ARGs overwinter in the gut of the snails?

2. Methods

2.1. SNAIL CULTURING

Stagnicola elodes and initial culture water were obtained from the Prairie Wetland Research Facility (PWRF) mesocosms located at the University of Manitoba. The snails in the mesocosms were originally sourced from Oak Hammock Marsh, Manitoba. *Stagnicola elodes* were cultured following similar procedures as described by Zakikhani et al. (1999). A 20-liter glass aquarium was filled with mesocosm water and replenished with City of Winnipeg dechlorinated tap water when water levels began to decrease. Sand, paper towel, and a few small pieces of PVC (polyvinyl chloride) pipe were placed in the aquarium to allow for mechanical digestion and more surface areas to lay egg masses. The culture was given fresh washed organic romaine lettuce daily (excluding weekends), kept on a 16:8 light-dark cycle and oxygenated (Zakikihani et al. 1999).

2.2. EXPERIMENTAL DESIGN

A laboratory study was conducted with a 14-day exposure period (S) to the antibiotic sulfamethoxazole, followed by either a 7-d depu-

ration period (introduction into clean media; S7D) or 7-d hibernation period (introduction into clean media at 4ºC to stimulate winter; S7F) (Figure 1). The test vessels were one liter glass Mason jars. The sulfonamide antibiotic sulfamethoxazole was added to the agarose (Fisher Brand reagents, ACS grade) followed by plating in the jars and inoculated with soil slurry from the PWRF mesocosms to allow growth of microorganisms containing ARGs. A nominal treatment level of 128 mg/L suflamethoxazole was introduced into the agarose as suggested by the European Committee on Antimicrobial Susceptibility Tests to ensure ARG formation (EUCAST, 2015). Sulfamethoxazole was introduced to the water column by passive diffusion from the agarose (days 4, 7, 14), plus three controls with no antibiotic added (Figure 1). Mason jars were filled with aged City of Winnipeg tap water (which is treated via chlorination and UV, reducing significantly the likelihood of pharmaceutical contamination) and aerated via pumps added to each to ensure adequate oxygenation and mixing. Jars were left for 24 hours to allow any excess ammonia to purge from the agarose. After 24 hours, half the water was replenished with aged tap water and then tested for levels of ammonia using API Ammonia Aquarium Test Strips and considered acceptable once ammonia levels were at < 3 mg/L. This was done twice before reaching target ammonia concentrations. Then 10 mL of a calcium and magnesium solution was added to increase the tap water to a medium hardness (80-100 mg/L) to ensure snails were not stressed in the jar environments. A total of five snails of various sizes were added to each test vessel with the aim to keep density sufficient to allow enough food and to limit possible overcrowdings. Test vessels were kept on a 16:8 light-dark cycle at 20ºC and randomly placed in a Conviro 2006 57/2 growth chamber for an exposure period of 14 days. Supplementary feeding of 1.5 g of fresh washed organic romaine lettuce was provided and water was topped up as needed to ensure jars remained full with aged tap water. At 14 days, snails from two replicates were transferred to new test vessels containing no sulfonamide antibiotic. One replicate followed a 7 day depuration period within similar growth chamber conditions to analyze if ARGs persist in the gut flora of the snail after removal from the environment. The other replicate followed a 7 day depuration period in the fridge (7F) at 4ºC to analyze if ARGs can over winter (through hibernation) in the gut flora. Water temperature (ºC), dissolved oxygen, conductivity and pH were taken every Monday, Wednesday and Friday from respective jars using a 6600 V2 Sonde YSI instrument.

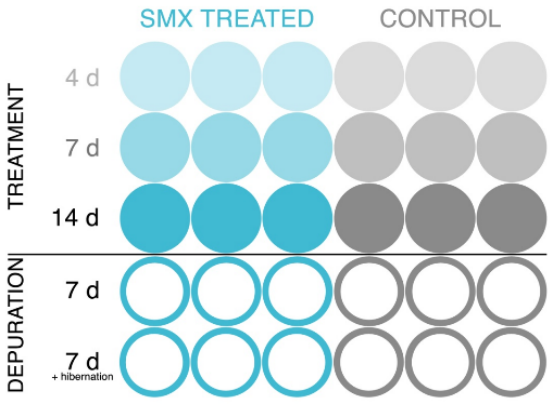

Figure 1: *Experimental design of Stagnicola elodes laboratory study with snails undergoing exposure for 14 days (S4, S7, S14) followed by a 7-d depuration period (S7D) or 7-d hibernation period (S7F), plus control (C).*

2.3. ANALYZING ARGs

Whole *Stagnicola elodes* (n=3) and feces were sampled from their respective test vessels at 4 days, 7 days, 14 days, and 7 days depuration (following the 14-day exposure) for ARGs (see "DNA Extraction" for methods). Water samples were taken at each time point from the respective jars for ARG analysis. As well, one extra jar was prepared and water samples taken at the time snails were introduced to the exposure jars to allow for initial ARG measurements. A zero hour sample of snail gut flora was taken from the culture to note level of ARGs prior to snail exposure. Laboratory snails were taken from their respective jars, the outer shells crushed and removed, and the entire snail dipped into a phosphate buffer saline solution, placed into a microcentrifuge tube, and frozen immediately for later DNA extraction and ARG analysis. Sampling *Stagnicola elodes* feces from laboratory jars consisted of extracting feces from the respective jar directly using transfer pipettes, placing into microcentrifuge tubes, and freezing immediately for later extraction and analysis. All water samples of 100 ml were filtered through 0.22 μm filter paper in Nalgene Analytical Test Filter Funnels and frozen immediately in microcentrifuge tubes for later ARG analysis.

2.3.1 DNA Extraction

A PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) was used for water filter DNA extraction and attempted feces DNA extraction. Prior to following the manufacturer's protocols, the filters were each cut in half in order to place in tubes. Chemical precipitation and centrifuge procedures followed manufacturer's protocols. Ultraclean Cell and Tissue Kit (MoBio Laboratories Inc., Carlsbad,CA) was used to attempt snail flora DNA extraction. Manufactures protocols were followed except Proteinase K was not used since ARGs in the bacteria associated with the snail were the question of interest and not DNA from snail tissue and cells. Proteinase K breaks down tissue to extract DNA from the organism and the bacterial DNA becomes lost in the large amounts of tissue DNA, leading to undetectable results of bacterial DNA. Extracted DNA was stored at -20^{o}C until further analysis.

2.3.2 Quantitative Polymerase Chain Reaction (qPCR)

Abundances of 16S rRNA (surrogate measure of total bacteria) and ARGs were quantified by qPCR using EvaGreen dye. Frozen water sample filters were thawed prior to sample processing. An 18 μL aliquot of working stock that included the EvaGreen dye, molecular grade water and primer solution was added to each 2 μL DNA sample. This study screened for the three most common sulfonamide resistant genes; *sul1, sul2*, and *sul3*; primers were not conserved among varying bacterial species since the study's main focus was the proportion of resistance. Sample dilution of 1:10 was determined to be the most efficient for reaction efficiencies. Gene standards were provided by Dr. Chuck Knapp at the University of Strathclyde and were tested to ensure that they had not been denatured from the freeze-thaw process during transportation. The primers and annealing temperatures had been previously established (Knapp et al. 2010; Ng et al. 2001; Pei et al. 2006). Molecular-grade water was used as a reaction negative control. There were two replicates analyzed per sample, and a template control for each plate analyzed. Analyzing the plate involved initial denaturation of two minutes at 94-95^{o}C, following 40 cycles of 10 seconds at 95^{o}C and 30 seconds at 47-60^{o}C for replication. For the 40 cycles, 60^{o}C was chosen to provide the optimal temperature for annealing, since the annealing temperatures range from 50^{o}C for *sul1* to 60^{o}C for *sul2* and *sul3* (Pei et al. 2006). Total abundance of genes was normalized to 16S rRNA values to represent resistance per total bacteria population.

2.4. SMX ANALYSIS

Sulfamethoxazole concentrations were measured in the overlying water throughout the experiment. Water samples were spiked

with isotopically labelled internal standard (sulfamethoxazole-d4) prior to direct-injection analysis. Concentrations were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). Chromatography was carried out using an isocratic method (70% H2O: 30 MeOH) with an Agilent 1200 Series (Agilent Technologies, Mississauga, ON) binary pump connected to a Phenomenex (Torrance, CA) Kinetex XB-C18 column (50 mm x 2.1 mm x 1.7 μm particle size) and C18 SecurityGuard ULTRA Cartridge (2.1 mm I.D.). Detections were achieved using an Agilent 6410B MS/MS equipped with an electrospray ionization source in positive mode. The following m/z transitions were monitored; 254.0 -> 156.1 (quantifier), 254.0 -> 108.1 (qualifier), and 258.0 -> 160.0 (internal standard).

2.5. DATA ANALYSIS

All analyses were conducted using SigmaStat 3.5 (Systat Software Inc. 2006). Statistical differences between the abundance of ARGs and water quality parameters (temperature, dissolved oxygen, conductivity, and pH) over time were determined by a one-way repeated measures ANOVA (p-value = 0.05). Normality and equal variance were tested. If data did not satisfy assumptions for a repeated measures one-way ANOVA following log transformation, a one-way repeated measured ranked ANOVA was performed.

3. RESULTS

Mortality of snails in our test system was observed. After four days, all snails remained alive. On the seventh day one snail died in S7-1 and two in S7-2. By day fourteen there were no snails remaining within S14-2, four snails remained in all the other jars with the exception of C14-3 having all five remain. After the 7 day depuration period C7D-1 and C7F-1 were the only jars that had three snails remaining for sampling. C7D-2, S7D-2, S7F-1, and S7F-3 had no snails remaining, C7F-2 had 1 snail remaining, and C7D-3, S7D-1, S7D-3, C7F-3, and S7F-2 had two snails remaining. Quantifying ARGs was successful for laboratory water samples, but not possible in the snail tissue and feces samples attempted from the laboratory assay. The amount of sul1, sul2, and sul3 sulfonamide resistant genes were added together to provide the total amount of ARGs in each sample. Abundance of ARGs was standardized to the abundance of 16S rRNA in each sample to provide indication of the proportion of the bacterial genes that could impart microbial resistance (Figure 2). As the study progressed, the greatest percentage of resistant genes was measured on day 14 with 6.6%, followed by Day 7, which was 4.9%. Day 0 and day 4 were relatively lower with measured proportions of 0.04% and 0.06%, respectively. Day 4 control had 0.02% ARGs. ARGs were present in depuration (S7D) and hibernation (S7F) jars with proportions of 1.4% and 3.9%, respectively. We observed greater proportions of resistant bacteria in later samples (6.6% and 4.9%), which are significantly greater than realistic environmental resistant bacteria proportions found in a wastewater lagoon in Dunnottar, Manitoba (i.e., 0.8%; Anderson et al., 2015), indicating that the levels of the sulfonamide antibiotic were more than sufficient to produce ARGs.

SMX concentrations within the treatment jars remained consistent from day 4 to 14 (Figure 3). The greatest concentration of SMX in the water was sampled on day 1 (5.7$\pm$0.7 mg/L) then continued to decrease until day 14 (2.9$\pm$0.7 mg/L). There was no quantifiable concentration of SMX measured within the control jars until day 7 (0.41 mg/L). This may be due to slight cross contamination from opening and closing treated and control jars in close proximity within the growth chamber.

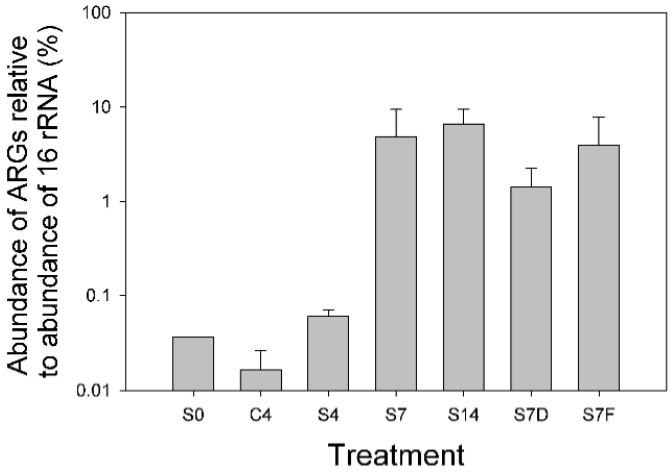

Figure 2: *Logged mean (SE) total abundances of sulfonamide antibiotic resistant genes standardized to abundances of 16S rRNA from laboratory jar water samples and analyzed using qPCR. C4 represents a control on day 4. S represents SMX treated jars. S4 (n=3), S7 (n=3), and S14 (n=3) represent the average abundance of ARGs in SMX treatment jars on day 4, day 7, and day 14. Snails from S14 treatment jars were transferred to untreated (no SMX) media in the seven day depuration (S7D) and hibernation jars (S7F). The average abundance of ARGs were then measured in the seven day depuration jar (S7D; n=3) and seven day hibernation jar (S7F; n=3).*

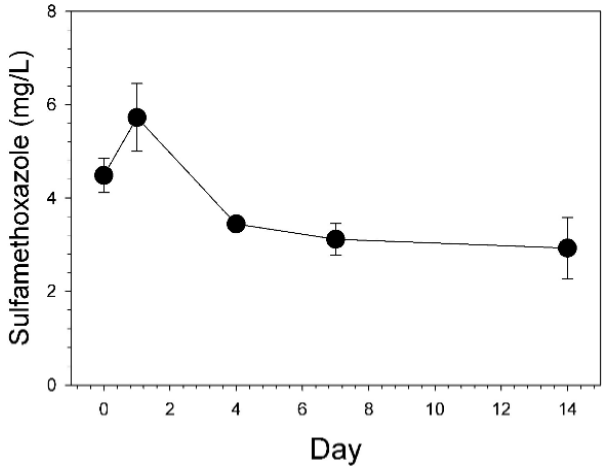

Figure 3: *Mean (SD) concentration of sulfamethoxazole in water from laboratory treatment jar s from day 0 to 14 (S0, S1, S4, S7, S14; n=3).*

Water quality remained relatively constant in the jars throughout the study. A one-way repeated measures ranked ANOVA found no statistical difference between controls and test jars over time for temperature (p=0.754; Figure 4), dissolved oxygen (p=0.344; Figure 5), pH (p=0.754; Figure 6) or conductivity (p=0.884; Figure 7).

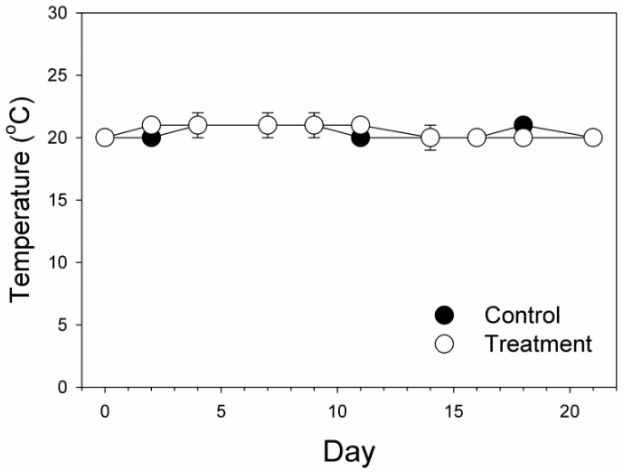

Figure 4: *Average temperature (^{0}C) in each sample jar (mean SD) over the 21 day study. Days 0 to 14 represent the temperature in sulfamethoxazole treatment jars (S), days 16 to 21 represent the temperature in depuration jars (D), plus control. No statistically significant differences were detected by repeated measures one-way ANOVA (p>0.05).*

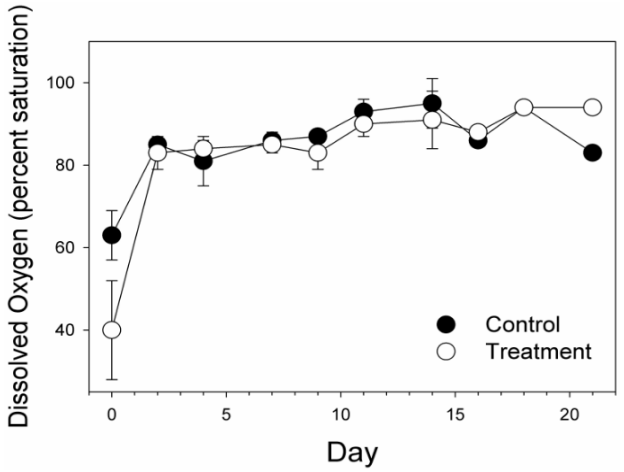

Figure 5: *Average dissolved oxygen (percentage saturation) in each sample jar (mean SD) over the 21 day study. Days 0 to 14 represent the dissolved oxygen in sulfamethoxazole treatment jars (S), days 16 to 21 represent the dissolved oxygen in depuration jars (D), plus control. Overlap was apparent on a number of days. No statistically significant differences were detected by repeated measures one-way ANOVA (p>0.05).*

4. DISCUSSION

We hypothesized that ARG-bearing microorganisms would become part of the snail gut microbiome, resulting in both a reservoir and a vector, allowing ARGs to be spread from their initial point of origin should snails be transported or the input of pharmaceuticals cease. Specifically, we attempted to address three questions 1) do ARGs become part of the snail gut microflora, 2) if ARGs do become part of the gut microflora, do they persist if ARGs are removed from the environment, and 3) can ARGs overwinter in the gut of the snails? Due to analytical issues it was not possible to observe whether ARGs had become part of the snail gut microflora. At the start of the study, the functionality of conventional DNA extraction procedures using a Tissue and Cell DNA

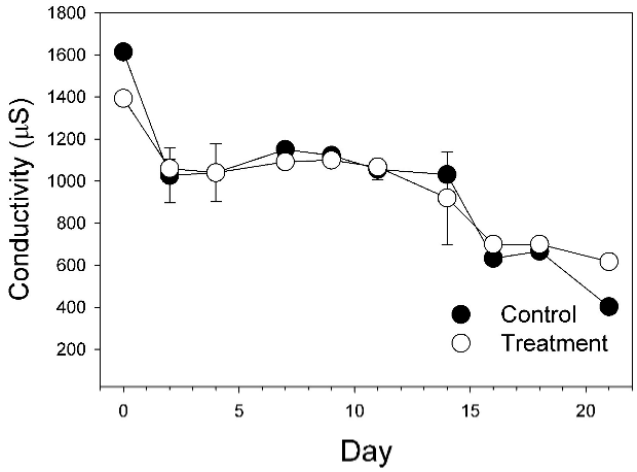

Figure 6: *Average conductivity (µS) in each sample jar (mean SD) over the 21 day study. Days 0 to 14 represent the conductivity in sulfamethoxazole treatment jars (S), days 16 to 21 represent the conductivity in depuration jars (D), plus control. Overlap was apparent on some days. No statistically significant differences were detected by repeated measures one-way ANOVA (p>0.05).*

Isolation Kit was not considered an issue. After trials of extracting various weights of snails for the extraction process, it was thought that using parts of snails 10-15 mg was effective for the extraction process. When running the DNA extractions, no ample amounts of 16s rRNA were detected. It is likely that conventional DNA extraction procedures have difficulty functioning on snails because of elevated concentrations of mucopolysaccharides; these are long chains of sugar molecules that are often found in mucus to assist snail movement (Davison et al., 1998). Mucopolysaccharides interfere with effective DNA extraction and polymerase chain reactions (PCR) amplification (Echevarria-Machado et al., 2005). Mucopolysaccharides have a viscous texture and make the DNA unmanageable in pipetting and unsuitable for PCR because they inhibit Taq polymerase activity (Echevarria-Machado et al., 2005). This inhibition results in insufficient amounts of 16s rNA (bacterial DNA) being detected through the qPCR process. In other words, because of the low efficacy with pipetting and inhibiting Taq polymerase, little to no DNA is extracted from the sample which leads to undetectable amounts of 16s rNA in qPCR analysis. Sulfamethoxazole was measured in zero hour water samples (4.5±0.4 mg/L; Figure 3), and generally remained at or above 3 mg/L over the 14 days, providing suitable conditions for aggregations of bacteria (biofilms) to develop ARGs. Though development of resistance was not immediate, the presence of ARGs was seen by day 7 with 4.9% resistant bacteria. Snails were surrounded by resistant biofilms to forage on and ARGs within the water column, providing ideal conditions for snails to develop ARGs within their gut microflora. There was a relatively high proportion of ARGs found within the water of the hibernation jars (S7F; 3.93.9%), but this could be an outlier as one of the test vessels had 11% while the other two had 0% resistant bacteria. Similar conditions were seen in the 7 day exposure jars (S7; 4.9±4.6%), one test vessel had 14% ARGs and the other two had <0.4%. These inconsistencies in the exposure jars may be a result of greater development of ARGs within the snails present in the outlier jar (S7-2; 14%), allowing for a larger portion to be excreted then sampled in water. ARGs measured in the depuration (S7D) and hibernation (S7F) jars could be caused by bacterial residue on the snails when transferred from treatment jars to depuration jars, allowing a higher portion of ARGs to be transferred in some jars but not others. Another possible cause of finding ARGs

in the depuration jars is because the snails released ARGs from their gut flora in their feces, since antibiotic resistance develops within the gut (Anderson et al., 2013). This may be preliminary evidence that ARGs can persist once removed from the environment and can overwinter within snail gut flora. Due to analytical issues, it was not possible to determine the concentrations of bacteria and resistant bacteria in snail tissue or feces, so this reasoning currently remains hypothetical and warrants further investigation. With the qPCR method of quantifying abundances of genes within samples, genes from both living and dead bacteria are included so the results may not truly represent the proportion of living bacteria that are resistant to antibiotics (Anderson et al., 2013). Therefore, the above should be taken into consideration when interpreting the observation of resistant bacteria in the laboratory assay.

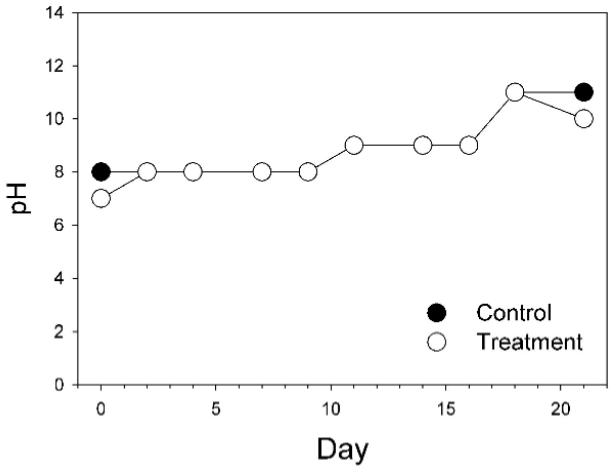

Figure 7: *Average pH in each sample jar (mean SD) over the 21 day study. Days 0 to 14 represent the pH in sulfamethoxazole treatment jars (S), days 16 to 21 represent the pH in depuration jars (D), plus control. Overlap was apparent on some days. No statistically significant differences were detected by repeated measures one-way ANOVA (p>0.05).*

The general test system has value to test our hypotheses, but the significant mortality observed needs to be addressed. Laboratory conditions stayed relatively consistent, meaning the growth chambers proved as an effective way to maintain consistent environmental conditions in a bioassay. The increase of dissolved oxygen in two days, from day 0 to day 2, is likely due to more time for oxygen pumps to aerate the water. Afterwards they remained consistent throughout the assay. The new depuration jars started on day 14 also remained relatively consistent. Elevated ammonia (>5mg/L) and pH (>9) were measured by the end of day 14 and day 7 depuration (day 21), contributing to observed algal growth and toxic conditions for snails. Leaching of ammonia likely occurred from the agarose over time, but ammonia is also generated by heterotrophic bacteria through nitrate reduction (Dai et al. 2012). The pH also affects the concentrations of ionized (NH_4^+) and un-ionized ammonia (NH_3); at a high pH a greater percentage of the total ammonia in water exists as NH_3, which is more toxic than NH_4 (Straus et al., 1991). When ammonia concentrations are high, it is difficult for organisms to sufficiently excrete the toxicant, leading to build up in internal tissues and blood which can potentially result in death (USEPA, 2013). The recommended water quality guideline for aquatic organisms to chronic exposure of un-ionized ammonia

is 1.9 mg TAN/L (Total Ammonian Nitrogen) at pH 7 and 20°C for 30 days (USEPA, 2013). Since concentrations of ammonia and pH levels by day 14 exceeded this chronic dose at a given pH recommended by USEPA, this likely caused our observed snail mortalities. Ammonia levels could have been reduced by refreshing half the water, though this would have led to a reduced exposure of ARGs in the exposure jars. It was also unknown that ammonia levels would spike to toxic levels by day 14. Therefore, it is recommended that ammonia levels be monitored closely with agarose experiments in water and when eutrophic conditions are observed. Many challenges presented themselves both methodologically and analytically throughout each tier of the study. Currently, microbial DNA extraction needed for qPCR analysis is unreliable in both gut and feces samples of snails. As a result, none of the hypothesized questions could be definitively answered because of the lack of analytical data. Still, with results indicating the presence of ARGs in depuration jars, further studies are recommended to determine freshwater snail's role in potentially degrading or transporting ARGs in aquatic environments.

5. ACKNOWLEDGEMENTS

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