GREEN ROOF TECHNOLOGY: INVESTIGATION OF CRUMB RUBBER EFFECTS ON MICROBIAL GROWTH

by

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ABSTRACT

Green roofs incorporate plant communities in the roof structure. The use of these structures is a growing practice worldwide, particularly in densely populated areas. In an attempt to find new methods for recycling crumb rubber, incorporation of this material into the growth medium of green roofs has become an attractive option. Though this approach decreases waste in landfills, there are concerns about the leaching of zinc, other heavy metals and nutrients into the environment.

The present study analyzed the impact of leachate from crumb rubber on the growth of *Salmonella enterica* subsp. *enterica* serovar Typhimurium. The study showed shown that microbes can colonize crumb rubber and that zinc-tolerant strains of *Salmonella* can be obtained after sub-culturing in increasing levels of zinc. Gene expression of *Salmonella* zinc efflux and influx pumps were measured using qPCR, and the potential of increased resistance to antimicrobial agents due to increased zinc tolerance was determined.

Crumb rubber extracts were obtained by incubation in synthetic rainwater at 28°C. The liquid was filtered from the crumb rubber after 1, 24, and 48 hours and microbes were exposed to these extracts. Results of growth studies involving *S.* Typhimurium and the 24-hour extract showed that crumb rubber contains compounds that were inhibitory to the bacterium, but when diluted at least 10-fold the extract

could serve as a source of nutrients, resulting in up to about 15-fold increased colony forming units compared to growth in the synthetic rainwater.

Salmonella were able to attach to crumb rubber as shown by the crumb rubber biofilm assays. Since crumb rubber is known to contain as much as 1% of its weight in zinc, *S.* Typhimurium were exposed to increasing amounts of zinc and strains with reduced susceptibility (SRS) capable of growing in the presence of 20 mM zinc or more were isolated.

qPCR was used to quantify the expression of the major zinc influx and efflux pumps in cells exposed to 0 mM and 20 mM zinc. The SRS strain exhibited a seven-fold increase in the gene expression of efflux pump gene *zntA* and a 2.5–fold decrease in the gene expression of the influx pump gene *znuA* compared to the parent when exposed to 20 mM added zinc.

In biofilm formation studies, the SRS strain was less able to attach to an abiotic surface, but was more motile than the parental strain as observed by motility assays on semisolid agar plates. The SRS strain did not show an increase in resistance to oxytetracycline, tylosin tartrate, fumagillin, DTAC, or triclosan. These data suggest that simultaneous addition of zinc and antimicrobial to cultures in TSB did not lead to cross-resistance.

Chapter 1

INTRODUCTION

1.1 Green Roofs

Green roofs incorporate plant communities in their structures. The use of these structures is a growing practice worldwide, particularly in densely populated areas.[1,2] Green roofs are classified as either intensive or extensive, based on soil depth and vegetation type.[1,2] Intensive green roofs have a deeper soil layer, generally at least 15 cm and larger plant species.[1,2] Extensive green roofs usually have a soil layer less than 15 cm and thus only permit growth of smaller and hardier plant species.[1,2] In either case, all green roofs provide a variety of benefits; for example, they mitigate storm water runoff, provide additional insulation which in turn regulates the inside temperature, attenuate noise, decrease air pollution, provide a habitat for wildlife, increase the life of the roof, decrease the amount of materials in landfills, and they are aesthetically pleasing.[1,2]

Green roofs are typically composed of a vegetation layer, soil layer, filter, and drainage material. The present study is concerned with the soil layer. The commercially used components of the soil layer are lightweight materials, including expanded slate or shale that can be supplemented with additional inorganic materials like pumice or crushed clay bricks and tiles.[3] Unfortunately, sources of lightweight

inorganic materials are limited; therefore, using shredded tires, termed "crumb rubber," is an appealing choice to supplement green roof growth media.

1.2 Crumb Rubber

Shredded tire, also referred to as "crumb rubber," is a waste product that is generated in high volumes. The Environmental Protection Agency (EPA) estimated that about 290 million scrap tires were produced in 2003 in the United States alone.[4] There are many markets for up to 86.5% of generated crumb rubber scraps; however, about 39 million tires remain unaccounted for. These unused tires are presumably sent to landfills each year, consuming large amounts of space and introducing additional health hazards.[4]

Dumping waste tires in landfills can cause health risks as they provide a favorable environment for disease-carrying pests to breed, harbor pathogens, catch fire, which is difficult to extinguish, release harmful chemicals, and leach heavy metals and nutrients.[4] Metals that have been found to leach from tires include zinc, selenium, lead, cadmium, magnesium, manganese, arsenic, strontium and barium.[5,6] Nutrients that are leached include nitrate, ammonium, and phosphate.[5]

A novel use for recycling crumb rubber is to incorporate it into the growth medium of green roofs; however, before using crumb rubber on a large scale, some concerns must first be addressed. The metal of interest in the present study is zinc, as it can make up approximately 1% of a tire's total weight.[5] Kanematsu found zinc in tire leachates at levels ranging from 2.0-2.8 mg/L.[5] The goal of the present study was

to determine the effects of crumb rubber and zinc on bacterial survivability, growth, cross-resistance, motility and attachment.

1.3 Zinc

Zinc is the second most common heavy metal present in living cells.[7,8] Although zinc is required as a cofactor for many proteins, excess zinc can be toxic. Lethal effects involve the inhibition of the electron transport system, inhibition of essential enzymes, and competition with other metal cations for binding sites.[9,10] Zinc is also used as a stabilizer for membranes and other macromolecules.[9] Bacteria have an intracellular concentration of zinc of around 0.2 mM.[11] In *Escherichia coli*, a microbe closely related to *Salmonella* Typhimurium, up to 3% of expressed proteins were identified as zinc-binding proteins.[12]

Specific and non-specific zinc transporters are present in bacterial cells.[9] Non-specific transporters are constitutively expressed, allowing zinc (sometimes along with other divalent ions) to enter the cell, even when the cell is reaching toxic levels of the ion(s).[9] Specific transporters are more highly expressed during times of low availability of zinc.[9]

The transporter ZnuABC is the most important influx pump for zinc in bacteria.[7,8,11] It is present when the cell is in conditions lacking zinc.[11] The zinc uptake regulator (Zur) is a metalloregulatory protein that has a DNA-binding domain and is zinc-bound when zinc levels are sufficient.[11] Zur is sensitive to zinc molarity as low as 10^{-15}.[7,11] ZinT is a secondary influx pump that works in addition to, but is not as important as ZnuABC.[7] ZinT is present during times of severe zinc deficiency in

Salmonella; however, it is not found in all bacteria that possess ZnuABC.[7,13] ZupT is constitutively expressed for preferential zinc uptake, but can facilitate Co^{2+}, Fe^{2+}, and Mn^{2+} import as well.[14-16]

ZntA is an ATP-driven zinc efflux pump that is associated with microbial resistance to the divalent cations, Cd^{2+}, Hg^{2+}, and Pb^{2+}.[10,17] Zn^{2+} is the most potent inducer of the promoter of *zntA* compared to the other divalent cations.[18] The transcription factor ZntR modulates *zntA* transcription, in the presence of high levels of extracellular zinc.[10] ZitB is responsible for zinc efflux via gradient diffusion; this pump also has a role in Cd^{2+} and Pb^{2+} resistance.[10,19,20]

ZntB is an additional zinc efflux pump. Less work has been done with this efflux pump; however, it is known to behave similarly to ZntA and ZitB, as it also contributes to interactions with Cd^{2+}, Pb^{2+}, and Ni^{2+}.[8] These associations were all discovered as a result of creating knockouts for *zntA*, *zitB,* and *zntB*.[8,17,19-23]

There are five general ways that bacteria overcome heavy metal toxicity. 1) They can produce a barrier to exclude the metal, 2) export the agent from the cell, 3) physically sequester it within the cell, 4) sequester it outside the cell, or 5) transform it into a non-toxic form.[9]

1.4 *Salmonella enterica* serovar Typhimurium as a Model Organism

Introduction of pathogens to a green roof could result from using animal or wastewater-derived fertilizer products or the introduction of fecal matter from birds and other animals.[24] Possible bacterial pathogens include *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli*.[24] *Salmonella* survival in fertilized soil

ranges from just a few days up to 332 days.[24] In the environment, bacteria often suffer periods of starvation. *Salmonella* do not form endospores, but do undergo physiological changes to respond to and survive in conditions lacking nutrients.[25]

The current study used *Salmonella* as the model organism. *Salmonella* spp. are facultative anaerobic Gram-negative rods, divided into two groups, typhoidal and non-typhoidal.[26] *Salmonella* species are the leading cause of bacterial foodborne diseases.[27] *Salmonella enterica* causes salmonellosis, which has symptoms that range from gastroenteritis, abdominal cramping, diarrhea, fever, bacteremia, and death.[28,29] Some individuals may develop long-term symptoms post-recovery.[29] These ailments include joint pain, eye irritation, and painful urination, which could ultimately lead to chronic arthritis.[29] Non-typhoidal *Salmonella* serovars, including Typhimurium, cause approximately 155,000 deaths each year and cost the United States an estimated $4.4 billion per year.[30,31]

Foodborne illness associated with fresh produce increased from 1% in the 1970s to over 6% during the 1990s.[32] There is evidence that *Salmonella* spp. are being internalized within plants, not just attached to the surfaces.[32] There are three modes of pathogen entry into plant tissue, including through the openings that are naturally part of the plant, entry through injury, and in the water taken up by the roots.[32] As green roofs are exposed to the outdoors, contamination with *Salmonella* is a potential problem. Therefore this bacterium was chosen as the test organism in the current study.

1.5 Development of Tolerance/Resistance and Cross-Resistance

Bacteria react rapidly to signals and substances in the surrounding environment.[33] There are numerous examples of cross-resistance between biocides and antimicrobials.[33] In a study by Nishino *et al*., it was found that the addition of 1.0 mM $ZnSO_4$ to LB broth led to increased induction of the *S.* Typhimurium multidrug efflux pumps MtdA and AcrD, which confer resistance to oxacillin, cloxacillin, nafcillin, β-lactams, novobiocin, and deoxycholate.[34] BaeSR is a signal transduction system that regulates the expression of *mdtABC* and *acrD*.[34] This group also found that an *S.* Typhimurium *baeSR* mutant strain was more susceptible to zinc and copper toxicity.[34]

Experiments were done by Peltier *et al.* using sludge reactors simultaneously supplemented with zinc (5.0 mg/L) and antibiotics (0.2 mg/L).[35] The overall conclusion was that zinc exposure led to increased resistance to tylosin tartrate, oxytetracycline, and ciprofloxacin.[35]

Copper-resistant microbes found in copper-amended soils have significantly higher instances of resistance to antibiotics than their copper-sensitive counterparts.[36] There are three ways that heavy metal exposure contributes to antimicrobial resistance; heavy metals directly interacting with the antimicrobial, or with genes or proteins responsible for antibiotic resistance, and they can also interact with microbial structures such as the cell membrane.[36]

For this study, oxytetracycline HCl (OTC), tylosin tartrate, fumagillin-B, dodecyltrimethylammonium chloride (DTAC), and triclosan were chosen to

investigate for potential cross-resistance. Tylosin, OTC, and fumagillin are antibiotics frequently used in agriculture.[35,37,38] Tylosin binds to the 23S rRNA to inhibit protein synthesis.[39] Oxytetracycline (also known as terramycin) interacts with bacteria similarly, but binds the 30S ribosomal subunit.[40,41] Fumagillin is an inhibitor of methionine aminopeptidase, which is the enzyme responsible for removing the N-terminal methionine from immature proteins.[42]

DTAC is a quaternary ammonium compound. It interacts with the bacterial membrane, rendering it more permeable.[43] Triclosan has a broad-spectrum of antimicrobial activity; therefore, it has been used in a wide variety of products over the past 45 years.[44,45] Triclosan acts by inhibiting fatty acid biosynthesis in bacteria by targeted NADH- or NADPH-dependent enoyl-acyl carrier protein reductase.[45] Mechanisms that foster resistance to triclosan may also lead to cross-resistance to antibiotics.[45] These antimicrobials were chosen because they are commonly used and have different modes of action.

1.6 Motility

Flagella are membrane-bound appendages used by bacteria to propel themselves, in liquid and on solid surfaces.[46] *Salmonella* Typhimurium typically has 5-15 peritrichious flagella that can be as long as 15 μm and are modulated by the bacterial cytoskeleton and proton or sodium motive force.[47-50] Flagella contain three architectural components; the filament (propeller), hook, and the basal body (motor and anchor). [49-52] Flagella components form a Type III secretion system (T3SS) for flagellar assembly, that functions similarly to the secretion system for effector

proteins.[52] The flagellar T3SS is used for the export of components that attach to the end of the flagellum, so that its newest part is furthest away from the cell.[51,52]

Motility is considered a virulence factor, as the presence of flagella increases virulence.[53] Aflagellate *Salmonella* were significantly less virulent than wild-type *Salmonella* when chicks were infected.[53] Flagellar genes are expressed until the bacterium reaches its destination, then *Salmonella* pathogenicity island-1 (SPI-1) T3SS genes and fimbrial genes are expressed.[54] In addition to movement, flagella are important for *Salmonella* during the early stages of biofilm formation.[53,55]

1.7 Biofilm Formation

Biofilm formation is ubiquitously present in nearly every environment.[56] Biofilms are bacteria that have securely attached to a surface that are protected by a polysaccharide-containing extracellular matrix.[56,57] It is advantageous for bacteria to exist in a biofilm as opposed to living in a planktonic state, and it is estimated that 99% of bacteria are living in biofilms.[58] When present in biofilms, cells have increased defense against antimicrobial agents and the host immune system, better accessibility to nutrients, enhanced gene transfer opportunities, and increased growth and cellular activity.[56,57,59-61] The existence of biofilms allows for a constant source of infection.[57] As a biofilm matures and increases in size, some cells detach and become planktonic. These cells may reattach to another surface and begin the cycle again.[57]

Biofilm matrices are composed of lipopolysaccharides, cellulose, and fimbriae. Fimbriae are adhesive, hair-like appendages that adhere to biotic and abiotic

surfaces.[62,63] Fimbriae expression is up-regulated under conditions of low nutrient levels, low osmolarity or temperature, and when stationary phase has been reached.[61]

1.8 Hypotheses

1. Zinc will leach from the crumb rubber into rainwater as it percolates through the green roof. The resulting extract will be inhibitory to the microbes present.
2. Bacteria in crumb rubber-containing green roofs can develop zinc resistance and cross-resistance.
3. Pathogens introduced into green roofs can form biofilms on crumb rubber, which can provide a constant source of re-contamination.

Chapter 2

METHODS AND MATERIALS

2.1 Bacterial Strains

2.1.1 Parental Strain

Salmonella enterica subsp. *enterica* serovar Typhimurium, ATCC® 14208S, was obtained from the American Type Culture Collection® (ATCC, Manassas, VA). This strain was used to develop the strains with reduced susceptibility (SRS) to $ZnSO_4$.

2.1.2 Development of Strains with Reduced Susceptibility to Zinc

The *Salmonella* parental strain was used to generate strains of *Salmonella* with reduced susceptibility (SRS) to zinc. The parent was exposed to increasing $ZnSO_4$ added to TSB, beginning with 5 mM and gradually increased. Cells capable of growing in 20 mM $ZnSO_4$ were ultimately obtained. The SRS cells obtained and used in our studies were capable of growing overnight in 20 mM zinc and produced at least 10^8 colony forming unites per mL when enumerated.

2.2 Media

2.2.1 Tryptic Soy Media

Overnight cultures were grown in Difco™ Trypic Soy Broth (TSB) (Becton, Dickinson and Company). Appropriate volumes of double concentrated TSB were used when needed to maintain consistent nutrient content in experiments that were amended with zinc or other chemicals. Tryptic Soy Agar (TSA) was used for enumeration plating. Both TSB and TSA were stored at 4°C.

2.2.2 M9 Minimal Salts Media

Difco™ M9 minimal salts, 5x was stored at 4°C until needed. Two milliliters of filter-sterilized 1.0 M $MgSO_4$, 100 µL of filter-sterilized $CaCl_2$, and 20 mL of 20% filter-sterilized glucose were added to 1x M9 media before use. This medium was used for the growth curves comparing survival in 24-hour extract, synthetic rainwater, only M9 and ddH_2O (section 2.4.2).

2.2.3 Rappaport-Vassiliadis Medium, Semisolid Modification (MSRV)

Difco™ MSRV was made as per the manufacturer's instructions with two modifications. An additional 1.3 g of agar was added to the medium and novobiocin was not added. The purpose of the added agar was to thicken the medium so that the plates could be overturned without disturbing the medium. Since pure cultures were used in this assay, novobiocin was not needed for differentiation between *Salmonella enterica* and *E.* coli. MSRV plates were stored at 4°C until needed. MSRV was used for comparing motility between the parent and SRS strains, as described in section 2.6.

2.3 Batch Crumb Rubber Extract Inhibition Assays

2.3.1 Synthetic Rainwater Preparation

Synthetic rainwater (SRW) with a final pH of approximately 4.3-4.6 was prepared as described in Davis *et al*.[64] SRW was filter-sterilized and stored in 1 L Pyrex glassware until needed.

2.3.2 Production of Crumb Rubber Extract

SRW was incubated with crumb rubber in 50-mL centrifuge tubes for either 1, 24, or 48 hours. A ratio of 2.0 g of crumb rubber per 10 mL of SRW was used. After incubation at 28°C, the mix was filtered through a 0.2-µm filter (Thermo Scientific Nalgene syringe filter cat. #195-2520). The resulting filtrate was placed in a new 50-mL centrifuge tube and used for survival assays and growth curves. The same lot of crumb rubber was used for all crumb rubber-related experiments.

2.3.3 Batch Extract Inhibition Assay with 1, 24, and 48-Hour Extracts

After collection, 1.0-mL aliquots of the 1, 24, and 48-hour crumb rubber extracts were placed in wells of a 24-well microtiter plate in triplicate (Corning, tissue culture-treated cat. #3524). A synthetic rainwater (SRW) control was used as well. Cells from overnight *Salmonella enterica* cultures were washed twice with and re-suspended in EPA dilution water (Hach) and used to make a 5% inoculum in each test and control well. Microtiter plates were incubated for 24 hours at 28°C

The overnight culture was enumerated by serially diluting and plating on Tryptic Soy Agar (TSA) to determine the number of organisms added to each well. All plates were incubated at 37°C. The TSA plates were counted after 24 hours.

To determine the survival of *Salmonella enterica* in each of the extracts, the extracts in each microtiter plate well was serially diluted and plated onto TSA. The plates were also counted after overnight incubation at 37°C

2.3.4 Batch Extract Inhibition in 24-Hour Extract Assay

Based on the results from the batch extract inhibition assays with the 1, 24, and 48-hour extracts, the 24-hour extract was diluted to 1:1, 1:10, 1:50, and 1:100 extract:synthetic rainwater. To determine survival in the diluted extracts, the preparation, incubation, and plating from microtiter plate wells was carried out as described in section 2.3.3.

2.3.5 Serial Washing Crumb Rubber

To determine if crumb rubber quickly releases its inhibitory material, 15-mL tubes containing 2.0 g of crumb rubber were incubated with 10 mL of SRW for 24 hours at 28°C, the SRW was collected and the procedure was repeated nine more times, in triplicate. Inhibitory effects were determined by enumeration of surviving bacteria as described in section 2.3.3.

2.4 Growth Curves

2.4.1 Growth Curves of Parent in TSB with Supplemented Zinc

Salmonella enterica was grown overnight in TSB at 37°C. Aliquots were inoculated in triplicate into six-well microtiter plates, containing 0, 5, 10, 20, 50, and 100 mM zinc dissolved in TSB (Becton, Dickinson, and Company, cat. #353846). Two-hundred-microliter samples were removed every 30 minutes from each well and transferred to a 96-well flat bottom non-tissue culture treated polystyrene microtiter

plate (Falcon cat. #351172). The optical density (O.D.) was recorded at 595 nm, using an MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, VA).

2.4.2 Growth Curves of Parent in M9 Minimal Salts Media with Crumb Rubber Extract

Salmonella enterica was grown overnight in TSB at 37°C. Aliquots were inoculated into six-well microtiter plates in triplicate, containing minimal salts media with supplemented glucose. Organisms in each well were allowed to grow for 5.5 hours at 37°C, with shaking until the $O.D._{595}$ reached was at least 0.1. Two-hundred-microliter samples were removed from each well and transferred to a 96-well plate to monitor absorbance. After 5.5 hours of growth, the well volumes were doubled by the addition of either 24-hour extract, SRW, M9, or ddH_2O. Growth was measured for an additional 3.5 hours to compare growth in media with the different amendments.

2.4.3 Growth Curves of Parent and SRS Strains in TSB Containing Variable Zinc Concentrations

Parental *Salmonella enterica* and strains with reduced susceptibility (SRS) were grown overnight in TSB at 37°C. Aliquots were inoculated in triplicate into six-well microtiter plates with TSB containing either 0, 5, 10, or 15 mM additional $ZnSO_4$. Two-hundred-microliter samples were removed every 30 minutes from each well and transferred to a 96-well plate and the O.D. was recorded at 595 nm.

2.5 Biofilm Assays

2.5.1 Attachment to Crumb Rubber

A half gram of crumb rubber was incubated in 1.0 mL of SRW containing a 5% *Salmonella enterica* inoculum for 24 hours, in a 2-mL centrifuge tube. After 24 hours of incubation at 28°C, the SRW was removed and the crumb rubber was rinsed three times with EPA dilution water to wash off planktonic cells.

The crumb rubber was incubated with the green nucleic acid dye SYTO 13 (1:250 dilution) for approximately 10 minutes before visualization using a Zeiss LSM 710 confocal microscope. Images were color-enhanced so that the nucleic acid fluoresced as a brilliant green and the crumb rubber appeared dark blue.

2.5.2 Twenty-Four-Hour Crystal Violet Assay

Overnight cultures of *Salmonella enterica* was grown at 37°C in TSB and inoculated into 96-well polystyrene non-tissue culture treated plates. The wells were prepared to have concentrations of 0, 1, 10 or 20 mM $ZnSO_4$, in TSB.

There were six replicate wells per sample. Incubation was at 37°C without shaking for 24 hours. At this time, three of the six replicate wells were pipetted vigorously to suspend the culture. The absorbance measured from these wells constituted the total cell density of the culture, including planktonic and biofilm cells. Liquid media were removed from the three remaining wells that were not suspended and were then double rinsed with ddH_2O and air dried. Two-hundred-microliters of 0.1% crystal violet in ddH_2O were added to each well.

The plates were incubated at room temperature for 30 minutes with shaking. The crystal violet was decanted and the wells were double rinsed with ddH_2O and air dried. Two-hundred-microliters of 95% ethanol were added to each well and the absorbance of the wells was measured at 620 nm after 10 minutes (Fisher Scientific Multiskan FC Microplate Reader). This is a modified assay from Boehm *et al*.[65]

To control for absorbance from TSB and zinc added to each well, TSB controls were treated in the same way as inoculated wells. The absorbance of the TSB controls was subtracted from the total cell density of wells. The crystal violet normalization was calculated by subtracting the O.D. of uninoculated wells that were treated in the same way as inoculated wells. This was to account for ambiguous crystal violet staining. The $O.D._{620}$ of the crystal violet solubilized from the biofilm was divided by the $O.D._{620}$ of the total cell density to quantify relative biofilm formation between the parent and SRS strains in different added zinc concentrations.

2.6 Motility of Parent and SRS Strains

Rappaport-Vassiliadis Medium, Semisolid Modified (MSRV) was used to compare parental and SRS motility. Six serial dilutions of overnight cultures of the parental and SRS strains were prepared in EPA dilution water in a 24-well microtiter plate. Ten-microliters of the overnight stock culture and the 10^{-1}, 10^{-2}, 10^{-3} serial dilutions of the culture were plated in triplicate onto the center of an MSRV plate. Plates were incubated at 37°C without shaking for 48 hours. After incubation, the diameter of the zone of growth was measured. Colony forming units (CFUs) for each dilution were determined by plating 10 μL of each of the six serial ten-fold dilutions of

the stock culture onto TSA plates. The number of cells in 1 µL of the inoculums was plotted against the zones of growth. A line of best fit was drawn through the data points. The size of the zone of growth using $1x10^5$, $1x10^6$, $1x10^7$, and $1x10^8$ cells as inoculums was determined.

2.7 Regulation of Zinc Pumps

2.7.1 Primer Design

Primers were designed using the SciTools application on the Integrated DNA Technology website (http://www.idtdna.com/scitools/scitools.aspx). The parameters used for the primer design were: 1) Primer dimers and hairpin structures had a ΔG value greater than -9 kcal/mole. 2) The Tm for all primers was between 59°C and 61°C. 3) No primer had a sequence similarity greater than 90% to non-target DNA regions as reported by Basic Local Alignment Search Tool (BLAST).[66] The genotype of *Salmonella enterica* subsp. *enterica* serovar Typhimurium *str.* 14028, complete genome was used in the BLAST (http://blast.ncbi.nlm.nih.gov/) search. Primers were stored as 100 µM stock solutions at -20°C.

2.7.2 Primer Testing

DNA was prepared using the BioRad Instagene™ Matrix and quantified using a NanoDrop RND-1000 Spectrophotometer and NanoDrop 3.1.2 Software (Thermo Fisher Scientific Inc. and (BioRad Hercules, CA)). A HotStarTaq® DNA polymerase kit was used in the polymerase chain reaction (PCR). Primers were tested by performing PCR using a PTC-100TM Programmable Thermal Controller (MJ

Research, Inc. Waltham, MA). PCR products were run on a 2% ethidium bromide agarose gel at 70 volts for approximately one hour. The band size of each product was determined using a 25 bp or 100 bp DNA ladder (Promega Madison, WI).

2.7.3 RNA Extraction with RNeasy Mini Kit

Salmonella enterica was grown overnight at 37°C and re-cultured the next day for at least three hours, to mid long phase. In a 2-mL centrifuge tube, 1.0 mL of RNAprotect Bacteria Reagent™ was added to 0.5 mL of the three-hour culture. This mixture was immediately vortexed for five seconds and centrifuged at 10,000 rpm for five minutes. The supernatant was removed and 50 µL of TE buffer with lysozyme was added to the pellet. Each reaction was incubated at room temperature for 20 minutes, while being vortexed for 10 seconds every two minutes. Buffer RLT, supplemented with 10 µL of β-mercaptoethanol per 1.0 mL of buffer (350 µL per reaction), was added and vortexed vigorously and centrifuged at 10,000 rpm for five minutes to pellet particulate matter. The supernatant was removed and placed in a 0.5-mL tube, to which 250 µL of 95% ethanol was added and mixed by pipetting. The remainder of the protocol was carried out as per the manufacturer's directions.

2.7.4 RNA Quantification

Isolated RNA was combined and measured in triplicate with a NanoDropR ND-1000 Spectrophotometer and NanoDropR 3.1.2 Software (Thermo Fisher Scientific Inc. Wilmington, DE).

2.7.5 Reverse Transcription

Reverse transcription was performed with the Omniscript RT Kit (Qiagen, Valencia, CA) on a PTC-100TM Programmable Thermal Controller (MJ Research, Inc., Waltham, MA). A random nonomer primer was used. A 1:100 dilution of the reverse transcription reaction was used in the real time PCR assay.

2.7.6 Quantitative PCR

Three replicates of each sample were run using the QuantiTect SYBR Green PCR Kit (Qiagen Valencia, CA). The cycling temperatures were 95°C for 15 minutes, then 40 rounds of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds. A dissociation curve was performed at the end of each run. SYBR® Green (Qiagen, Valencia, CA) was used to detect the levels of cDNA. The ABI Prism 7300 Sequence Detection System was used to perform quantitative real time PCR. ABI Prism 7000 SDS Software was used to analyze the results (Applied Biosystems, Carlsbad, CA). In each well, five µL of cDNA (diluted 1:100 from the RT reaction) was added and the final concentration of primer was 0.5 ng/µL. The gene *gyrB* was used as an internal control.

2.8 Cross-Resistance to Commonly Used Antimicrobial Agents

Salmonella enterica was grown overnight in 0 mM $ZnSO_4$. To test basal tolerance to each antimicrobial, *Salmonella enterica* cultures were placed in each well of a 24-well microtiter plate and exposed to a range of increasing concentrations of the antimicrobials. After a range of antimicrobial was determined, parent and SRS strains were grown overnight in TSB with 0 mM additional zinc and then re-cultured in the

presence of 0, 1, 10, and 20 mM additional zinc, as well as antimicrobial. After growth overnight in microtiter plates, the O.D. of both strains in all conditions was measured at 620 nm. Each well was corrected for absorbance due to additional zinc and antimicrobial, by subtracting wells treated in the same way without culture inoculation. The resulting O.D.s were reported in bar graphs.

2.8.1 Cross-Resistance to Oxytetracycline

Oxytetracycline (OTC) was prepared by dissolving 0.1 g of OTC powder in 50 mL of ddH_2O, to make a 2,000-parts per million (ppm) stock. The stock was diluted to 500-ppm when needed. The parent and SRS strains were grown overnight in the absence of additional zinc. The following day, zinc was added at concentrations of either 0, 1, 10, or 20 mM zinc, with 0, 0.5, 1, 2, 3, and 4 ppm OTC.

2.8.2 Cross-Resistance to Tylosin Tartrate

Tylosin tartrate was prepared by dissolving 0.2 g of tylosin powder in 50 mL of ddH_2O, to make a 4,000-ppm stock. The parent and SRS strains were grown overnight in the absence of additional zinc. The following day, zinc was added at concentrations of either 0, 1, 10, or 20 mM zinc, with 0, 381, 571, 762, 952, and 1,143 ppm tylosin

2.8.3 Cross-Resistance to Fumagillin-B

Fumagillin-B was prepared by dissolving 0.1 g of fumagillin powder in 50 mL of 70% ethanol, to make 2,000-ppm stock. The parent and SRS strains were grown overnight in the absence of additional zinc. The following day, zinc was added at

concentrations of either 0, 1, 10, or 20 mM zinc, with 0, 10, 50, 100, 150, and 200 ppm fumagillin.

2.8.4 Cross-Resistance to Dodecyltrimethylammonium Chloride (DTAC)

DTAC was prepared as a 50 mg/mL of ddH_2O stock solution in ddH_2O and diluted to 5 mg/mL (5,000-ppm) for the working stock solution. The parent and SRS strains were grown overnight in the absence of additional zinc. The following day, zinc was at concentrations of either 0, 1, 10, or 20 mM zinc, with 0, 119, 238, 357, 476, and 595 ppm DTAC.

2.8.5 Cross-Resistance to Triclosan

Triclosan was prepared as a 50 mg/mL solution in 70% ethanol and diluted to 0.25 mg/mL (equivalent to 0.25-ppm) for the working stock solution. The parent and SRS strains were grown overnight in the absence of additional zinc. The following day, zinc was added at concentrations of either 0, 1, 10, or 20 mM zinc, with 0, 0.25, 0.5, 0.75, 1.0, and 1.25 ppm triclosan.

Chapter 3

RESULTS

3.1 Crumb Rubber Extract Batch Inhibition Assays

3.1.1 Batch Extract Inhibition Assay

The extracts obtained from contact between synthetic rainwater (SRW) and crumb rubber were toxic to *Salmonella enterica* when compared to just SRW. After as little as one hour of contact between crumb rubber and synthetic rainwater (termed one-hour extract) there was less survival of *Salmonella enterica* (P=0.0415) when compared to the SRW control, shown in **Figure 3**. The data for the 24-hour extract was not statistically different from the 48-hour extract, but was more inhibitory than the one-hour extract (P=0.0361) and SRW (P=0.0157). Inhibition was determined by exposing *Salmonella enterica* to each of the extracts and SRW for a period of 24 and 48 hours and enumeration of surviving cells.

A similar pattern was seen after 48 hours of *Salmonella enterica* incubation in each extract as before; however, there was no difference between the one-hour extract and 24-hour extract (P=0.0514) for this extended time point (**Figure 4**). SRW compared to all three extracts had significantly more CFUs when enumerated

(P<0.0001). The 48-hour extract continued to be more inhibitory than the one-hour extract (P=0.0286).

3.1.2 Dilutions of Crumb Rubber Extract

Survival of *Salmonella enterica* in 24-hour extract diluted to 1:1, 1:10, 1:50, and 1:100 was significantly greater than in SRW and the undiluted 24-hour extract. (The ratios represent extract:synthetic rainwater). *Salmonella enterica* survived more in 1:50 and 1:10 diluted extract (P=0.007 and P=0.0085, respectively) than in SRW. The 1:100 diluted extract resulted in increased survival, but acted similarly as SRW (P=0.0596). Significantly higher survival was observed in all dilutions as compared with the half and fully concentrated extract (**Figure 5**). This finding is in agreement with earlier findings that the full concentrated extract was significantly more inhibitory than the SRW.

3.1.3 Serial Washing Crumb Rubber

Serial washes conducted for 24 hour periods showed that the inhibitory material derived from crumb rubber was not leached rapidly. The same 2 g of crumb rubber was serially exposed to fresh 10-mL aliquots of synthetic rainwater (SRW) 10 times. The first wash permitted some bacterial survival, but less than the synthetic rainwater control (P=0.0455). The remaining washes allowed no detectable microbial survival (P=0.0453). The leaching of inhibitory materials is likely to be a long process, as demonstrated in **Figure 6**.

3.2 Growth Curves

3.2.1 Growth Curves of Parent in TSB Supplemented with Zinc

Salmonella enterica can grow in TSB to which up to 10 mM zinc has been added. *Salmonella enterica* grown in 5.0 mM additional zinc grew similarly to the control, but did not reach as high an O.D. *Salmonella enterica* in TSB with 20, 50, and 100 mM added zinc did not grow well. Some growth apparently occurred in the presence of 20 mM $ZnSO_4$ by the end of the experiment, whereas the cells in 50 and 100 mM $ZnSO_4$ may have lysed, as evidenced by the decrease in O.D. (**Figure 7**). Based on these results, it was decided that the SRS strain gaining tolerance to 20 mM of zinc would serve as a sufficient comparison to the parent strain for future studies, including biofilm formation assays, tests of motility on semisolid agar, qPCR of genes relevant to zinc regulation, and analyses of cross-resistance to antimicrobial agents.

3.2.2 Growth Curves with Parent in M9 Minimal Salts Media with 24-Hour Extract

Salmonella enterica was grown in M9 minimal salts media for 5.5 hours until the absorbance at 595 nm was more than 0.1. All wells of the six-well microtiter plate contained the same volume of M9 media (**Figure 8B**). At this time, the volume in each well was doubled with the addition of either 24-hour extract, SRW, additional M9, or ddH_2O (**Figure 8C**). The growth curves established following the amendments showed that the salts in the SRW facilitated growth more than addition of M9 and ddH_2O after 3.5 hours. Addition of the 24-hour extract fostered the most growth, shown in **Figure 8A.**

3.2.3 Growth Curves of Parent and SRS Strains in TSB with Variable Zinc Concentrations

The growth curves of the parent and the SRS strain in the presence of additional 5, 10, or 15 mM zinc dissolved in TSB were established (**Figure 9A**). Interestingly, the parent grew slower than the SRS strain in media without the addition of zinc; however, both strains ultimately grew to an absorbance of over 0.5 (**Figure 9B**). Growth curves in 5 mM additional zinc showed the converse of the 0 mM curves. The parent grew faster than the SRS strain in the beginning, but both strains reached an ending absorbance of over 0.4 (**Figure 9C**).

The SRS strain grew faster than the parent in media to which 10 mM zinc was added. By the end of the study, the O.D.at 595 nm of the SRS strain was over 0.3, while the parent O.D. was below 0.2 (**Figure 9D**). The SRS was able to reach an absorbance of almost 0.2, in the presence of 15 mM added zinc, while the parent did not exceed an absorbance of 0.05 (**Figure 9E**). The results of the growth curves are shown in a single graph (**Figure 9A**), as well as individual graphs comparing the strains in each zinc concentration (**Figure 9B-E**).

3.3 Biofilm Assays

3.3.1 Attachment to Crumb Rubber

Salmonella enterica (5% inoculation) was incubated for 24 hours in synthetic rainwater and crumb rubber. Microscopy indicated that in the uninoculated control few bacteria were associated with crumb rubber and they appeared to be dead (**Figure 10A**). Crumb rubber inoculated with *Salmonella enterica* showed substantial

attachment even after washing three times with SRW to remove unattached cells (**Figure 10B)**. The figure is color-enhanced and displays the surface of crumb rubber as blue, while nucleic acid is green, due to SYTO 13 dye.

3.3.2 Twenty-Four-Hour Crystal Violet Assay

The addition of 1 mM zinc to *Salmonella enterica* grown in TSB did not significantly affect biofilm formation (P=0.5265), though there was a slight increase, shown in **Figure 11**. The parent formed significantly less biofilm in the presence of 10 mM and 20 mM additional zinc, when compared to the parent grown in 0 mM (P=0.0065 and P=0.0055, respectively). Zinc levels did not significantly affect biofilm formation of the SRS strain.

The parent formed significantly more biofilm than the SRS strain in TSB with 0 mM and 1 mM additional zinc (P<0.001). Biofilm formation was not different between the parent and SRS at 10 and 20 mM additional zinc. Based on these results, the SRS strain appeared to be less capable of forming biofilm than the parent, independent of added zinc.

3.4 Motility of Parent and SRS Strains

Parent, SRS, and *Staphylococcus aureus* were grown overnight in TSB with 0, 1, or 10 mM of added zinc. Ten-microliters of stock culture, 10^{-1}, 10^{-2}, and 10^{-3} dilutions were used to inoculate the center of MSRV plates. After incubation at 37°C for 48 hours, without shaking, the zone of growth was measured for each plate. The SRS strain spread through the entire plate very quickly, compared to the parent. This trend was seen in all three zinc concentrations, with 10 mM additional zinc allowing

the SRS strain to be the most motile. The parent had increased motility in 1 mM additional zinc, while the parent showed similar motility on plates with 0 and 10 mM additional zinc. The known non-motile control *Staphylococcus aureus* in **Figure 12** did not survive or spread on the plates, as expected.

3.5 Quantitative Polymerase Chain Reaction

The parent and the SRS strains were compared for expression of zinc influx, efflux pumps, and a transcription factor, using qPCR. Expression of *zntA*, *zntB*, *zitB*, *znuA*, and *zntR* were compared and the housekeeping gene *gyrB* was used as a control. ZntA, ZntB, and ZitB are zinc efflux pump proteins, while ZnuA allows zinc influx, and ZntR is a metallosensory transcription factor that regulates *zntA* transcription. **Table 2** summarizes the function of these proteins in more detail.

The expression of these genes was compared between the parent and SRS strains in the absence of additional zinc and in 20 mM additional zinc. Gene expression was also analyzed between zinc concentrations for the same strain (0 mM and 20 mM results were compared to each other in the SRS strain and in the parent).

When grown without the addition of zinc, expression of *zntA*, *zntB*, *zitB*, *znuA*, and *zntR* were not statistically different between the parent and SRS strain (**Table 3)**. The addition of 20 mM zinc caused up-regulation of *zntA* by seven-fold in the SRS strain and *znuA* was significantly down-regulated by 2.5-fold. *zntB*, *zitB* and *zntR* expression were not affected significantly compared to growth without added zinc (**Table 4)**.

When the parent was grown in the presence of 20 mM additional zinc *zntA*, *zntB*, and *zitB* were up-regulated 7.8, 4.7, and 8.4-fold, respectively when compared to growth in TSB without additional zinc. *znuA* was down-regulated 1.8-fold. Though *zntR* expression was not affected significantly (**Table 5**).

The SRS strain grown in TSB with 20 mM additional zinc significantly up-regulated *zntB* and *zitB* whereas *zntA* and *zntR* expression was not significantly affected compared to that in TSB with 0 mM added zinc. As expected, *znuA* was down-regulated in cells grown in TSB with 20 mM additional zinc (**Table 6**).

3.6 Cross-resistance to Commonly Used Antimicrobials

The zero zinc/zero antibiotic grown cultures served as controls for these experiments. In all of these, growth was highest for the parental and SRS strains grown in the absence of zinc. All strains showed sensitivity to all of the antibiotics. Differences in inhibition of the parental and SRS strains were observed in some of the antibiotic treatments. In some cases, at the higher concentrations of antibiotic the parental and SRS strains grown in 20 mM added zinc showed no growth, possibly indicating a synergism between zinc and the antibiotic. No cross-resistance was observed.

3.6.1 Cross-Resistance to Oxytetracycline

The parent and SRS strains were both grown in 0 mM additional zinc overnight and were then transferred to a 24-well microtiter plate containing either 0, 1, 10, or 20 mM of zinc in TSB. Each zinc concentration was amended with range of oxytetracycline (OTC) and the O.D.s were recorded. At 2 to 4 ppm oxytetracycline

parental and SRS strains grown in the presence or absence of zinc were inhibited to the same extent. The results are shown in **Figure 13**. Additional information on each antimicrobial is summarized in **Table 7**.

3.6.2 Cross-Resistance to Tylosin Tartrate

The parent and SRS strains were both grown in 0 mM additional zinc overnight and were then transferred to a 24-well microtiter plate containing either 0, 1, 10, or 20 mM of zinc in TSB. Each zinc concentration was amended with a range of tylosin and after overnight growth the O.D.s were recorded. SRS in 10 mM additional zinc was less inhibited by tylosin at all concentrations tested when compared with the parent in 10 mM additional zinc. Parent and SRS strains in 20 mM added zinc were similarly and almost completely inhibited at the lowest concentration of antibiotic tested. The results are shown in **Figure 14**.

3.6.3 Cross-Resistance to Fumagillin-B

The parent and SRS strains were both grown in 0 mM additional zinc overnight and were then transferred to a 24-well microtiter plate containing either 0, 1, 10, or 20 mM of zinc in TSB. Each zinc concentration was amended with a range of fumagillin and the O.D.s were recorded. Both parental and SRS strains in 10 and 20 mM zinc were totally inhibited at 150 and 200 ppM of the antibiotic. The parental strain in 0 mM added zinc appeared to be slightly less inhibited than the SRS strain in 0 mM added zinc strain at the higher concentrations of the antibiotic. The results are shown in **Figure 15**.

3.6.4 Cross-Resistance to Dodecyltrimethylammonium Chloride (DTAC)

The parent and SRS strains were both grown in 0 mM additional zinc overnight and were then transferred to a 24-well microtiter plate containing either 0, 1, 10, or 20 mM of zinc in TSB. Each zinc concentration was amended with a range of DTAC and the O.D.s were recorded. Both the parent and SRS stains in 20 mM added zinc were totally inhibited at 119 ppm, the lowest concentration of antimicrobial tested. The parental strain in 0 mM added zinc appeared to be slightly less inhibited than the SRS stain in 0 mM added zinc at 595 ppm, the highest concentration of DTAC tested. The results are shown in **Figure 16**.

3.6.5 Cross-Resistance to Triclosan

The parent and SRS strains were both grown in 0 mM additional zinc overnight and were then transferred to a 24-well microtiter plate containing either 0, 1, 10, or 20 mM of zinc in TSB. Each zinc concentration was amended with a range of DTAC and the O.D.s were recorded. Both the parental and SRS stains in 10 and 20 mM added zinc were totally inhibited at 0.5 ppm and higher concentrations. Parental and SRS strains grown in the absence of zinc and 1 mM added zinc were similarly inhibited at the triclosan concentrations tested. The results are shown in **Figure 17**.

Chapter 4

DISCUSSION

4.1 Crumb Rubber Extract Inhibition Batch Assays

4.1.1 Batch Extract Inhibition Assay

Two grams of crumb rubber in contact with 10 mL of synthetic rainwater for 24 hours yielded an extract that was inhibitory to survival and growth of *Salmonella* Typhimurium ATCC 14208S. It is known that crumb rubber contains a variety of heavy metals, which leach out of the rubber more readily when exposed to a lower pH.[5,67] The SRW pH of ~4.4 is similar to that of Delaware rainwater, indicating the likelihood that zinc and other heavy metals would leach from green roofs using this material.[68]

Our collaborators at Pennsylvania State University, Harrisburg compared zinc leaching from a green roof with a growth matrix composed of recycled medium containing 15% crumb rubber to a commercial medium (expanded shale). They found that the recycled medium did not leach significantly more zinc than the commercial medium. The average amount of zinc that they found in the crumb rubber-amended roof was over 0.2 mg/L and up to over 0.8 mg/L. The commercial medium was found to have an average of about 0.15 mg/L and up to 0.4 mg/L (K. H. Baker, personal communication).

4.1.2 Dilutions of Crumb Rubber Extract

Mixing 2 g of crumb rubber with 10 mL of SRW for 24 hours resulted in the leaching of materials that were inhibitory to bacteria. It was expected that the crumb rubber to rainwater concentration that would be present in a green roof is lower than the concentrations used in these studies. To address this, the effect of diluted extract was assessed and it was observed that the crumb rubber leached material that fostered survival, when diluted at least 10-fold.

4.1.3 Serial Washing Crumb Rubber

In order to decrease or prevent heavy metal leaching from crumb rubber for future applications, prewashing with a low pH may be necessary. Using the synthetic rainwater with a pH of about 4.4, washing crumb rubber repeatedly showed that inhibitory materials leach for at least ten washes in 24 hour increments. Heavy metal leaching has a wide range of environmental effects; therefore, this has implications for human health.

Since crumb rubber is incorporated into asphalt and disposed of in landfills, there are opportunities for metal leaching. In both situations, the crumb rubber would be exposed to rainwater, which has a local pH of ~4.4.[68] Though washing the crumb rubber would help reduce the issue of metals leaching into the environment, another issue arises of how to dispose of the washings.

4.2 Growth Curves

4.2.1 Growth Curves in TSB Supplemented with $ZnSO_4$

Growth curves were established to determine the basal tolerance of the parent to zinc, when grown in a rich medium. *Salmonella enterica* was able to grow in 5 and 10 mM added zinc, but seemed to lyse in 20, 50, and 100 mM added zinc. Based on these growth curves, 20 mM was chosen to be a suitable level of zinc to train the SRS strain up to. Because this strain was unable to survive in 20 mM added zinc, it was the desired concentration minimum concentration to train up the organism to. The SRS organisms tolerant of such a high concentration of zinc were expected to be genetically different than the parent.

4.2.2 Growth Curves of Parent in M9 Minimal Salts Media with Extract

Crumb rubber extract indeed contains usable nutrients, as the cells in the presence of the extract grew more than those only exposed to M9, ddH_2O and SRW. Unexpectedly, the addition of SRW did contribute some salts that enhanced *Salmonella enterica* growth compared to addition of M9. This can be attributed to the fact that SRW contains inorganic salts from the acids used to acidify the solution.

4.2.3 Growth Curves of Parent and SRS strains in TSB containing Variable Zinc Concentrations

It was expected that the SRS strain would grow better than the parent in TSB containing an additional 5, 10, and 15 mM $ZnSO_4$; however, the SRS strain initially grew faster than the parent in 0 mM additional zinc and the parent initially grew better in the presence of 5 mM additional zinc. Both strains grew to a similar ending

absorbance by the end of the growth curves. As expected, the SRS strain grew to an absorbance to twice that of the parental strain and the parent was not able to grow in 15 mM additional zinc.

The growth of the SRS strain was impaired by the presence of increasing dissolved zinc concentrations, but has been shown to overcome toxicity by 24 hours. This was demonstrated by enumeration of SRS organisms after contact with high levels of dissolved zinc, which grew to concentrations of at least 10^8 CFUs. These results indicate that the SRS strain is indeed resistant to higher levels of additional zinc than the parent strain.

4.3 Biofilm Assays

4.3.1 Attachment to Crumb Rubber

It was not surprising that *Salmonella enterica* was able to attach to crumb rubber, since biofilm formation occurs on a range of surfaces; including, but not limited to plastic, cement, glass, stainless steel, rubber, and plant and animal cells.[55] Since addition of zinc has been shown to increase bacterial attachment to eukaryotic cells *in vitro*, crumb rubber may actually promote bacterial biofilm formation as bacteria in the proximal area would be exposed to an elevated level of zinc and other heavy metals.[69]

4.3.2 Twenty-Four-Hour Crystal Violet Assay

Crystal violet was used to quantify attachment of the parent and SRS strains to an abiotic surface. The results suggest that the SRS strain is much less capable of forming biofilm, independent of zinc levels. Fimbriae are the organelles used for

initial attachment. In future studies, it would be interesting to characterize the differences in expression of fimbrial genes between the SRS and parental strain.

To further determine differences in fimbriae between the strains, transmission electron microscopy would allow observation of individual cells to determine if fimbriae are present. Furthermore, decreased fimbriae and subsequent biofilm formation has been associated with decreased invasion of eukaryotic cells; therefore, down-regulation of fimbriae could yield less virulent organisms.[43,70]

4.4 Motility of Parent and SRS Strains

The SRS strain was more motile than the parent, which may be due to up-regulation of flagella. Motility is a classic virulence factor in bacteria; however, studies have shown that overexpression of flagella or increased motility actually attenuates virulence in bacteria.[43,71-73] To confirm that this is the case in the SRS strain future work in determining flagella expression and staining would be necessary. Another interesting study would be to compare invasion of HeLa or Caco-2 cells between the SRS strain and the parent, as done in other studies.[43,74]

4.5 Quantitative Polymerase Chain Reaction

Since *zntA*, *zntB,* and *zitB* are responsible for zinc efflux pump expression, it was expected that these genes would be up-regulated in high zinc levels (20 mM). Particularly, we expected that there would be an up-regulation in the SRS strain when compared to the parent. We had the same expectation of *zntR*, as it is a metallosensory protein. The only one anticipated to be down-regulated was *znuA*, since it is a zinc influx pump.

It was not surprising that the parent and SRS had similar expression of all the zinc-related genes studied in 0 mM added zinc. If efflux pump expression was high in the SRS strain even without excess zinc, this could impair the organism's growth since zinc is required for many intracellular functions. In the presence of 20 mM zinc *zntA* expression was increased seven-fold, which is in agreement with previous findings that *zntA* was important for zinc detoxification.[10,21] The constitutively expressed zinc influx gene *znuA* was down-regulated by 2.5-fold in the SRS strain in the presence of 20 mM zinc. This differential expression could play a large role in how the SRS strain handles higher zinc levels. When grown in the presence of 20 mM zinc, *zitB* and *zntR* expression were only up-regulated in the SRS strain by 1.8- and 1.7-fold, respectively.

As expected, parent *zntA*, *zntB*, and *zitB* expression were all significantly up-regulated in the presence of 20 mM zinc compared to no zinc and *znuA* expression was down-regulated. *zntR* expression was not affected significantly. Similar results were observed in the comparison of SRS strain gene expression in 0 mM and 20 mM additional zinc.

The results from these experiments help explain the differences between mechanisms for dealing with zinc toxicity between the parent and SRS strain. ZntA seems to be the most important efflux pump, in agreement with the literature. The gene expression for this pump is up-regulated by nearly eight-fold. The gene expression of the most important zinc influx pump, ZnuA, was significantly down-regulated in the SRS strain by 2.5-fold. The combination of these two differences may be the most important to allow the SRS strain to withstand higher zinc levels than the

parent, provided that mRNA levels are reflected in the protein levels and activity of the pumps.

4.6 Cross-resistance to Commonly Used Antimicrobials

4.6.1 Cross-Resistance to Oxytetracycline

It was expected that there would be cross-resistance to OTC in the parent and/or SRS strains when the medium was supplemented with 1 mM zinc. A previous study showed that bacterial communities in sludge reactors that were amended with 5.0 mg/L of zinc were more resistant to subsequent 0.2 mg/L OTC exposure, compared to reactors that were not initially supplemented with zinc.[35] The sludge reactor study and other papers that have reported similar findings mostly involved bacterial communities, where genetic transfer can freely occur. Another reason that there was increased resistance in the reactors could be that the zinc previously selected for hardier organisms.[35] However, a pure culture of *Pseudomonas aeruginosa* was able to gain resistance to a β-lactam antibiotic, imipenem, when it was simultaneously treated with copper.[75]

A study published in 2007 used *Salmonella* Typhimurium to determine the relationship between heavy metal resistance and multidrug efflux pump activity.[34] They found Luria-Bertani broth supplemented with 1 mM zinc or 2 mM copper induced activity of multidrug efflux pumps. Because of these findings, 1 mM zinc was included in our cross-resistance studies as well.

Our studies used pure cultures; therefore, genetic transfer could not occur as in the sludge reactor studies. We did not observe cross-resistance to OTC when the

cultures were simultaneously supplemented with zinc. It may be that zinc does not interact with the efflux pumps or genes or operons responsible for OTC expulsion until very high levels of zinc are present.

4.6.2 Cross-Resistance to Tylosin Tartrate

The same sludge reactor study by Peltier *et al.* examined development of resistance to tylosin tartrate as was done with oxytetracycline.[35] The basal tolerance to tylosin in TSB was tested in preliminary studies. *Salmonella enterica* tolerance to tylosin was rather high compared to the other antimicrobials tested. In the Peltier study, organisms had the highest basal tolerance and highest increase in resistance to tylosin compared to the other two antibiotics that they studied; oxytetracycline and ciprofloxacin.[35]

Our parent strain was innately able to tolerate tylosin on the parts per thousand (ppt) scale, while the other antimicrobial tolerances were on the parts per million (ppm) scale. However, addition of zinc did not appear to play a role in promoting tolerance to tylosin in either the parent or the SRS strain.

Possible explanations for this may also be that these studies were conducted on pure cultures, where no genetic exchange can occur as in community studies. Also, the environment may have lower levels of available zinc ions, which may be more likely to lead to cross-resistance to other antimicrobial agents.

4.6.3 Cross-Resistance to Fumagillin-B

Fumagillin is a commonly used antibiotic in environmental applications, particularly in the control of disease in honey bees.[76] It is also effective against drug-

resistant plasmodia *in vitro* and in a mouse model.[77] The mode of action of fumagillin is inactivation of methionine aminopeptidase.[42,76,77] Given this information, fumagillin was chosen for the antibiotic cross-resistance study.

The simultaneous addition of zinc and antibiotic was not observed to foster cross-resistance to fumagillin, but caused complete inhibition of bacterial growth when 10 and 20 mM of zinc was added with at least 150 ppm of fumagillin added. The SRS strain had overall decreased survival when exposed to higher levels of fumagillin compared to the parental strain.

4.6.4 Cross-Resistance to Dodecyltrimethylammonium Chloride (DTAC)

DTAC is a quaternary ammonium compound that causes the membrane to become more permeable. Zinc is responsible for stabilizing membranes; therefore, it was hypothesized that the simultaneous addition of zinc and DTAC would lead to cross-resistance. We did not observe cross-resistance between zinc and DTAC. The parent and SRS strains grown in 20 mM added zinc were totally inhibited at the lowest concentration of DTAC tested (119 ppm). It would be interesting to add a lower concentration of zinc, such as 5.0 mg/L as used in the Peltier study.[35]

4.6.5 Cross-Resistance to Triclosan

Triclosan is a compound frequently incorporated into a variety of products, including soap, toothpaste, which acts by blocking lipid synthesis. Cross-resistance was not observed between zinc and triclosan. Both the parent and SRS strains were completely inhibited by as little as 0.5 ppm, when grown in 10 and 20 mM added zinc; however, both strains were similarly inhibited at the concentrations tested when grown

in the presence of 0 or 1 mM added zinc. Using a lower concentration of zinc than 1 mM would be interesting to test for potential cross-resistance to triclosan.

4.7 Conclusions

- Crumb rubber contains materials that can leach into rainwater and are inhibitory to *Salmonella enterica* serovar Typhimurium. These inhibitory effects are overcome when the extract is diluted.
- The inhibitory material in crumb rubber leaches out over an extended period of time.
- *Salmonella* are able to attach to crumb rubber in synthetic rainwater.
- *Salmonella* organisms tolerant of increasing concentrations of zinc can be isolated.
- A strain with reduced susceptibility (SRS) to zinc was more motile than the parental strain it was derived from and was significantly less capable of forming a biofilm.
- The qPCR results help explain the differences between the parent and SRS strain for overcoming zinc toxicity. ZntA and ZnuA are the key players in zinc tolerance and homeostasis, based on the gene expression between the parent and the SRS strain. The *zntA* zinc efflux gene was up-regulated in the SRS strain, while the zinc influx gene *znuA* was down-regulated.
- Cross-resistance was not observed between zinc and the antimicrobials oxytetracycline, tylosin, fumagillin, DTAC, and triclosan.

Table 1: Primer sequences used for qPCR.

Gene	Primer sequences
zntA forward	5'-CGC CAT GAA CAC GCT GTT AAC CAA-3'
zntA reverse	5'-TCG CTT CGG AGT TCA CGT AGT TGT-3'
zntB forward	5'-ATG AAA TTG CCC AGG TGA TGC AGG-3'
zntB reverse	5'-TAC CGC CCA GGT TAA CGC CAA ATA-3'
zitB forward	5'-GCG AAC CTG TTT GCG TTC TGG ATT-3'
zitB reverse	5'-AGC AAA TCG CCC ATC ACA TGT AGC-3'
znuA forward	5'-ACG TAC ATC ACC ATC ACG GCG AAT-3'
znuA reverse	5'-ATC CTT CAG GTT GGC GTC GAG TTT-3'
zntR forward	5'-TAT CGC ATT GGT GAG CTG GCA AAG-3'
zntR reverse	5'-ATA CAG ACG AAA CCC GCC TTC AGT-3'
gyrB forward	5'-AAT GAC AGT TCA CGC AGG CGT TTC-3'
gyrB reverse	5'-ACT GGT TAT CCA GCG AGA TGG CAA-3'

Table 2: Functions of zinc-related proteins examined in this study.

Protein	Function	Direction of zinc transport
ZntA	P-type ATPase, confers resistance to Pb(II), Zn(II), Cd(II), efflux homeostasis in high zinc concentrations, up-regulated for sustainable zinc detox[10,21]	Efflux
ZntB	ZntB is a bitopic integral membrane protein[23]	Efflux
ZitB	P-type ATPase, confers resistance to Pb(II), Zn(II), Cd(II), Ni(II) efflux homeostasis in low zinc concentrations, constitutive first line defense[19]	Efflux
ZnuA	Substrate-binding component of ABC transporter[11]	Influx
ZntR	Transcription factor that induces ZntA[10]	Transcription Factor

Table 3: Gene expression of five zinc-related genes measured via qPCR comparisons between the parent and SRS strain in TSB without added zinc.

Parent and SRS 0 mM Zinc				
Gene	Average fold change	S.D. SRS	S.D. Parent	P-Value
zntA	-1.4006	0.0671	0.3687	0.2063
zntB	1.2583	0.1648	0.4616	0.5465
zitB	-1.2260	0.1911	0.3366	0.3837
znuA	-1.2897	0.0767	0.1585	0.0840
zntR	1.2535	0.6449	0.3556	0.6453

*S.D. indicates standard deviation

Table 4: Gene expression of five zinc-related genes measured via qPCR comparisons between the parent and SRS strain in TSB with 20 mM added zinc.

Parent and SRS 20 mM Zinc				
Gene	Average fold change	S.D. SRS	S.D. Parent	P-Value
zntA	7.0087	0.6964	0.0928	0.0001
zntB	1.3501	0.2852	0.3391	0.2852
zitB	1.8187	0.3335	0.3654	0.0536
znuA	-2.5129	0.0406	0.0807	0.0003
zntR	1.7259	0.4335	0.2317	0.0674

*S.D. indicates standard deviation

Table 5: Gene expression of five zinc-related genes measured via qPCR comparisons between the parent in TSB with and without 20 mM added zinc.

Parent 0 and Parent 20 mM Zinc				
Gene	Average fold change	S.D. SRS	S.D. Parent	P-Value
zntA	7.8571	0.0117	0.3687	0.0128
zntB	4.6991	0.0697	0.4616	0.0333
zitB	8.4395	0.0189	0.3366	0.0073
znuA	-1.8639	0.0432	0.1585	0.0077
zntR	1.5210	0.3460	0.3556	0.1699

*S.D. indicates standard deviation

Table 6: Gene expression of five zinc-related genes measured via qPCR comparisons between the SRS strain in TSB with and without 20 mM added zinc.

SRS 0 and SRS 20 mM Zinc				
Gene	Average fold change	S.D. SRS	S.D. Parent	P-Value
zntA	1.2496	0.1241	0.0943	0.0520
zntB	4.5063	0.0468	0.1317	0.0006
zitB	8.4953	0.0215	0.2384	0.0029
znuA	-3.6273	0.0281	0.0993	0.0003
zntR	2.2351	0.5614	0.5594	0.0662

*S.D. indicates standard deviation

Table 7: Chemical groups and functions of antimicrobials used in cross-resistance experiments.

Name	Class/Chemical group	Function
Fumagillin-B	2-(2-pyridinyl)-pyrimidine core	Methionyl aminopeptidase inhibitor[42]
Oxytetracycline	Tetracycline	Inhibition of protein synthesis, by binding 30s ribosomal protein[40]
Tylosin Tartrate	Macrolides	Inhibition of protein synthesis, by binding the 23s rRNA[37,39]
Dodecyltrimethyl ammonium chloride (DTAC)	Quaternary Ammonium Compound (QAC)	Increase in permeability of the cytoplasmic membrane via OmpE[43]
Triclosan	Trichloro derivative of two hydroxydiphenyl ether	Block lipid synthesis by inhibiting a NADH-dependent enoyl-acyl carrier protein reductase[44]

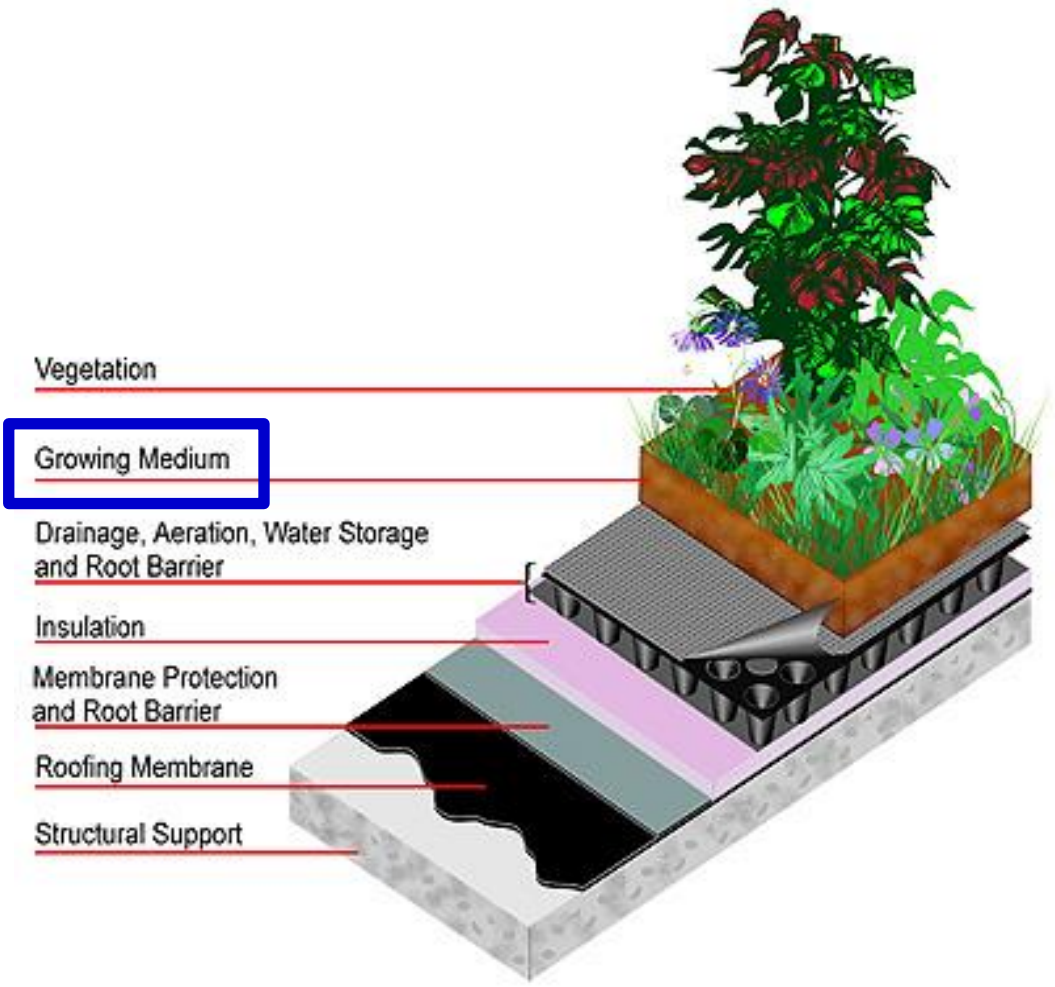


Figure 1: Typical green roof components. The growing medium is focused on in this study.
Reference: www.lid-stormwater.net/greenroofs_home.htm

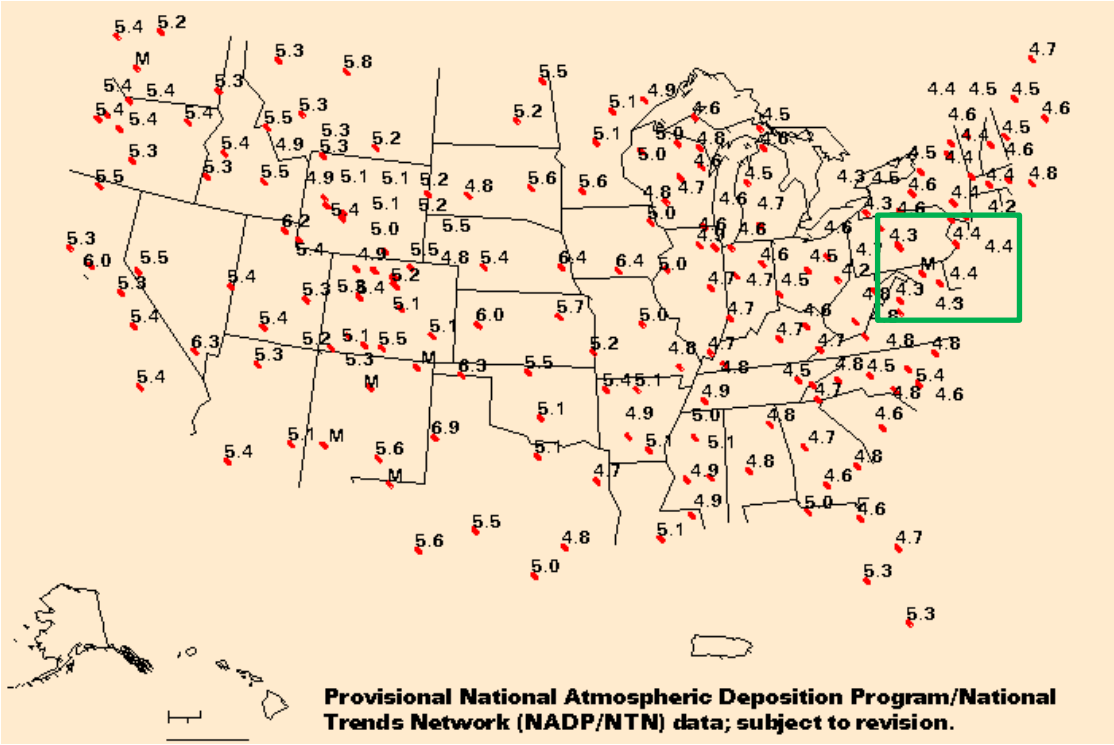


Figure 2: National rainwater pH. As of 2001 the local environment is 4.3-4.4, which was simulated by the synthetic rainwater used in this study.
Reference: water.usgs.gov/nwc/NWC/pH/html/ph.html

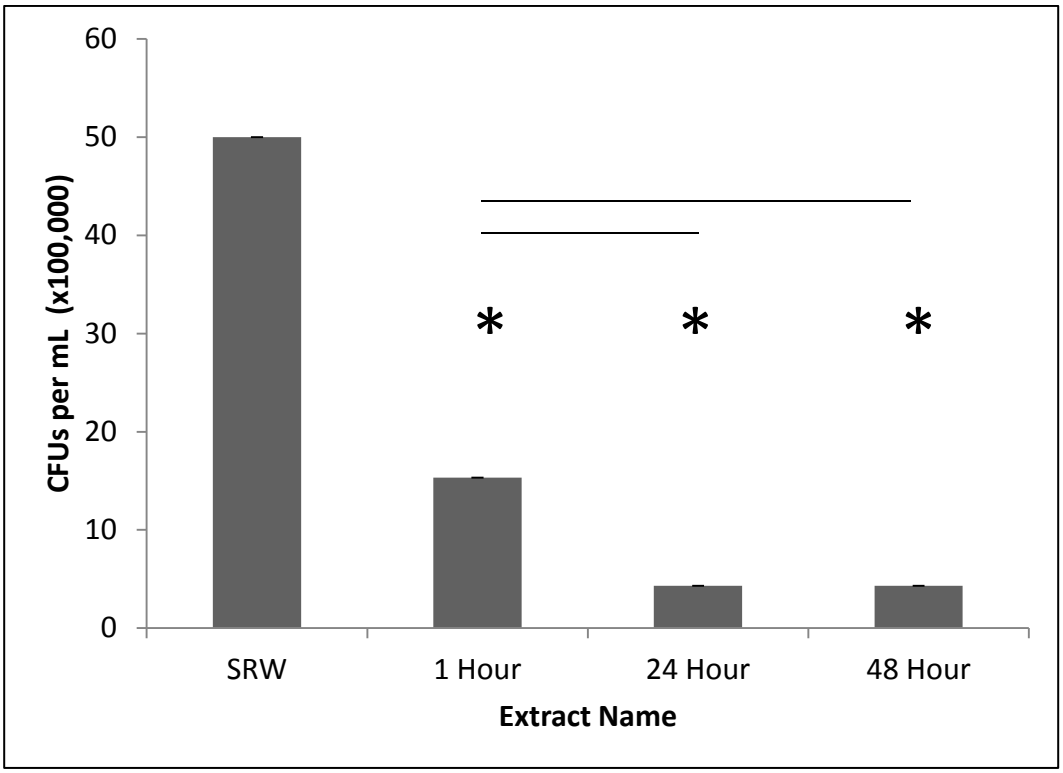


Figure 3: *Salmonella* survival after 24 hours of incubation. Enumeration of *Salmonella* after 24 hours of incubation with synthetic rainwater, 1-hour extract, 24-hour extract, and 48-hour extract. Bars represent SEM. Asterisks represent $P<0.05$ between extract and synthetic rainwater. Black horizontal bars represent $P<0.05$ between the extracts.

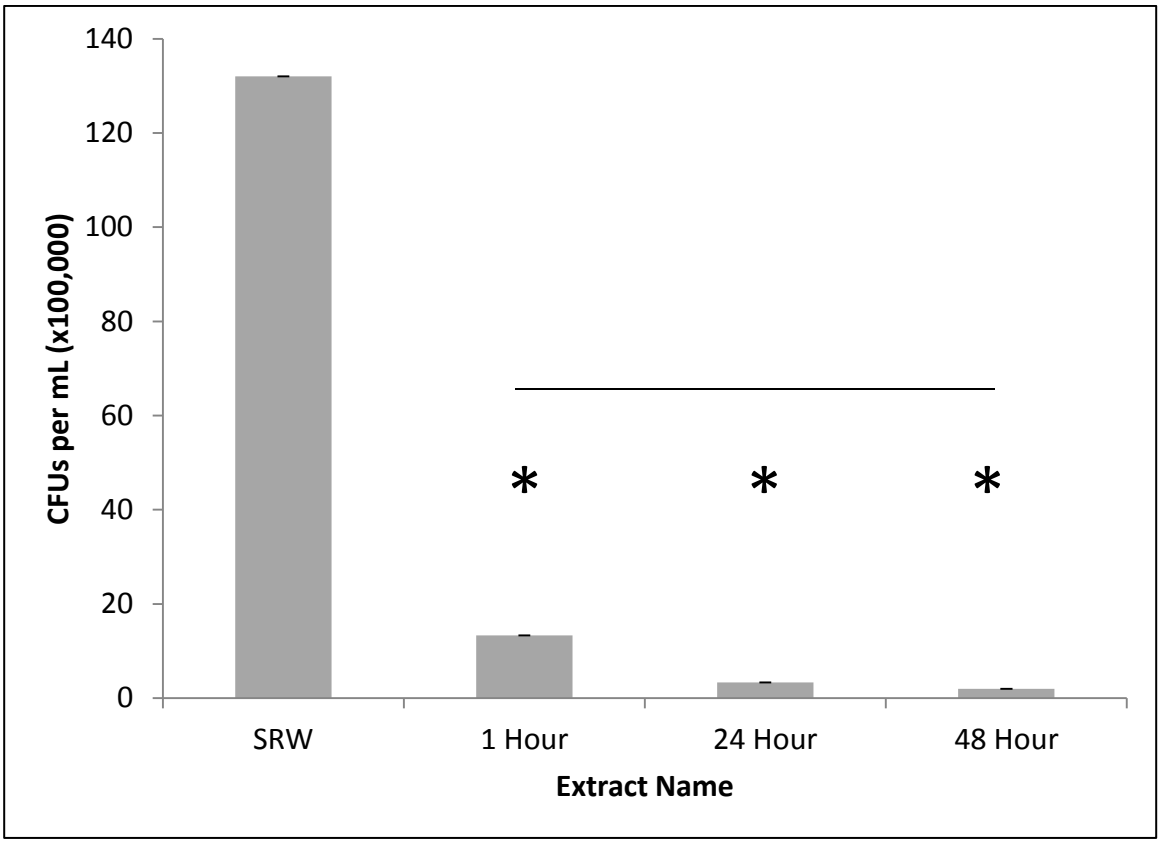


Figure 4: *Salmonella* survival after 48 hours of incubation. Enumeration of *Salmonella* after 48 hours of incubation with synthetic rainwater, 1-hour extract, 24-hour extract, and 48-hour extract. Bars represent SEM. Asterisks represent $P<0.05$ between extract and synthetic rainwater. Black horizontal bar represent $P<0.05$ between the extracts.

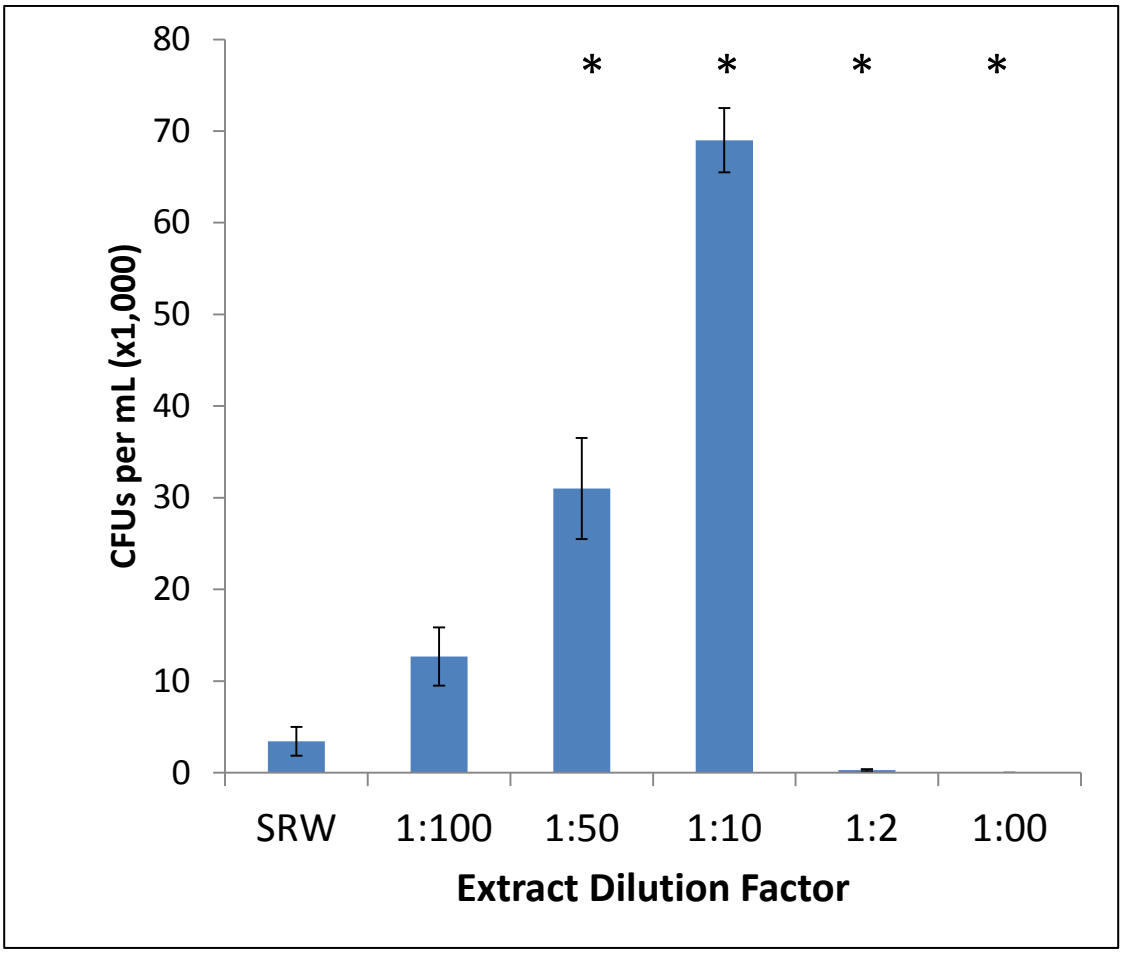


Figure 5: *Salmonella* survival after 24 hour incubation in diluted 24-hour extract. Enumeration of *Salmonella* after 24 hours of incubation with synthetic rainwater and diluted extract. Bars represent SEM. Asterisks represent $P<0.05$ between synthetic rainwater and extract dilutions.

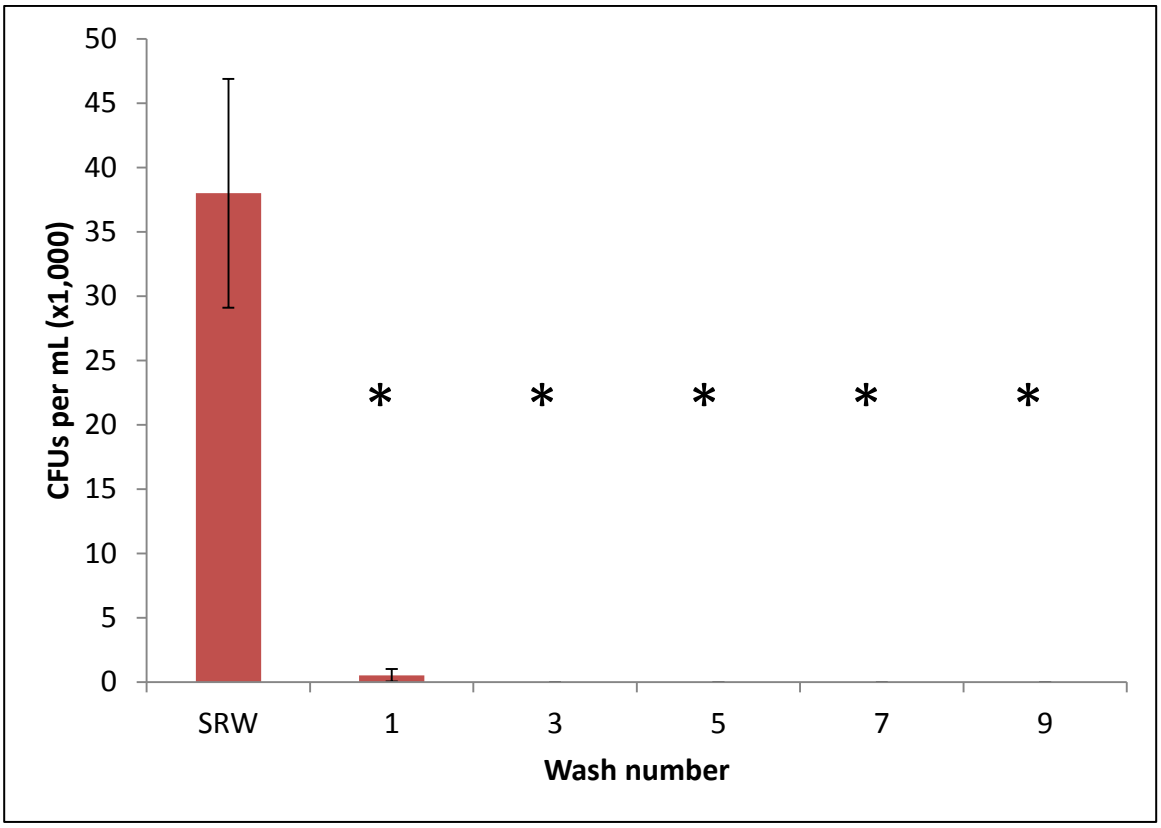


Figure 6: Serial washes of crumb rubber effect on *Salmonella.* Enumeration of *Salmonella* after 24 hours of incubation with crumb rubber washes. Bars represent SEM. Asterisks represent $P<0.05$ between synthetic rainwater and washes.

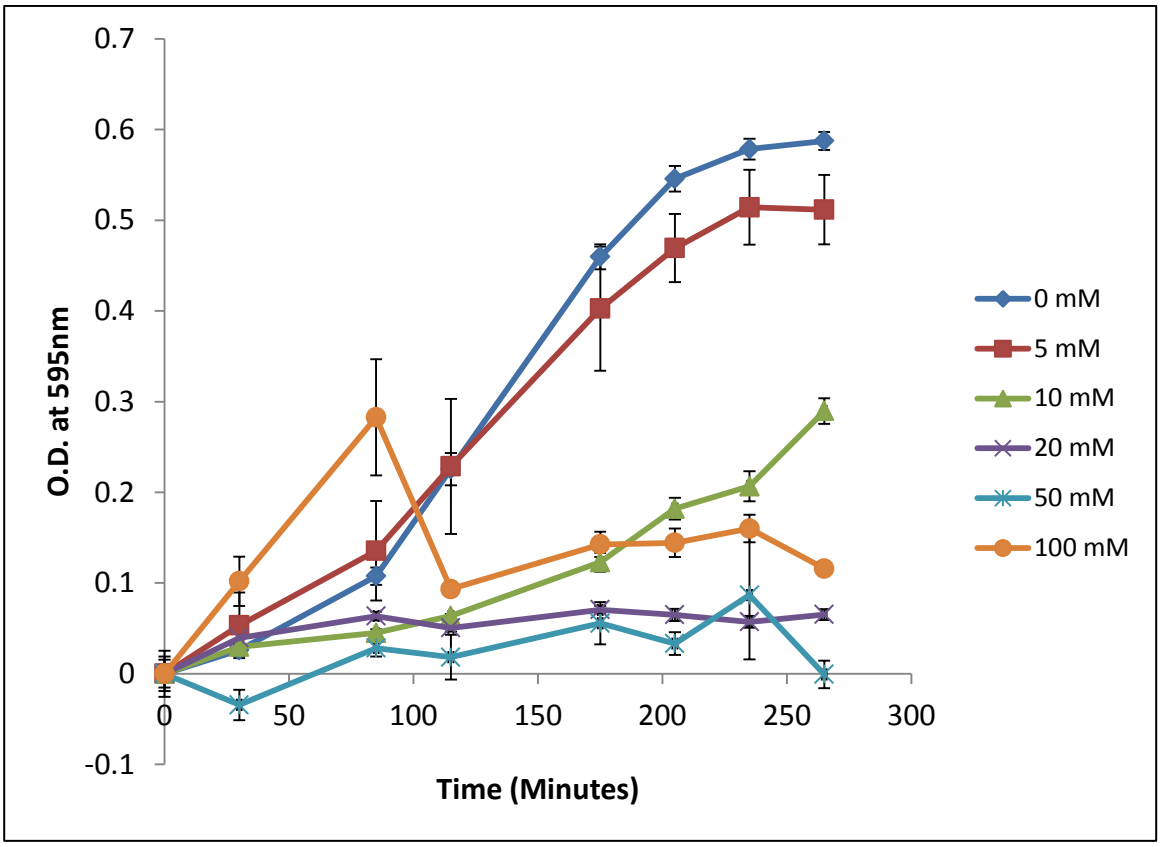
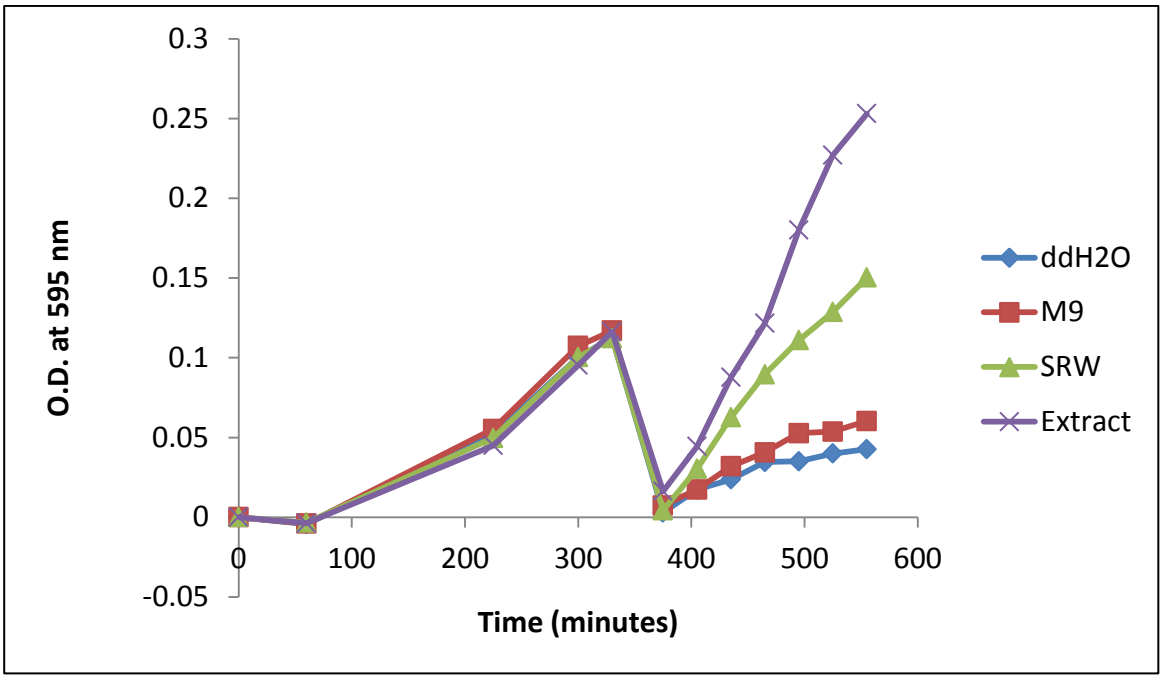
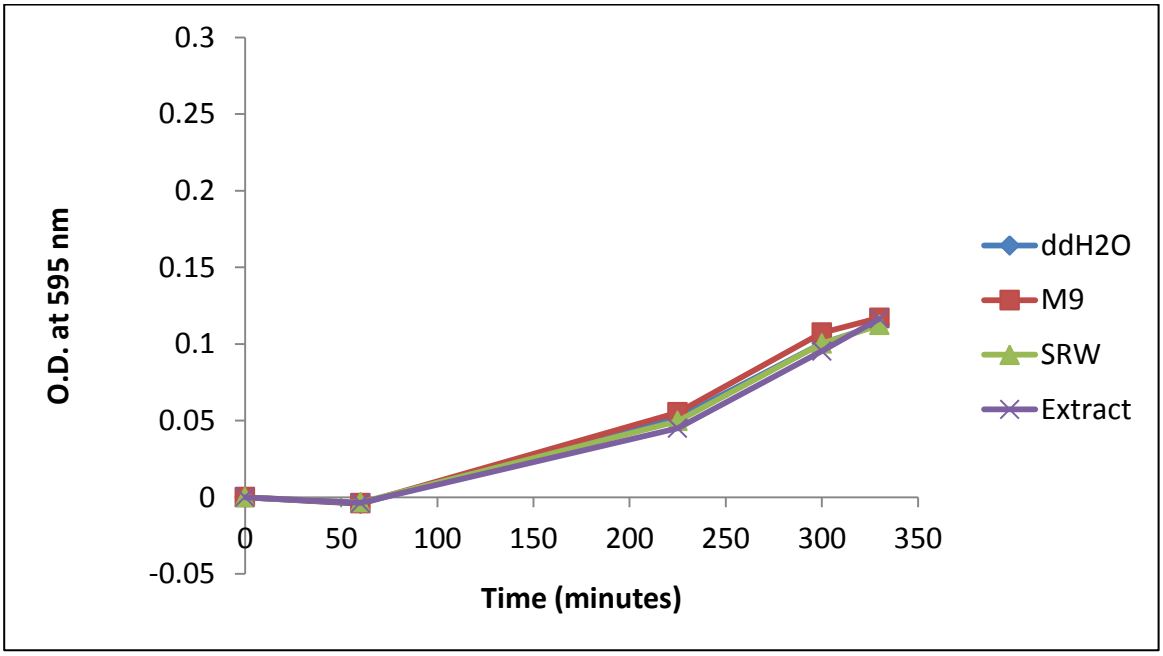


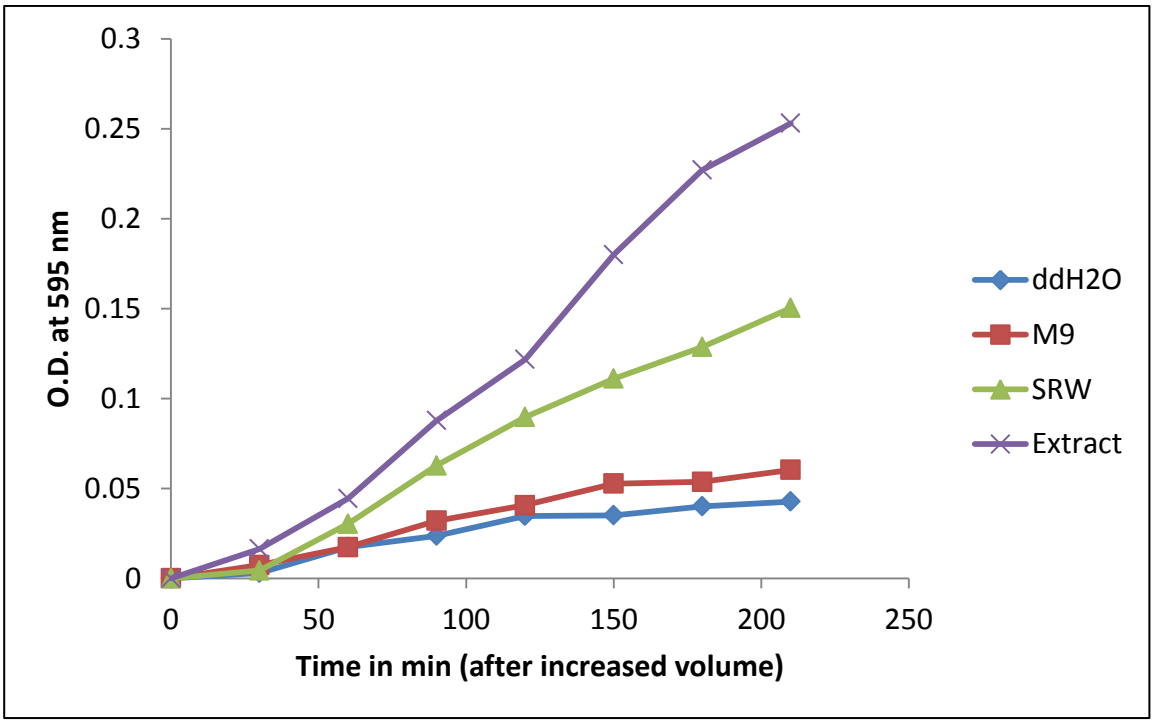
Figure 7: *Salmonella* were grown in increasing levels of zinc. Bars represent SEM.


0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
O.D. at 595 nm
0
100
200
300
400
500
600
Time (minutes)
ddH2O
M9
SRW
Extract

A

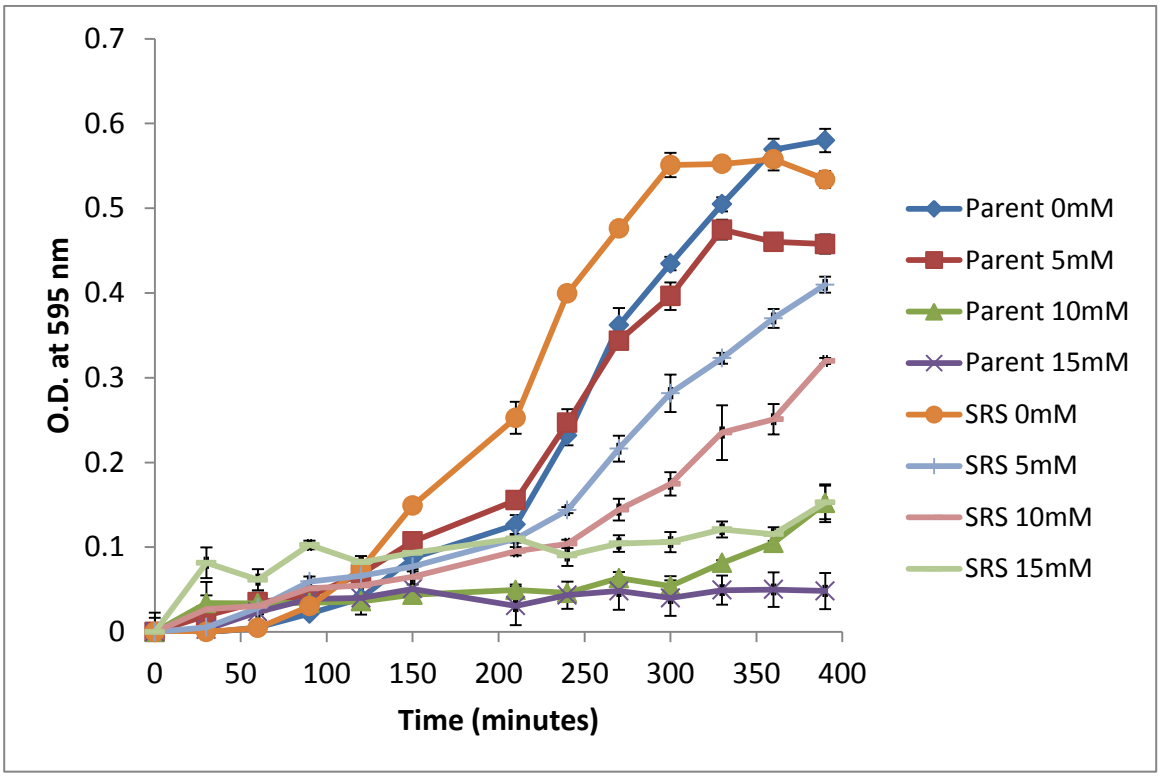

0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
O.D. at 595 nm
0
50
100
150
200
250
300
350
Time (minutes)
ddH2O
M9
SRW
Extract

B

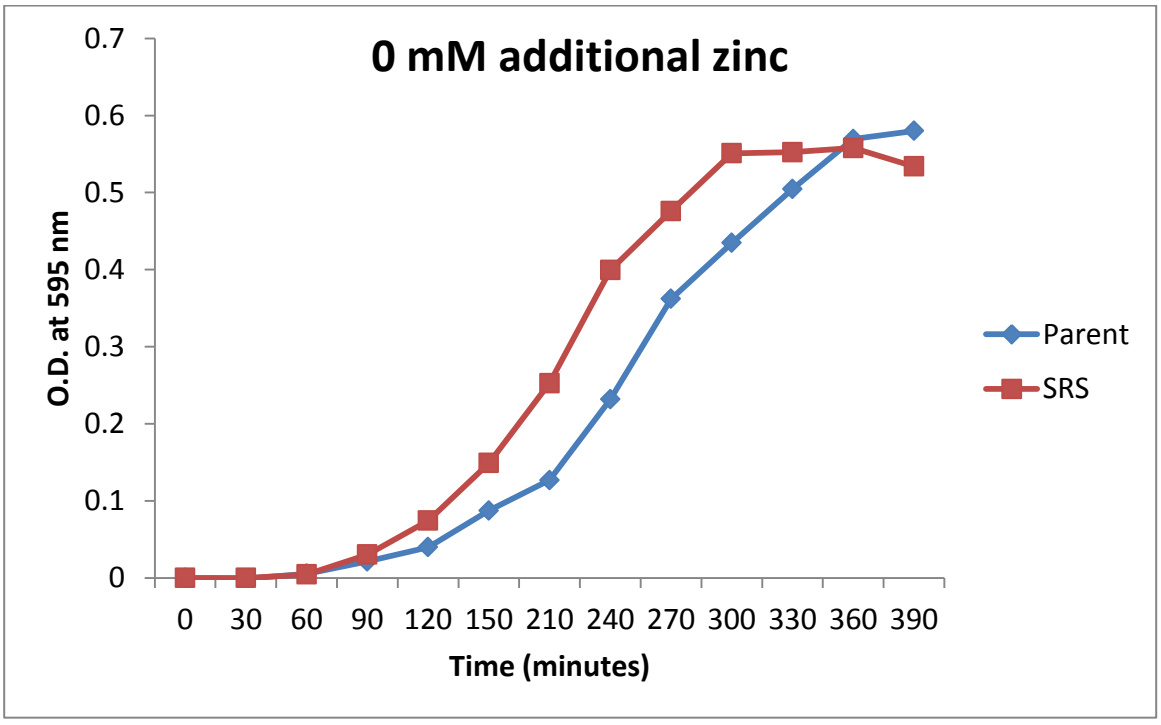


C

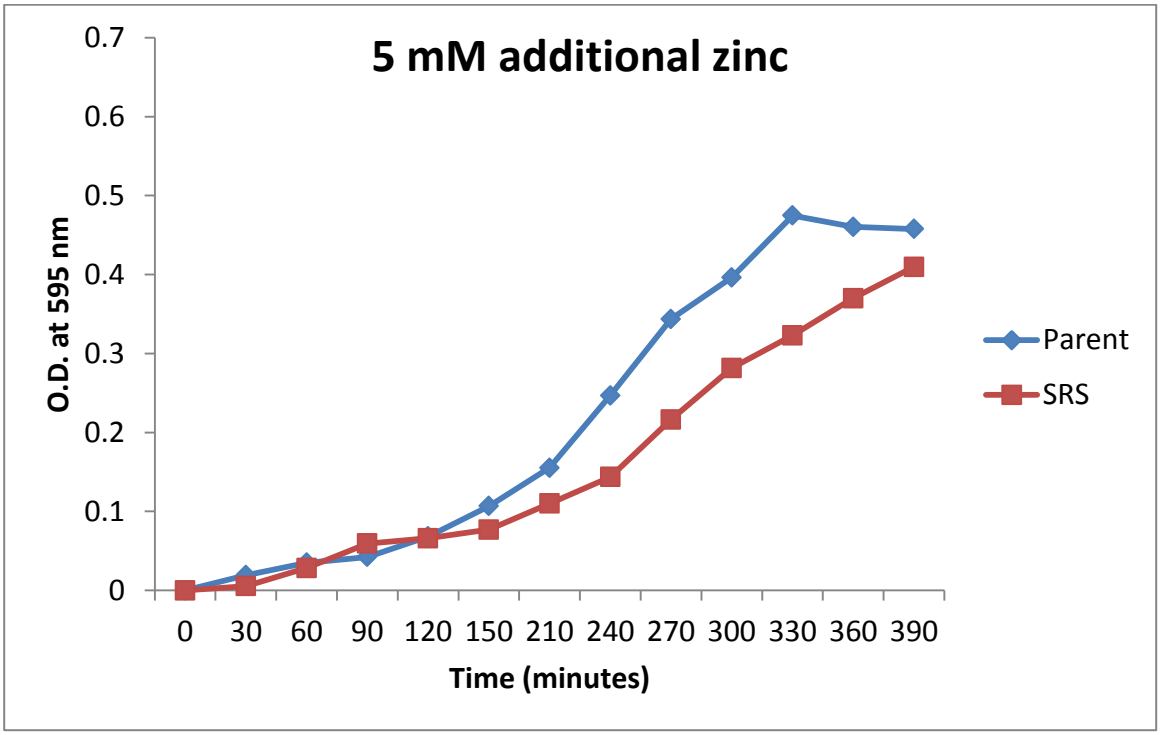
Figure 8: Growth curves of *Salmonella* in M9 with the additional amendments. **A** shows the blanked absorbances for the full 9 hour time course. **B** shows that all the wells grew to the same O.D. before the addition of amendments. **C** shows the growth curve beginning after the first 5.5 hours with the addition of the 24-hour extract, SRW, additional M9, and ddH_2O.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. at 595 nm
0
50
100
150
200
250
300
350
400
Time (minutes)
Parent 0mM
Parent 5mM
Parent 10mM
Parent 15mM
SRS 0mM
SRS 5mM
SRS 10mM
SRS 15mM

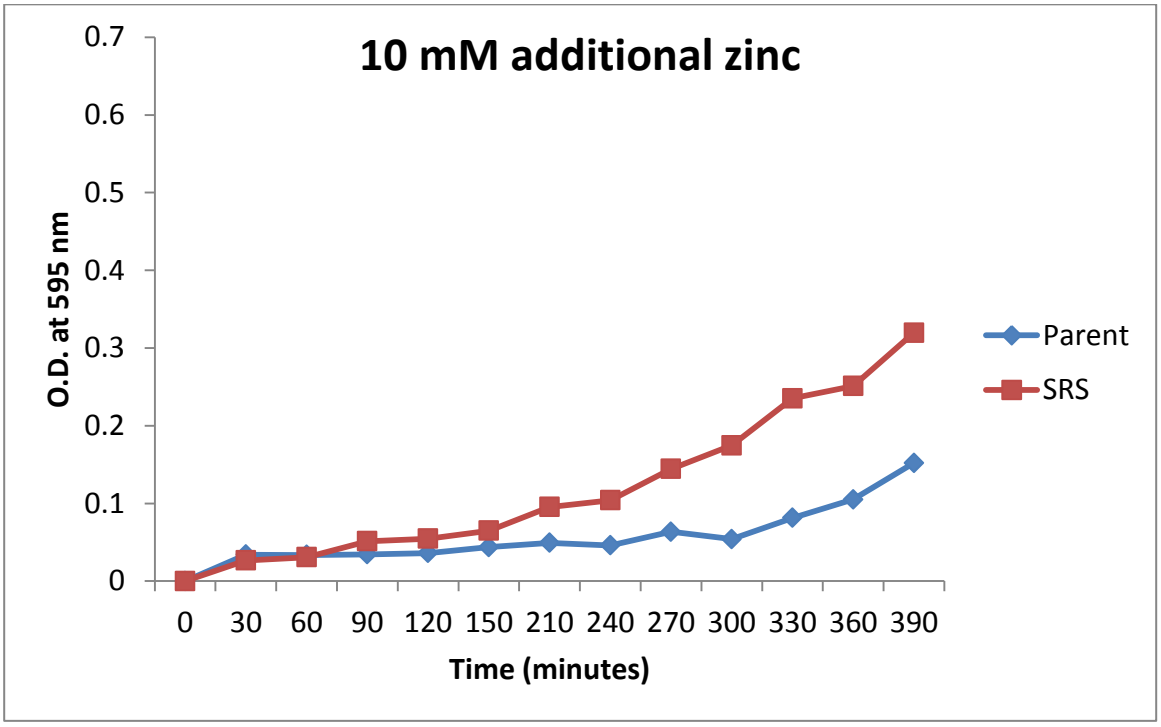
A


0 mM additional zinc
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. at 595 nm
0
30
60
90
120
150
210
240
270
300
330
360
390
Time (minutes)
Parent
SRS

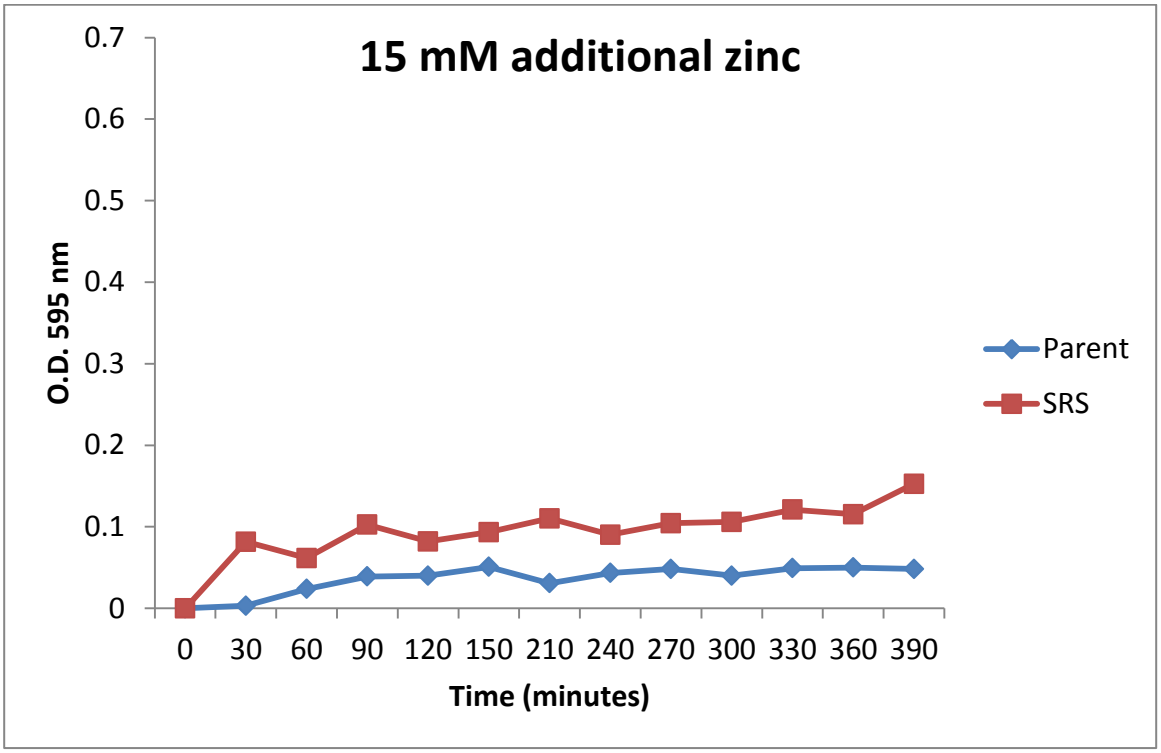
B


5 mM additional zinc
O.D. at 595 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 30 60 90 120 150 210 240 270 300 330 360 390
Time (minutes)
Parent
SRS

C

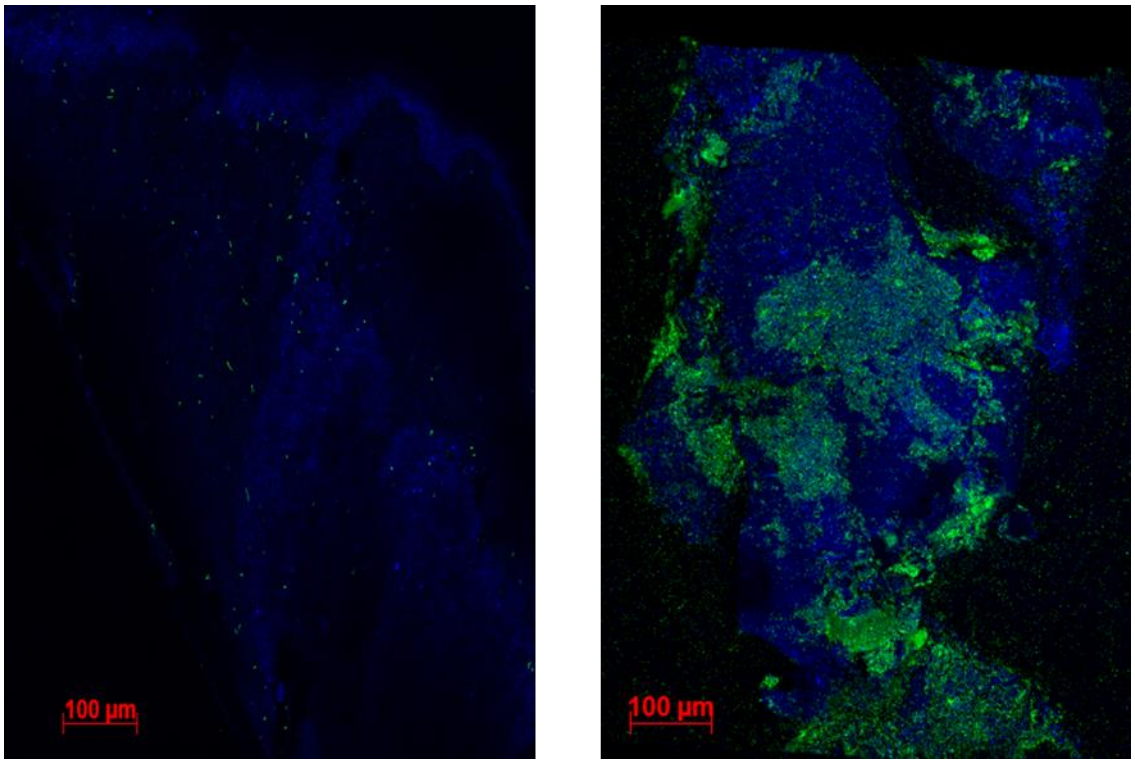

10 mM additional zinc
O.D. at 595 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 30 60 90 120 150 210 240 270 300 330 360 390
Time (minutes)
Parent
SRS

D



E

Figure 9: Parent and SRS growth curves in TSB with added zinc. The parent and SRS strains were grown in the presence of 0, 5, 10, or 15 mM additional $ZnSO_4$. **A** shows all the growth curve results on one graph. **B** compares growth of the parent and SRS strains in 0 mM additional zinc. **C** compares the growth of the parent and SRS strains in 5 mM additional zinc. **D** compares the growth of the parent and SRS strains in 10 mM additional zinc. **E** compares the growth of the parent and SRS strains in 15 mM additional zinc. Bars represent SEM.



A B

Figure 10: *Salmonella* biofilm on crumb rubber. A is crumb rubber that was not inoculated with bacteria. B is crumb rubber inoculated with a 5% inoculation of *Salmonella*. The color enhancement is the blue is the surface of crumb rubber and green is the nucleic acid (stained with SYTO 13) of bacterial cells.

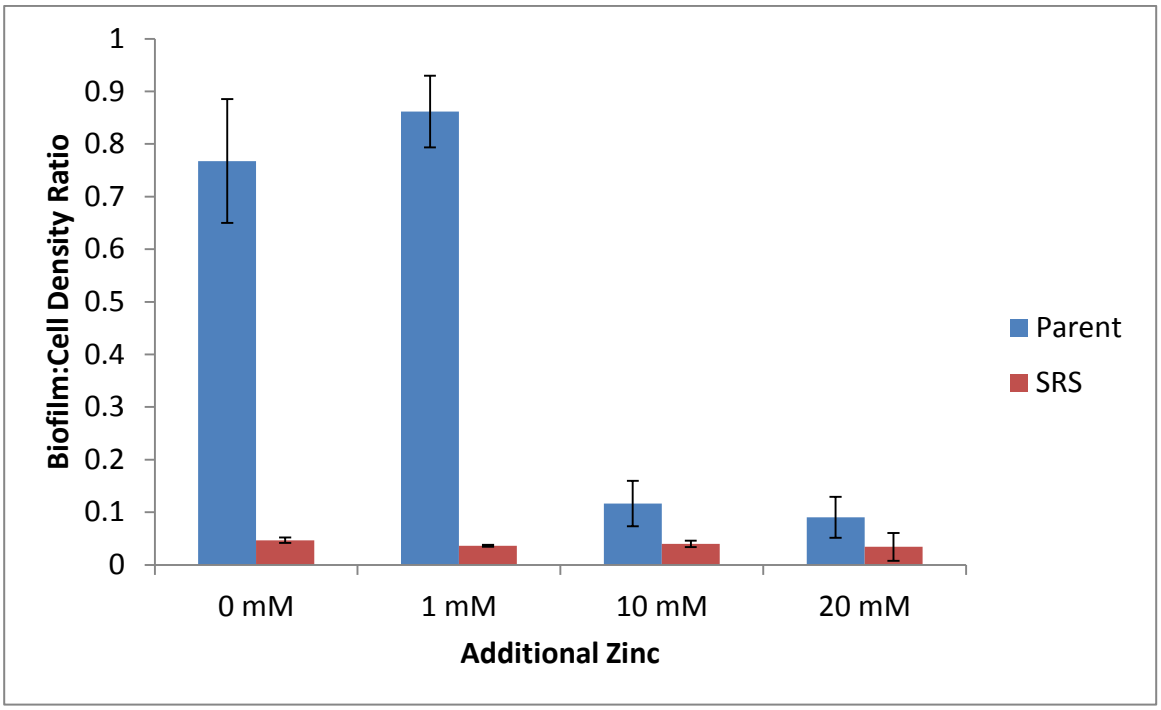


Figure 11: Biofilm formation of parent and SRS strains. Biofilm formation is shown as an O.D. ratio of crystal violet solubilized from biofilm:density ratio. Biofilm formation was measured by crystal violet after 24 hours and divided by the total cell density. The ratio between biofilm O.D. and total cell density O.D. is shown. The bars represent SEM.

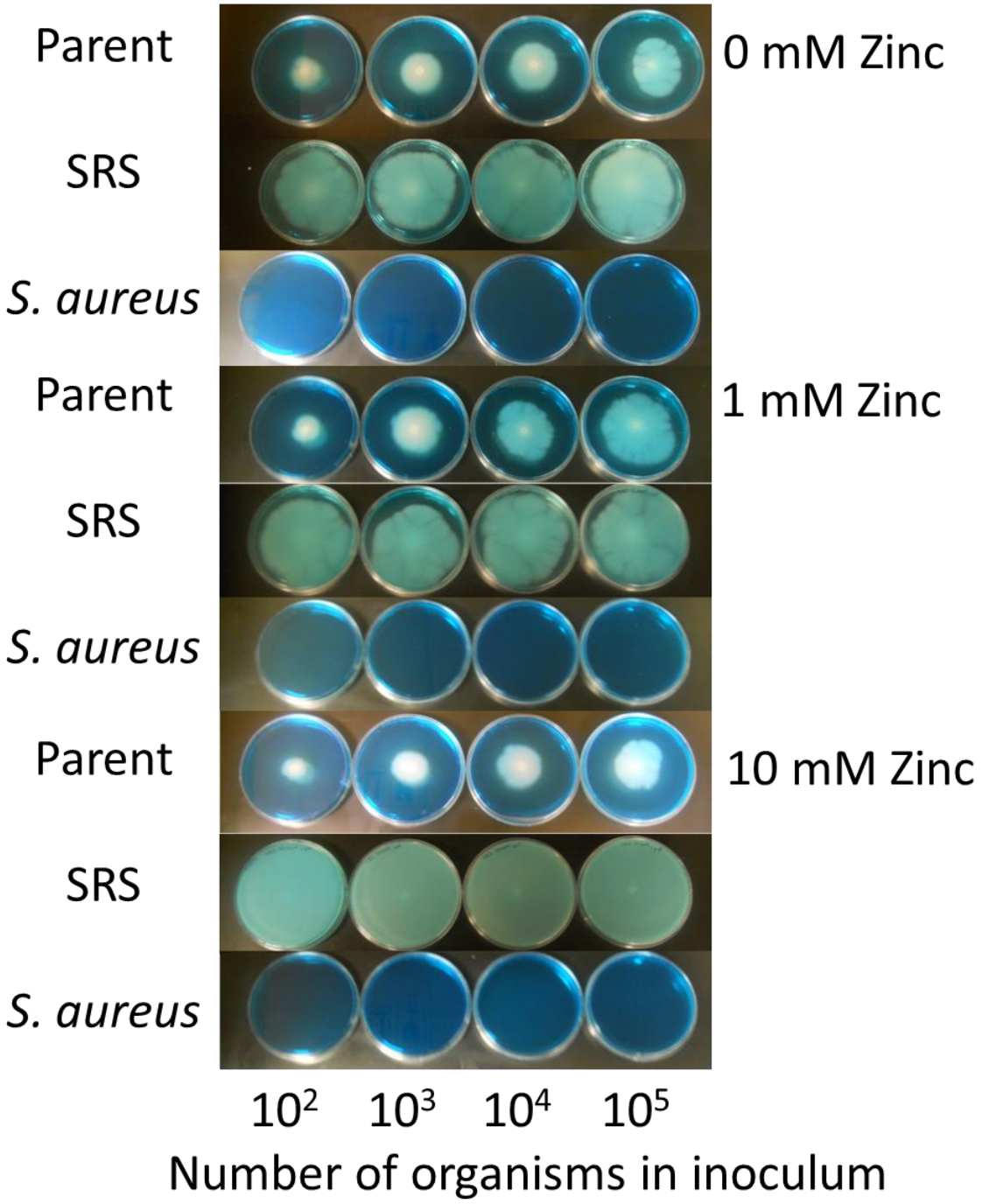
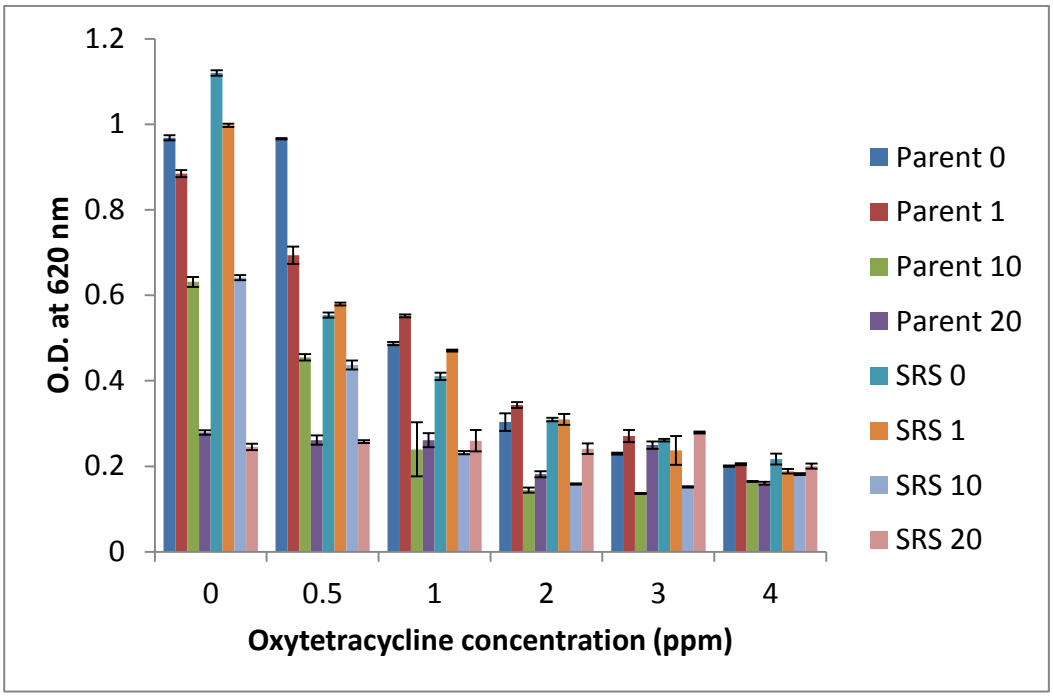
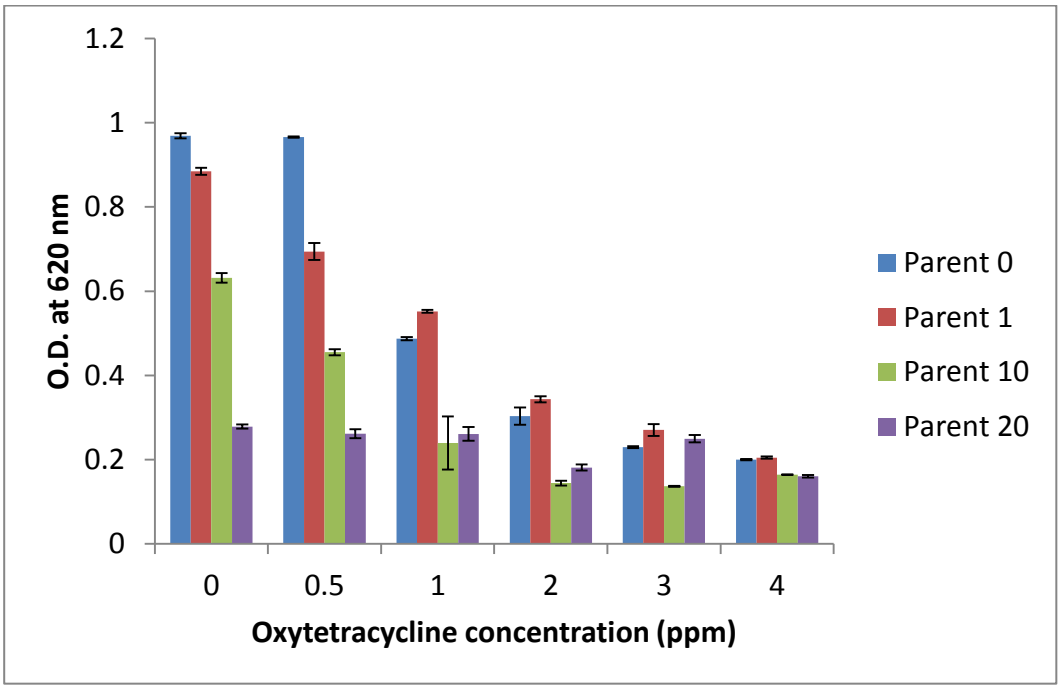


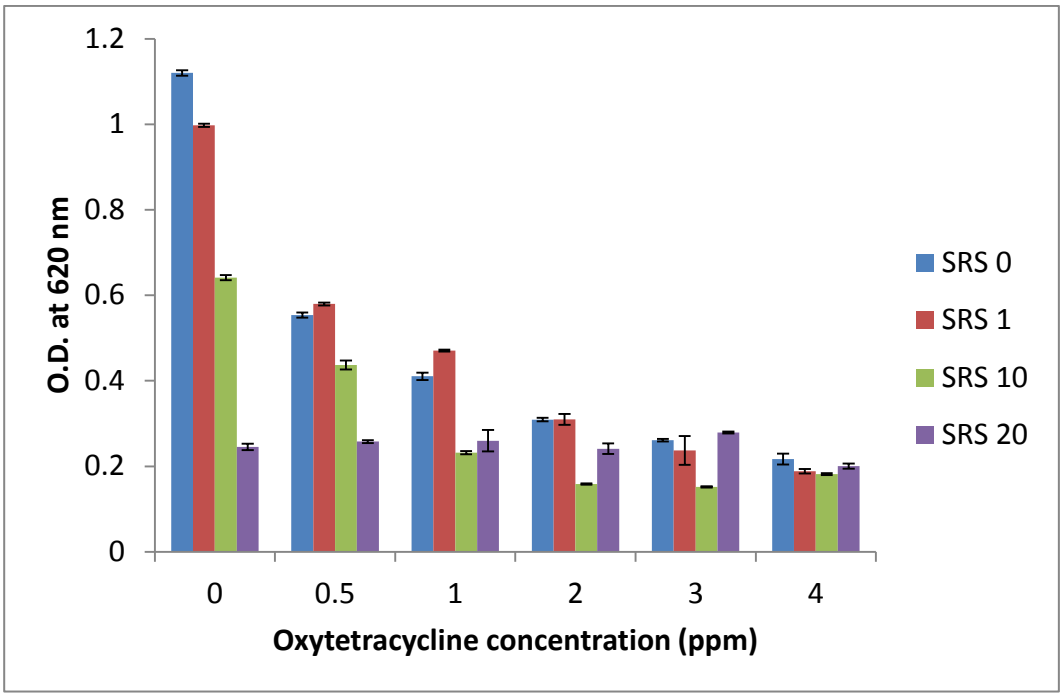
Figure 12: Motility of parent and SRS strains. MSRV motility plates were inoculated and grown at 37°C for 48 hours. The zone of growth was measured for the parent, SRS strain, and *S. aureus* non-motile control in 0, 1, and 10 mM zinc.


1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 620 nm
0
0.5
1
2
3
4
Oxytetracycline concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20
SRS 0
SRS 1
SRS 10
SRS 20

A

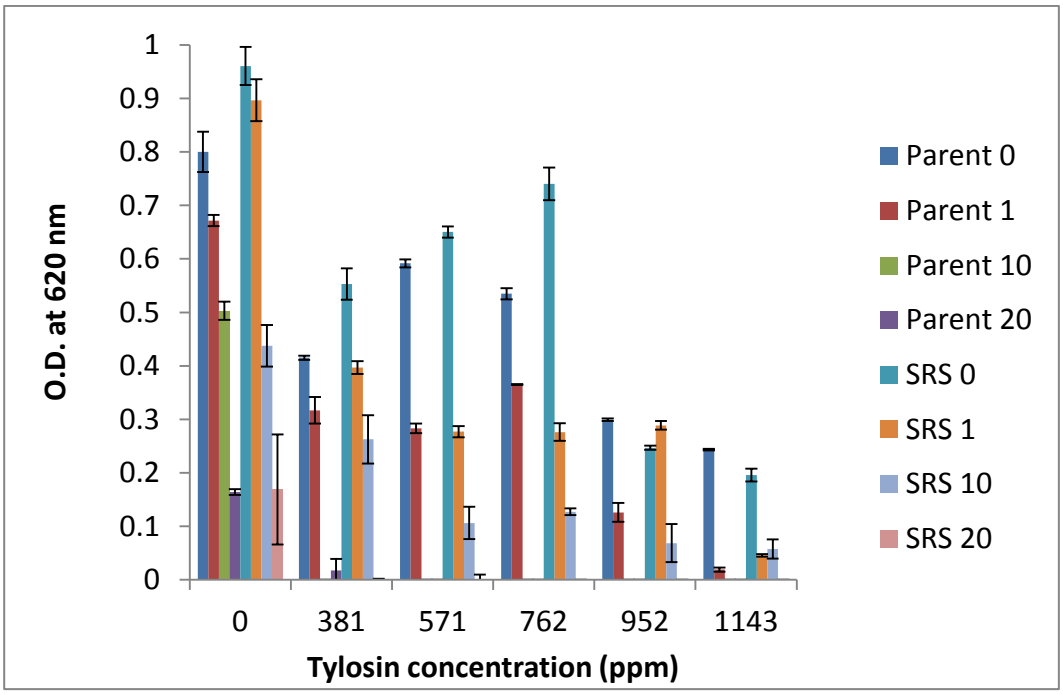

1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 620 nm
0
0.5
1
2
3
4
Oxytetracycline concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20

B

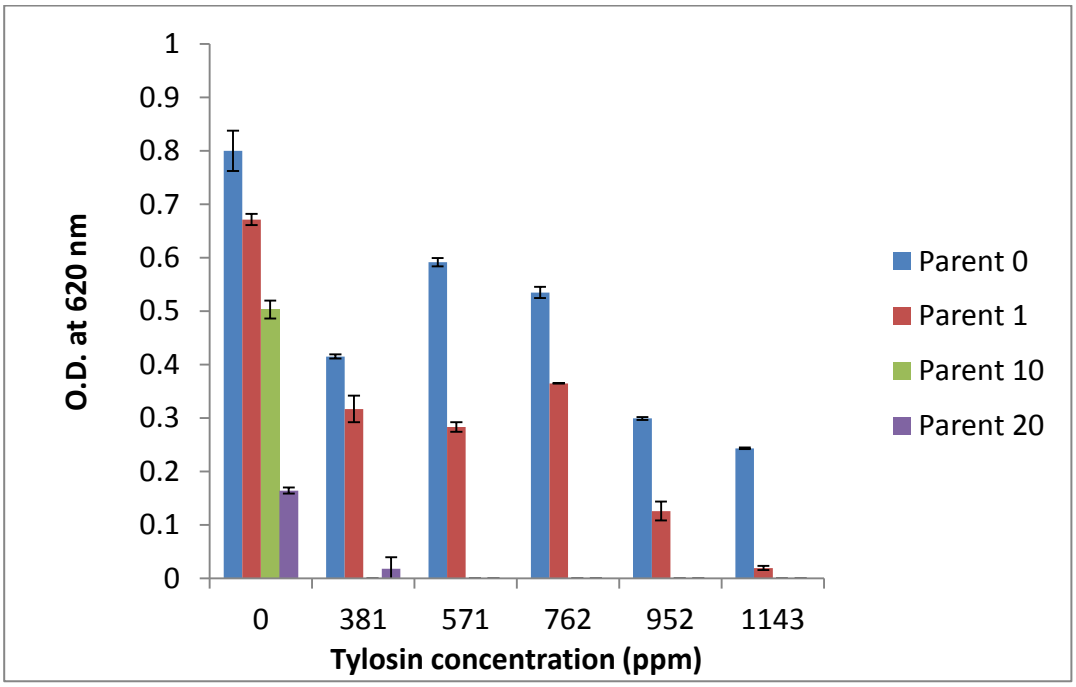


C

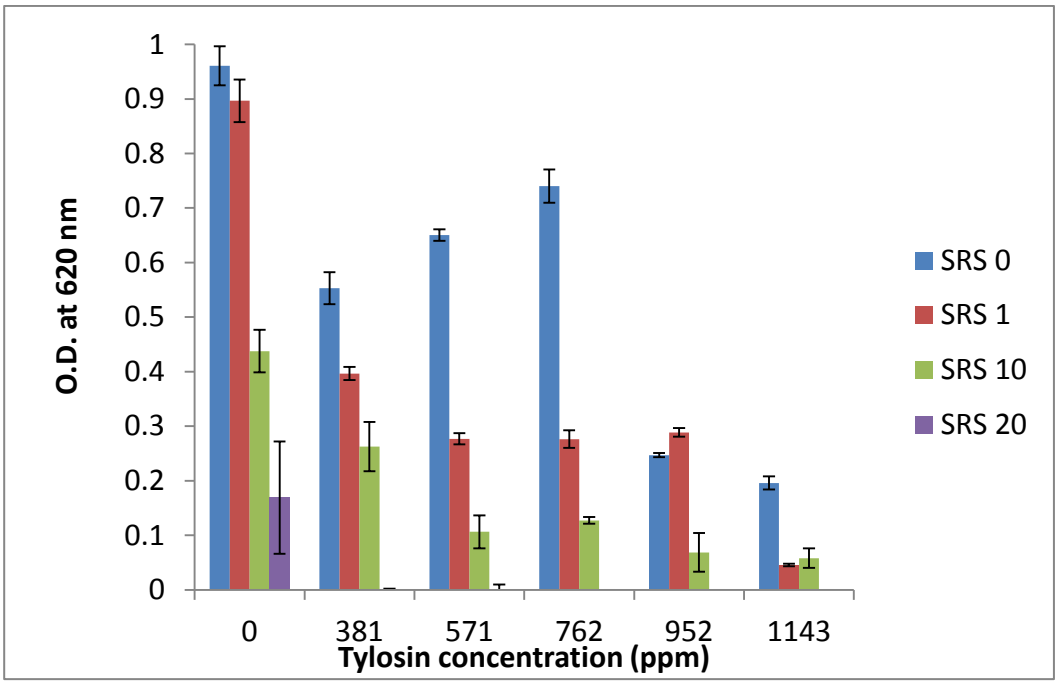
Figure 13: The O.D. for the parent and SRS strains grown in 0, 1, 10, and 20 mM of zinc and increasing OTC concentrations overnight are shown. **A** shows results of the parent and SRS strains in a single graph. **B** shows only the parent. **C** shows only the SRS strain. Bars represent SEM.


O.D. at 620 nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
381
571
762
952
1143
Tylosin concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20
SRS 0
SRS 1
SRS 10
SRS 20

A

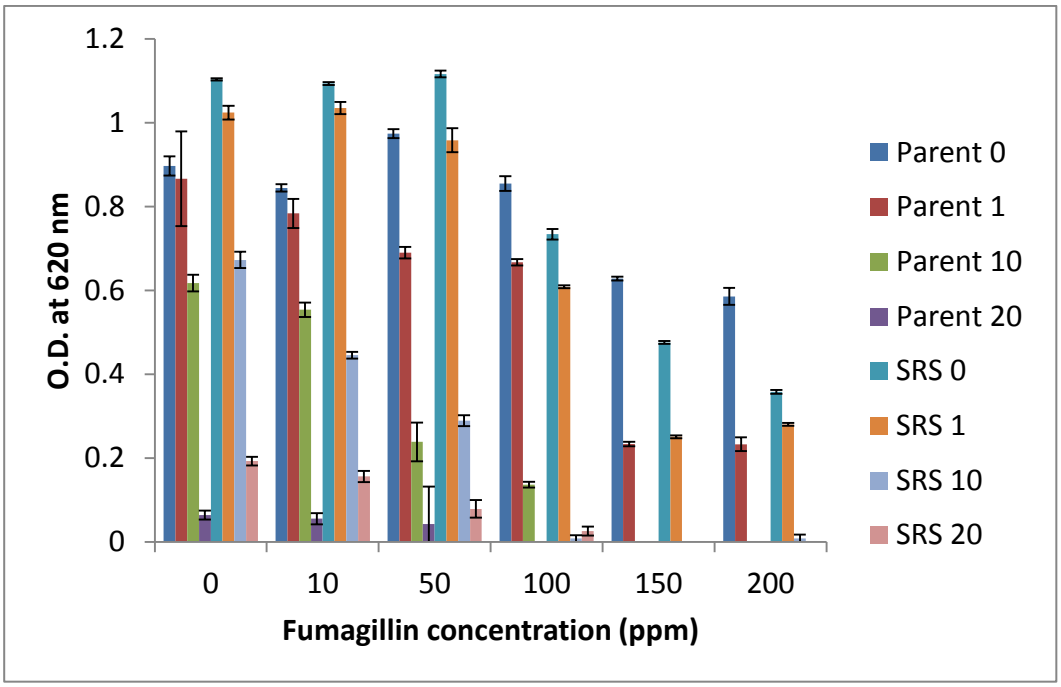

O.D. at 620 nm
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
381
571
762
952
1143
Tylosin concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20

B

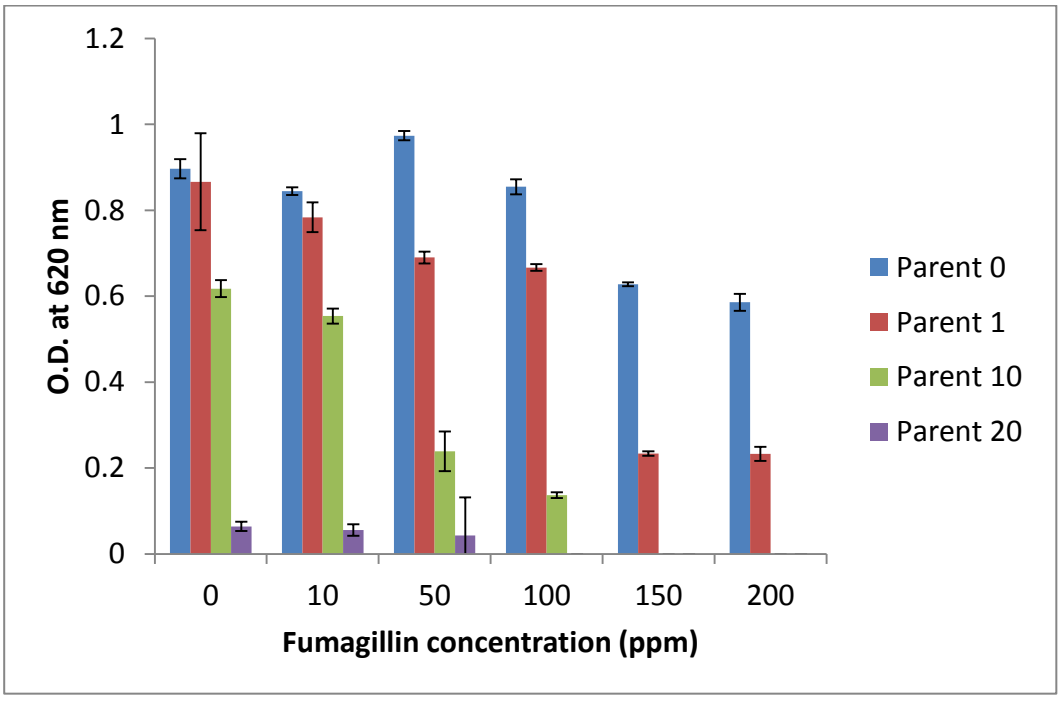


C

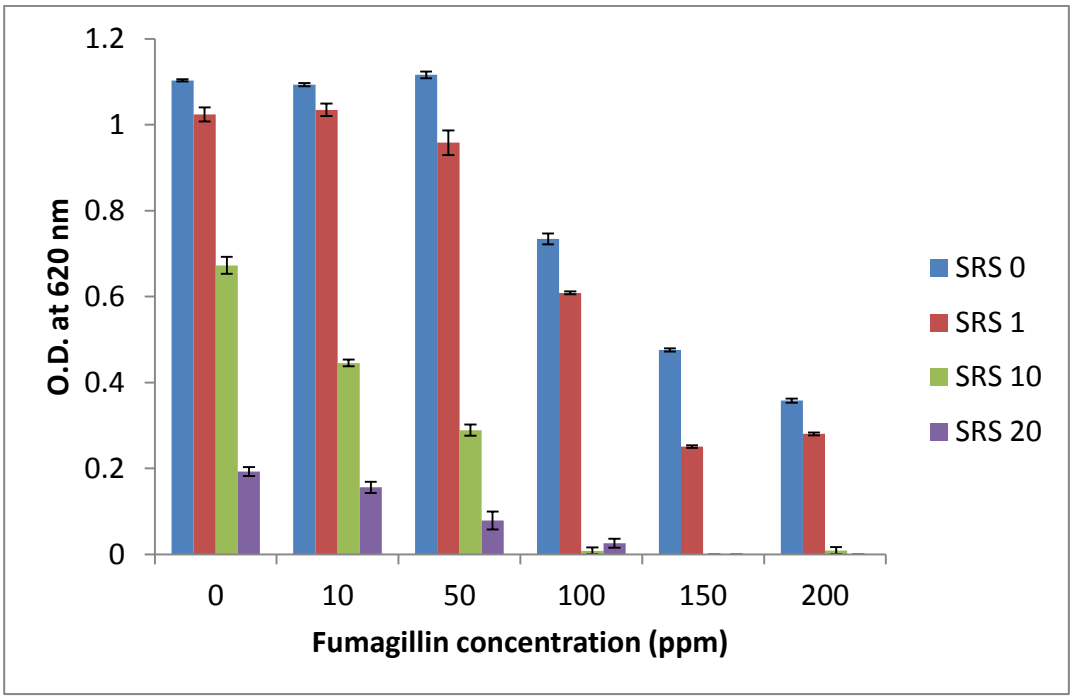
Figure 14: The O.D. for the parent and SRS strains grown in 0, 1, 10, and 20 mM of zinc and increasing tylosin concentrations overnight are shown. **A** shows results of the parent and SRS strains in a single graph. **B** shows only the parent. **C** shows only the SRS strain. Bars represent SEM.


1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 620 nm
0
10
50
100
150
200
Fumagillin concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20
SRS 0
SRS 1
SRS 10
SRS 20

A

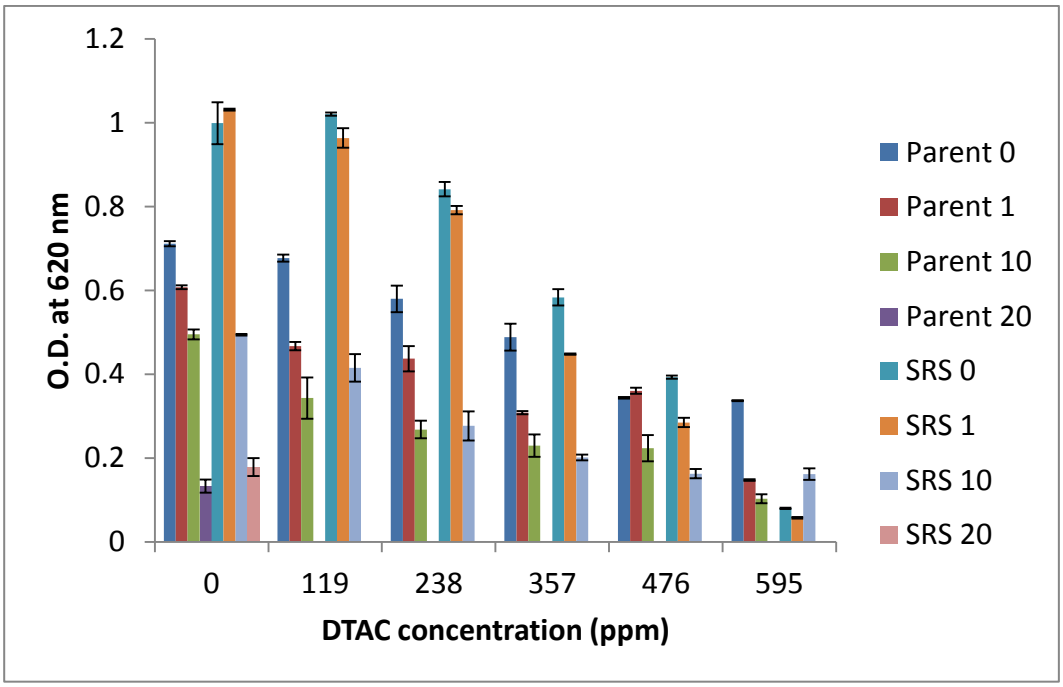

1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 620 nm
0
10
50
100
150
200
Fumagillin concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20

B

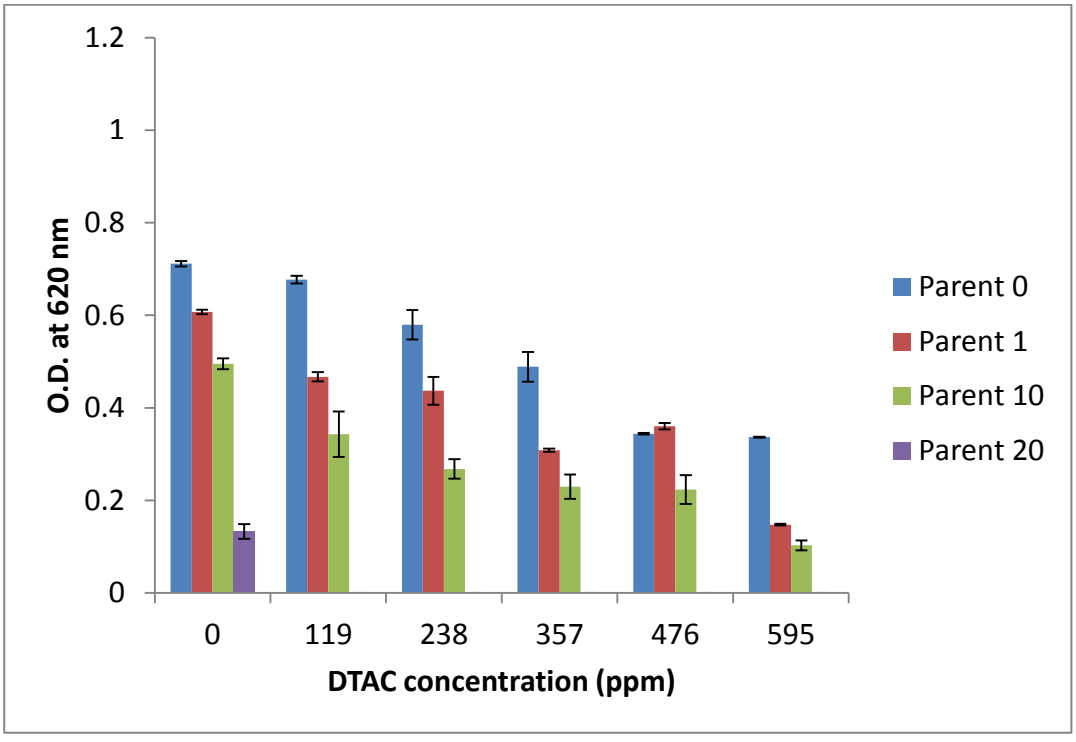


C

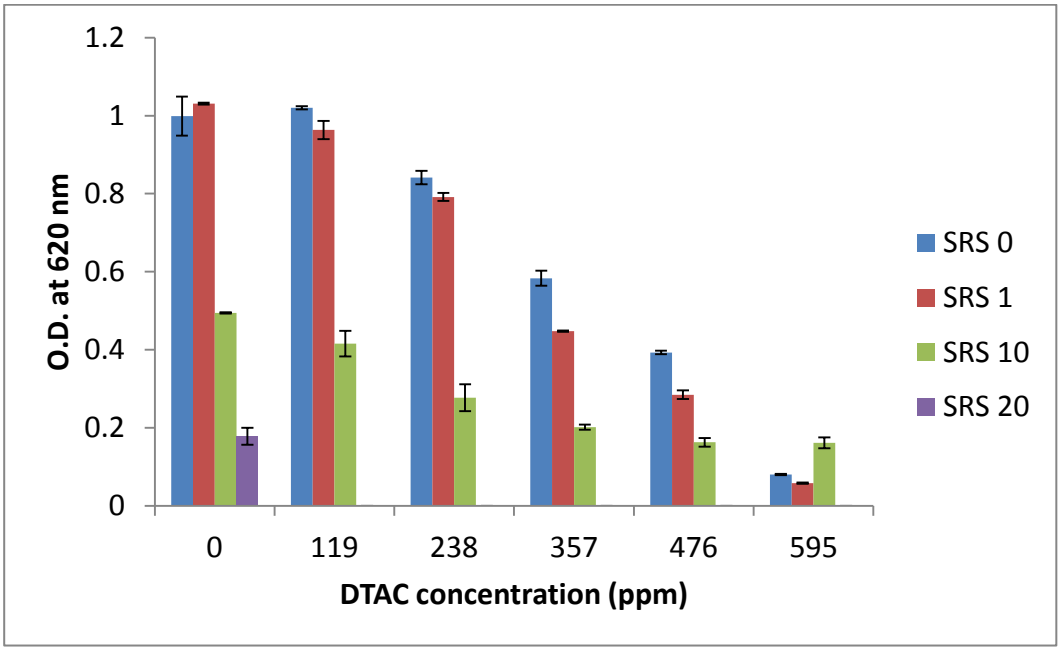
Figure 15: The O.D. for the parent and SRS strains grown in 0, 1, 10, and 20 mM of zinc and increasing fumagillin concentrations overnight are shown. **A** shows results of the parent and SRS strains in a single graph. **B** shows only the parent. **C** shows only the SRS strain. Bars represent SEM.


1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 620 nm
0
119
238
357
476
595
DTAC concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20
SRS 0
SRS 1
SRS 10
SRS 20

A

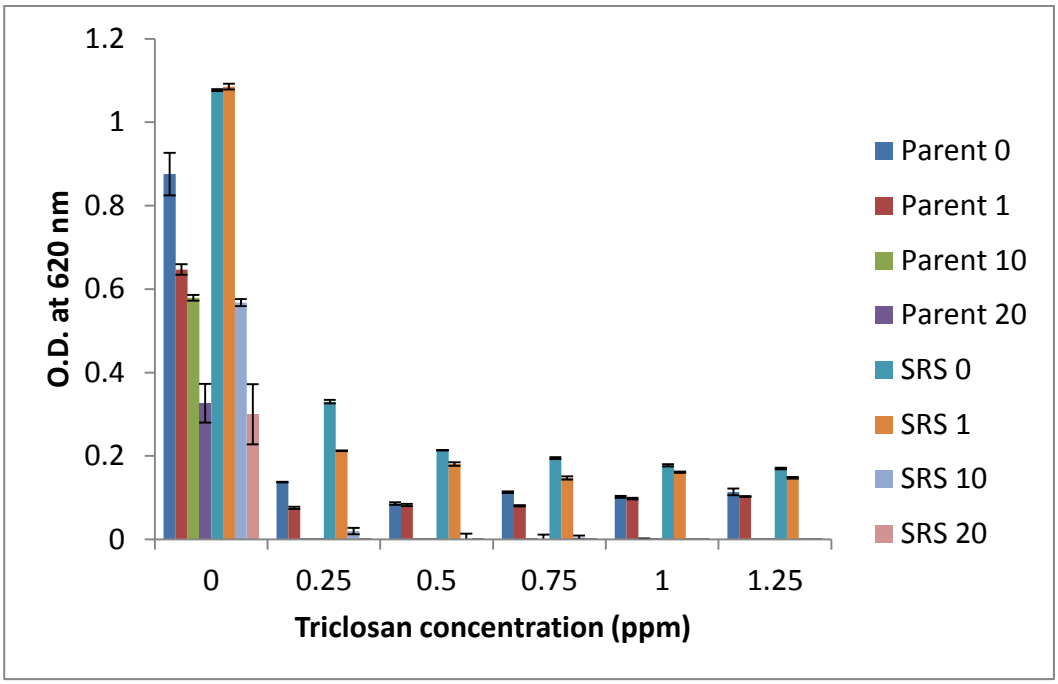

1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 620 nm
0
119
238
357
476
595
DTAC concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20

B

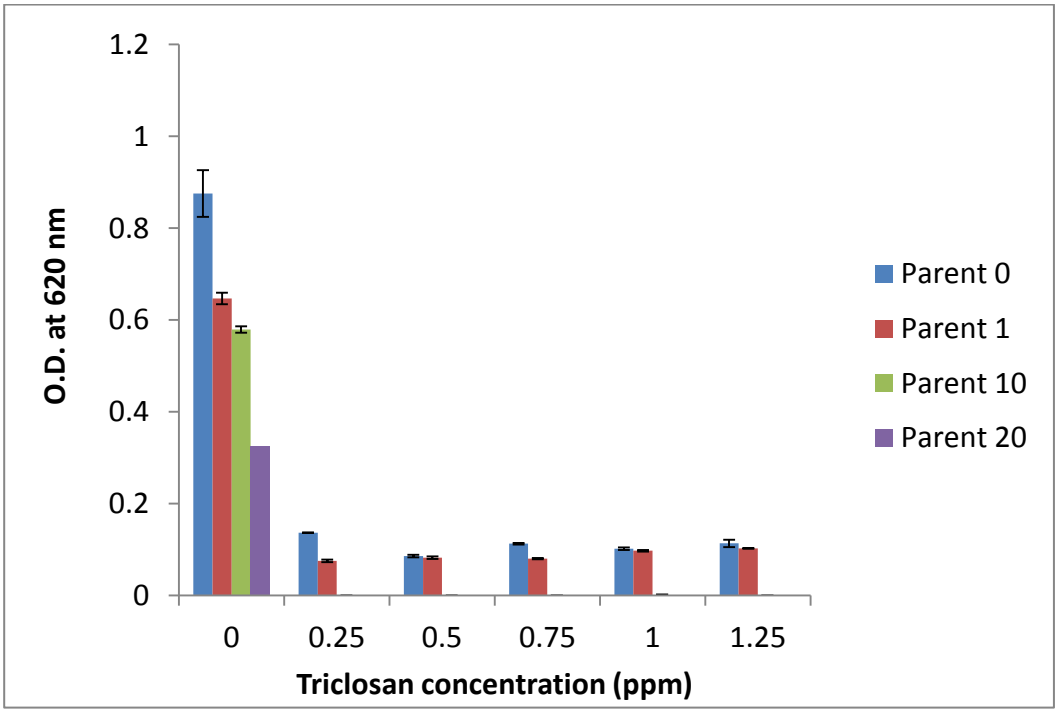


C

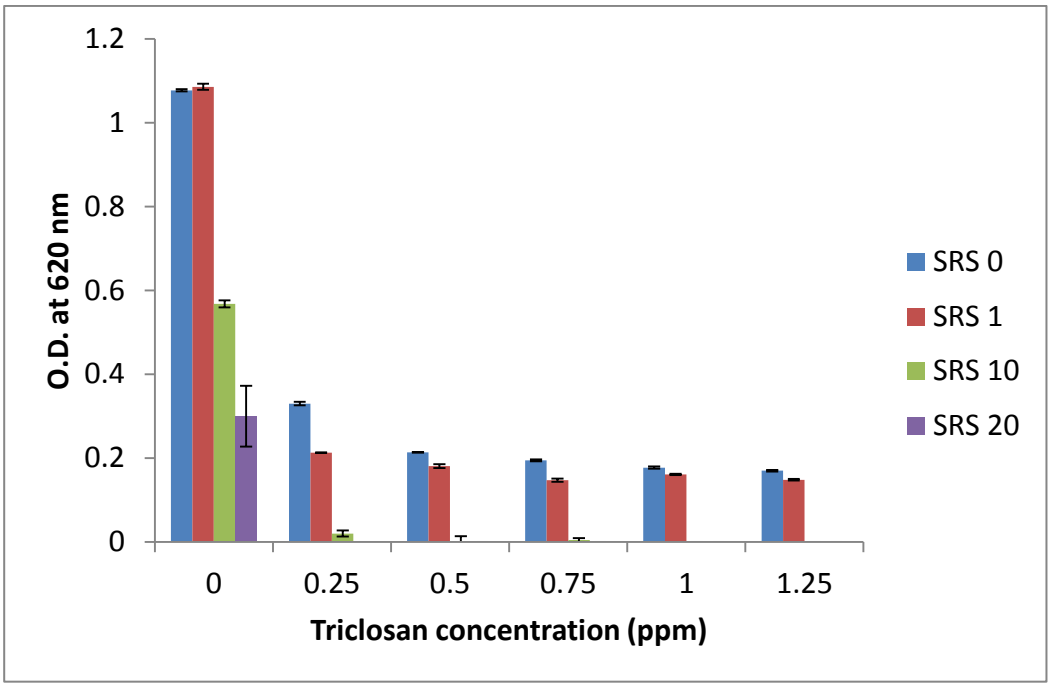
Figure 16: The O.D. for the parent and SRS strains grown in 0, 1, 10, and 20 mM of zinc and increasing DTAC concentrations overnight are shown. **A** shows results of the parent and SRS strains in a single graph. **B** shows only the parent. **C** shows only the SRS strain. Bars represent SEM.


1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 620 nm
0
0.25
0.5
0.75
1
1.25
Triclosan concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20
SRS 0
SRS 1
SRS 10
SRS 20

A


1.2
1
0.8
0.6
0.4
0.2
0
O.D. at 620 nm
0
0.25
0.5
0.75
1
1.25
Triclosan concentration (ppm)
Parent 0
Parent 1
Parent 10
Parent 20

B



C

Figure 17: The O.D. for the parent and SRS strains grown in 0, 1, 10, and 20 mM of zinc and increasing triclosan concentrations overnight are shown. **A** shows results of the parent and SRS strains in a single graph. **B** shows only the parent. **C** shows only the SRS strain. Bars represent SEM.

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