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PRESENCE OF THE *tet*A GENE IN BACTERIA FROM SOIL IRRIGATED WITH GREYWATER CONTAINING TRICLOSAN

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ABSTRACT

Products with triclosan are being used in great amounts. The overuse and misuse of triclosan products may be influencing bacteria to have cross-resistance to antibiotics, such as tetracycline. Hand soaps and toothpastes are common triclosan-laced products with residues that are discharged into household greywater. Environmental studies are needed to assess the effects of triclosan on microbial soil communities, when the soil is irrigated with greywater containing triclosan. This study helps fulfill missing environmental data, by determining if the presence of the *tet*A gene in bacteria in soil is influenced by triclosan. This study may be the first to examine the possible increase of antibiotic resistant microorganisms associated with the use of greywater used for irrigation. The *tet*A gene in soil bacteria was isolated, amplified by PCR, and detected by electrophoresis, in this study. There was a significant increase in the presence of the *tet*A gene in samples irrigated with greywater containing triclosan. This finding suggests that soil bacteria are succumbing to selective pressures and incorporating life sustaining *tet*A into their genome. Additional testing should be done with larger sample sizes and additional *tet* gene sequences.

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CHAPTER 1

INTRODUCTION

A combination of misrepresentation and propaganda has led society to misunderstand bacteria. Advertisements and commercials have misled consumers into thinking that every microbe is detrimental to their health. Consumers have turned to antimicrobials to annihilate any bacteria, regardless of their pathogenic tendencies. Antimicrobial products fill grocery shelves, and consumers are buying them, by the shopping cart load. Personal hygiene products, soaps, and cleaning supplies are only a few examples of antimicrobial products being used on a daily basis. "Consumers buy antibacterial products, thinking they are actually helping to protect themselves from deadly bacteria and viruses" (Levy, 2000). The obsession with antimicrobials may actually be negatively affecting society. The overuse of these products may be selecting for resistant bacteria to reproduce and flourish. These bacteria are increasingly antibiotic resistant. Certain chemicals that are used as antimicrobial agents may be perpetuating resistance.

Antibiotic Resistance as a Public Health Problem

Overuse and Overprescription of Antibiotics

The overuse and overprescription of antibiotics has become a public health problem, due to increased antibiotic resistance. Microorganisms are able to withstand antimicrobial medications at an alarming rate (WHO, 2011). Once effective, antibiotics and antivirals are increasingly becoming ineffective (WHO, 2011). The misuse of antimicrobial medications and products may be spreading antibiotic resistance. The overuse and overprescription of antibiotics may be a leading cause of antimicrobial resistance. Most patients are uneducated in antibiotic use, and expect doctors to prescribe antibiotics for most respiratory illnesses (WHO, 2001). "Prescriber's

perceptions regarding patient expectations and demands substantially influence prescribing practices" (WHO, 2001). Prescribers may feel obligated or fearful of negative clinical outcomes and prescribe unneeded antibiotics (WHO, 2001). Misperceptions contribute to the failure of patients from finishing a complete prescription of antibiotics, when the patient feels well again (WHO, 2001). There are no indications that these misunderstandings will be rectified any time soon.

Agriculture Use of Antibiotics

The uses of antibiotics in agriculture have become a public health issue and may be contributing to antibiotic resistance in the environment. There are two reasons large quantities of antibiotics enter the environment through agriculture purposes. One issue involves the use of antibiotics for growth enhancements. Large amounts of antimicrobials are regularly added as supplements to feed for animals and birds (WHO, 2001). "Animal husbandry is an important user of antibiotics and in some countries, tetracylines, are in subtherapeutic levels as feed additives for growth promotion in a range of animals including poultry, cattle, salmon, and catfish" (Schwarz et al, 1998). "This practice exposes large numbers of animals to frequently subtherapeutic concentrations of antimicrobials" (WHO, 2001). Animals exposed to low concentrations of antibiotics "frequently contain bacteria that are resistant to the antimicrobial used" (WHO, 2001). Antibiotics used for growth promotion for food producing animals are not regulated, because they are viewed as feed additives (WHO, 2001).

Antibiotic treatment, for illness, is another source of large amounts of antibiotics to enter into the environment, from agricultural purposes. Large numbers of animals, even whole herds, are treated with considerable quantities of antibiotics. This practice is initiated to avoid large stock

losses. Entire herds are often treated without diagnostic tests, and with broad-spectrum antibiotics, at any hint of illness (WHO, 2001). "Inadequate understanding about and training on appropriate usage guidelines and the effects of an appropriate antimicrobial use on resistance are common among farmers, veterinary prescribers and dispensers" (WHO, 2001). There are vast discrepancies in the prescribing of antibiotics for food producing animals (WHO, 2001). Antibiotics in animal waste may be a source of concern. There may be a risk of transferring resistant microorganisms to the human population through contaminated water and food products (WHO, 2001). Water runoff from agricultural animal food production may be a significant source of resistant microorganisms (Stachowiak et al, 2010). Regardless to as why animals are treated with large quantities of antibiotics, there may be an environmental impact that affects human health.

Biocides, Cross-Resistance, and Efflux Pumps

Resistant bacteria are more likely to reproduce and pass along their genetic code to other bacteria, increasing the total resistant microorganisms. Environments that have low, constant levels of a toxic substance are ideal habitats to allow resistant bacterial population numbers to increase. The populations may not only acquire resistance to a specific substance, but possibly multiple substances, due to cross-resistance. Environments with low levels of antimicrobials and biocides may be contributing to the antibiotic resistance due to cross-resistance. "Several studies have shown that the use of biocides may select for organisms resistant to these compounds with cross-resistance to antibiotics associated with efflux pumps" (Stachowiak et al, 2010). Efflux pumps allow bacteria to transport toxic substances, from their cells, so lethal levels of substrates do not accumulate in the organism (Webber and Piddock, 2003). Efflux pump genes are found on both chromosomes and plasmids, and can be easily acquired by other bacteria. Mutations can

cause over-expressions of efflux pumps (Webber and Piddock, 2003). A single efflux pump is capable of transporting multiple substrates, including various classes of antibiotics, such as disinfectants, biocides, and dyes (Webber and Piddock, 2003). A broad range of substances may be able to turn on some efflux pumps (Webber and Piddock, 2003). Bacteria, with specific over-expressed efflux pumps, are prone to survival in environments that have toxic substrates (Webber and Piddock, 2003). The combinations of biocides, cross-resistance, and efflux pumps have significantly increased the probability of antibiotic resistance as a public health concern. Environmental impact studies are critically needed in this area.

Impacts of Increased Antibiotic Resistance

"Resistance costs money, livelihoods and lives and threatens to undermine the effectiveness of health delivery programmes" (WHO, 2001). Resistant microorganisms are difficult to treat and often "fail to respond to standard treatment, resulting in prolonged illness and greater risk of death" (WHO, 2011). Mortality depends on proper clinical diagnosis and treatment of resistant bacteria. Patients with antibiotic resistance infections may have to undergo invasive medical procedures, treatments, and hospitalizations to recover from their illnesses. This can lead to substantial financial difficulties and lost productivity. If resistant infections spread within the human population, this may put a considerable burden on our medical industry and impact our economy. Multi-resistant tuberculosis (MDR-TB), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and gonorrhea are only a few examples of resistant microorganisms and illnesses affecting the human population around the world. With the continuation of the spread of these antibiotic resistant microorganisms, there may be no effective treatments available to treat these illnesses. "Antimicrobial resistance threatens to return the world to the pre-antibiotic era" (WHO, 2011).

Greywater

Greywater Use

Greywater is comprised of the used water from households, with the exception of sewage from toilets (Baker, Harrow, and Ritchey, 2010). Greywater can be between 50 to 80% of wastewater coming from individual households (Harrow et al, 2010). "The use of wastewater in agriculture is occurring more frequently because of water scarcity and population growth" (WHO, 2004). Greywater is commonly used for ornamental landscape irrigation, specifically for nonedible landscape plants (Waskom and Kallenberger, 2009). Global climate change and droughts have increased the need to reuse water. "The use of greywater for the irrigation of lawns, ornamental plants, and other landscape vegetation has become an accepted practice in the Southwest United States, the Middle East and the Australian dry lands" (Harrow et al, 2010). In the western United States, greywater reuse is becoming a popular topic due to water rights issues, and rainwater harvesting restrictions (Waskom and Kallenberger, 2009). In rural areas in the United States, greywater may be piped directly outside of households, directly onto the ground, due to the lack of adequate sewage treatment facilities in the vicinity. The use of greywater may be a potential human health issue, because of the potential for pathogens in it. "Research on the public health hazards of greywater use is limited, with no data indicating problems, or nonproblems for that matter" (Waskom and Kallenberger, 2009). The lack of data suggests a critical need for research in greywater use.

Possible Contaminants in Greywater

Besides the risk for microorganisms, other contaminants can be also found in greywater. Greywater has been found to have high concentrations of personal care products, residential

pharmaceuticals, and soaps (Harrow et al, 2010). "Triclosan is the most commonly used antibacterial agent in the United States with an estimated use of 0.6-10 million kg yr^{-1}" (Harrow et al, 2010). Triclosan is found in numerous products, on the United States market. Triclosan has been put into items such as clothing, toys, toothbrushes, rubber, hand soaps, toothpaste, deodorants, and laundry detergents. (U.S. EPA, 2008). A concentration of 0.1-0.3% of triclosan can typically be found in products (Liu et al., 2009). In this study, the personal care products containing triclosan is a key concern, because they may end up in greywater. "The use of greywater for irrigation, therefore, may result in the release of significant quantities of triclosan into the soil environment" (Harrow et al, 2010). Concentration levels of triclosan in greywater have been found in the range of $0.075 \mu g L^{-1}$–$16.6\ \mu g L^{-1}$ (Almqvist and Hanaeus, 2006). Terrestrial environments may be significantly impacted, with the release of low levels of triclosan in the use of greywater irrigation. There is a significant lack of data in this area, and research is needed to assess if antimicrobial resistance impacts can be seen with greywater irrigation containing triclosan.

TRICLOSAN

Triclosan is added to products to inhibit the growth of bacteria, mildew, and even fungi (U.S. EPA, 2010). Triclosan is also used as a pesticide, because of the wide range of bacteria that it impedes growth (U.S. EPA, 2010).

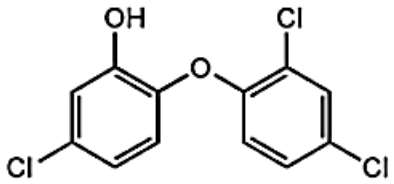
Physical and Chemical Properties of Triclosan:

"Triclosan is a chlorinated aromatic compound" (U.S. EPA, 2010). See Table 1 for physical and chemical properties.

Table 1 Physical and Chemical Properties of Triclosan

Property	Finding	Reference
Molecular Weight	289.54	(U.S.EPA, 2008)
Melting Point	56.5°C	(U.S.EPA, 2008)
Water Solubility	12mg/L	(Lyndall et al, 2010)
Dissociation Constant	pKa=8.14@20°C	(U.S.EPA, 2008)
Octanol-water Partition Constant	4.76@25°C	(Lyndall et al, 2010)
K_{oc}	9,200	(U.S.EPA, 2008)
Henry's Law Constant	$1.5X10^{-2}$ atm-m^3/mole	(U.S.EPA, 2008)

Chemical Structure: (U.S. EPA, 2008.)



Common Names: (U.S. EPA, 2008.) Triclosan
Irgasan

Chemical Names: 2, 4, 4'-trichloro-2'-hydroxydiphenyl ether (U.S. EPA, 2010)
5-chloro-2-(2, 4-dichlorophenoxy) phenol (U.S. EPA, 2008)

Empirical Formula: (U.S. EPA, 2008.) $C_{12}H_7C_{13}O_2$

CAS Registry Number: 3380-34-5

Environmental Fate of Triclosan

Triclosan is formed as white powder (U.S. EPA, 2008.) Triclosan has hydrophobic and ionizable characteristics (Lyndall et al., 2010). Triclosan is able to be biodegraded, and can also be photodegraded (Lyndall et al., 2010). Triclosan can degrade into 2,4-dichlorophenol (2,4-DCP) and 2,8-dichlorodibenzo-*p*-dioxin (2,8-DCDD), which are also susceptible to further

degradation, if conditions are favorable (Lyndall et al., 2010). "The dioxin photoproducts is a concern because chlorinated dioxins are known to be toxic and 2,4-DCP is an EPA priority pollutant" (U.S. FDA, 2005). Triclosan is chemically similar to the dioxin family, which may raise concerns. This similarity is alarming, because dioxins are distributed throughout the environment in low concentrations and can be bio-accumulated, increase risk of cancer, and cause hormone alterations in mammals (U.S. EPA, 2010). "Dioxins are of concern because of their highly toxic potential" (WHO, 2010). The methylation of triclosan has been observed in the bacterial methylation of triclosan, resulting in methyltriclosan (Lyndall et al., 2010). The methylation of triclosan changes a phenol to ether, causing methyltriclosan to be slightly more hydrophobic than regular triclosan (Lyndall et al., 2010). Although more research is needed to assess methyltriclosan, its properties suggest a high bioaccumulation potential. Alarmingly, triclosan reacts with chlorine in tap water, to form chloroform, 2,4-DCP and other chlorinated intermediates (U.S. FDA, 2005). The Department of Health and Human Services (DHHS) classified chloroform as a carcinogen (ATSDR, 1997). The Environmental Protection Agency allows 100µg/L of chloroform in drinking water (ATSDR, 1997). Laboratory studies show that triclosan containing soap and chlorinated water tap water could combine and form 15,000ng/L of chloroform after five minutes and 49,000ng/L within 120 minutes (U.S. FDA, 2005). "The authors conclude that the potential exists for substantial chloroform production to occur via daily household use of triclosan-containing products" (U.S. FDA, 2005).

Triclosan has a K_{oc} value of 9,200 and is expected to be immobile in soil (U.S. EPA, 2008). A Henry's Law constant of $1.5x10^{-7}$ atm-m^3/mole suggests that triclosan will not volatize from soil or water surfaces (U.S. EPA, 2008). In aquatic environments, triclosan may attach to suspended solids and has the potential to accumulate (U.S. EPA, 2010). When triclosan is within

sediments, degradation is slowed (U.S. FDA, 2005). As stated before, triclosan is photolytic and degrades in artificial light lab studies, creating 2,4-DCP (U.S. EPA, 2008). Aqueous photolysis of triclosan can take less than an hour in ideal abiotic conditions or up to ten days in lake water (U.S. EPA, 2008). Aerobic biodegradation has been found to be the most efficient way to degrade triclosan (U.S. EPA, 2010). In the atmosphere, triclosan has a half-life of approximately eight hours (U.S. EPA, 2008). Other studies suggest that triclosan is pH dependent for photolytic degradation (U.S. FDA, 2005). Triclosan may degrade quickly in summer months with a pH of 8.0, but not at a pH of 5.6 (U.S. FDA, 2005). The dynamic environment of large surface waters, such as lakes variability in sunlight, pH, season, and sedimentation could significantly increase triclosan's half-life (U.S. FDA, 2005). Computer models estimate that the triclosan half-life can vary from 2 to 2000 days, depending on the real world variables (U.S. FDA, 2005).

Environmental Toxicity of Triclosan

See Table 2. Toxicity of Various Organisms to Triclosan.

Table 2. Toxicity of Various Organisms to Triclosan

Organism	Toxicity	Reference
Green Algae	(EC_{50}) 0.0007mg/L	(U.S.EPA, 2008)
Blue-Green Algae	(EC_{25}) 0.00067mg/L	(U.S.EPA, 2008)
Water Flea	(EC_{50}) 0.39-0.42mg/L	(U.S.EPA, 2008)
Zebrafish	(IC_{50}) 220µg/L	(U.S.FDA, 2005)
Fathead Minnows	(LD_{50}) 260-440µg/L	(U.S.FDA, 2005)
Medaka	(IC_{50}) 400µg/L	(U.S.EPA, 2008)
Rainbow Trout	(LD_{50}) 0.26-0.288mg/L	(U.S.EPA, 2008)
Bobwhite Quail	(LC_{50}) 825mg/Kg	(U.S.EPA, 2008)
Rat (Acute Oral)	(LD_{50}) >5000mg/Kg	(U.S.EPA, 2008)
Rat (Acute Inhalation)	(LC_{50}) >0.15mg/L	(U.S.EPA, 2008)
Rabbit (Acute Dermal)	(LD_{50}) >9300mg/Kg	(U.S.EPA, 2008)

Triclosan is highly toxic to aquatic organisms, including algae, invertebrates, and fish. (See Table 2.) Green algae, Blue-Green algae, Water flea, Zebrafish, Rainbow Trout, Fathead minnows, and Medaka studies show only trace amounts of triclosan in aquatic environments may cause devastating results. These organisms are extremely important to the aquatic ecosystem. Population dynamics could be greatly affected by triclosan in the food chain. Detrimental levels of triclosan could affect food availability throughout the food chain. Triclosan is hydrophobic and insoluble in water. This is unfortunate for bottom-dwelling aquatic organisms, because triclosan would quickly leave the water column and concentrate in the sediment. Special consideration should be applied to allowing triclosan into aquatic environments. Studies for vertebrate organisms signify low to moderate toxicity levels (U.S. EPA, 2008). There is a significant lack of comparative acute toxicity studies with triclosan. Further testing involving various organisms is needed. Organisms such as guinea pigs, hamsters, cats, and monkeys would be beneficial studies to expand our understanding of triclosan.

Carcinogenicity

The EPA has classified triclosan as "Not Likely to be Carcinogenic to Humans" (U.S. EPA, 2008). This classification was based on outdated studies that were completed before the widespread use of personal care items containing triclosan in the United States. A more comprehensive assessment needs to be done using updated studies.

Behavioral and Endocrine Disruption

Behavioral and endocrine disruption may occur to a wide-range of vertebrates. Studies suggest that tadpoles and fish have behavioral changes that affect the way they feed and evade predators, in environments with triclosan (U.S. FDA, 2005). "In frogs, triclosan can disrupt

thyroid hormone-associated gene expression and induce changes in the thyroid hormone-mediated metamorphosis process" (Calafat et al., 2008). These studies suggest that a human's endocrine systems may also be affected by triclosan, in some way. "There is some evidence that triclosan disrupts thyroid hormone homeostasis and interacts with the androgen and estrogen receptors" (U.S. EPA, 2008). Triclosan has been found to interact with cytochrome P450-dependent enzymes, UDP-glucuronosyltransferases and the human pregnane X receptor; but the significance of these findings still needs to be established (Calafat et al., 2008). More studies are needed to fully assess triclosan's endocrine effects on humans.

Regulatory Ambiguity

The use of triclosan, in the last few years, has overwhelmed the regulatory systems of the FDA and EPA. Triclosan once was added to only a few products, but it now has increased in popularity. The FDA regulates the consumer products that contain triclosan, and the EPA is responsible for triclosan pesticide products (U.S. EPA, 2010). The "FDA does not monitor or regulate biocides in the environment" and bases regulatory conclusions on data compiled in the EPA's risk assessment reports (U.S. FDA, 2005). The EPA risk assessment reports commonly minimize the evaluation of products and applications that are not EPA-registered products. Although this may seem insignificant, exposure limits are being set on a small number of studies. The EPA seems to be setting dietary exposure limits of triclosan, for the entire United States, based on one study, completed in Southern California (U.S. EPA, 2010). The dietary exposure to triclosan is also based on the dietary exposure of EPA regulated products, such as adhesives, pulp and paper, ice-making equipment, which are not FDA regulated products that may be ingested (U.S. EPA, 2010). Information on triclosan pesticide residual levels left on crops, and the dietary effects of FDA products, such as toothpaste, are left out of the exposure limit

analysis. Additional studies need to be accomplished to have a true comprehensive exposure limits. Therefore, there may be a gap in gathered and analyzed data due to the multiple governmental agencies trying to regulate triclosan.

Triclosan in the United States Population

One of the first comprehensive studies that incorporated a large number of participants was the National Health and Nutrition Examination Survey (NHANES) triclosan study of 2003-2004 (Calafat et al., 2008). Approximately 75% of the 2,517 individuals tested had detectible levels of triclosan concentrated in their urine (Calafat et al., 2008). Higher triclosan levels were found in individuals in their 30's and participants with high household incomes (Calafat et al., 2008). Age and socioeconomic status may influence the amount of triclosan exposure to humans (Calafat et al., 2008). The EPA has used this study as the basis of the aggregate risk data used in the risk assessment determination of triclosan (U.S. EPA, 2008). "This study suggests that the main source of triclosan exposure to humans is by personal care products" (Calafat et al., 2008). These findings suggest that triclosan is prevalent in our environment, including personal care products and possibly concentrated in our food and water supplies.

Triclosan in Wastewater

Personal care products and other chemicals are not specifically treated in wastewater treatment. Consequently, minute amounts of triclosan can potentially be left discharged into the environment. European studies find that triclosan can be found in low amounts, even after wastewater treatment and the biodegradation in surface waters and sediments (U.S. FDA, 2005). Triclosan in wastewater influents can have concentrations ranging from 62 to 21,900 ng/L (U.S. FDA, 2005). The total amount of triclosan removed from influent is dependent on what

wastewater method is used (U.S. FDA, 2005). The activated sludge removal of triclosan has been shown to be the most effective with 95% efficiency, other methods, such as trickling filter, are not as efficient (U.S. FDA, 2005). With the use of current wastewater treatment plant methods in the United States, approximately 58 to 97% of triclosan can be removed (U.S. FDA, 2005). Triclosan in effluents from wastewater treatment plants, in the United States, have amounts ranging from 10-2700 ng/L (U.S. FDA, 2005). Whatever method that is used, a substantial amount of triclosan may be released into the environment. A study from the United States Geological Survey collected stream samples from 30 states and of the 95 compounds studied; triclosan was the sixth most detected compound (U.S. FDA, 2005). Not only did this study cover a wide-span of the United States streams, but the streams were "considered susceptible to contamination from various wastewater sources" (U.S. FDA, 2005). Triclosan was found in approximately 60% of the samples collected (U.S. FDA, 2005). This is a comprehensive study suggesting that triclosan contamination of surface water may be contributed to wastewater sources (U.S. FDA, 2005). "The effluents of wastewater treatment plants have been shown to be significant pathways for hazardous substances to enter an aquatic environment" (Almqvist, and Hanaeus, 2006).

Cross-Resistance of Triclosan and Tetracycline

Cross-resistance to triclosan and tetracycline is an enormous concern. Both compounds are structurally and chemically similar. Phenolic ringed chemicals have been found to lower antibiotic resistance by escalating the over-expression of the efflux pumps (Randall et al., 2004). Resistance caused by phenolic rings is found in both biocides and antibiotics (Randall et al., 2004). Triclosan and tetracycline target cells in similar manners. Triclosan works as a biocide, by blocking lipid synthesis in bacteria (McMurry et al., 1998). Tetracycline works as an

antibiotic, by targeting protein synthesis in bacteria (Griffin et al., 2010). The structural and chemical similarities do suggest that cross-resistance is probable.

Cause for Concern

Triclosan has become readily available in the environment. A U.S. Geological Survey found that "triclosan was the sixth most common chemical found in streams" (U.S. FDA, 2005). Triclosan may be found in constant, low levels throughout the environment. Resistant bacteria are being selected by the overuse or misuse of biocides and antibiotics in households, hospitals, agricultural areas, and effluent from wastewater treatment plants (Galvin et al, 2010). The resistant, mutant bacteria are then released into the natural environment and share mutant genes. In environments with low levels of a toxic substance, like triclosan, mutant bacteria will thrive. Bacteria without over-expressed efflux mutations will die off. The selective pressure of the low levels of triclosan is creating an important issue that needs action and research immediately. By commonly using triclosan personal care products, environmental conditions are primed for selection for triclosan resistance and antibiotic resistance.

***tet*A Gene and This Study**

The *tet*A gene is a protein encoding for the over-expression of single component tetracycline efflux pumps (de Cristo′bal et al., 2006). There are approximately eight classes of tetracycline efflux genes, known as the *tet* family (Speer et al., 1992). Classes A to E are located on plasmids and use tetracycline efflux as a resistance mechanism (Speer et al., 1992). There is a correlation between tetracycline resistance and triclosan resistance. "The key genes in *E.coli* involved in this form of resistance are a regulatory gene, *marA*, and an efflux gene complex *acrAB*" (Levy, 2000). "Triclosan resistance occurs through multidrug efflux pumps, such as *AcrAB* in *E.coli*

and the *Mex* proteins in *P. aeruginosa"* (Levy, 2000). It has been clinically proven "that the overexpression of the *AcrAB* pump produced not only antibiotic resistance, but also cross-resistance to triclosan" (Levy, 2000). Bacteria "that overproduce the *marA* or *SoxS* protein (which is a *marA* homologue) upregulate the *AcrAB* multidrug efflux pump which pumps out pine oils, organic solvents, triclosan, quaternary ammonium compounds, chloroxanol, and chlorhexidine" (Levy, 2000). The clinical studies suggest that there could be a significant impact to the environment and possible emerging resistance in ecological environments (Levy, 2000). Research is lacking in the impact of triclosan in environmental microorganisms.

This study focuses specifically on *tet*A. Looking for the *tet*A gene sequence helps determine if over-expression of tetracycline efflux pumps is present in the microbial DNA. This study will determine if there is an effect of triclosan on soil bacteria. This will be accomplished by assessing the presence of *tet*A in soil with frequent, low concentrations of triclosan. Real environmental conditions will be simulated in order to assess how soil bacteria react to the exposure of greywater from residential households. This study may be the first to examine the possible increase of antibiotic resistant microorganisms associated with the use of greywater for irrigation.

CHAPTER 2

MATERIALS AND METHODS

Pots and Greywater Solution

This experiment was done in two time periods. Time period one was started in November 2009 and concluded in February 2010. Time period two began in May 2010 and concluded in October 2010. Fifty-four replicate plastic pots were filled with a soil mixture, consisting of potting soil, sand, and soil from a Pennsylvania farm. Approximately 100g of the soil mixture was used in each pot. The pots were used to simulate real environmental soil conditions. The pots were divided into two groups. One group was irrigated weekly with a greywater mixture (control group), while the other group was watered with greywater with triclosan. The greywater composition was synthesized according to Baker's composition of solutions (Baker, Harrow and Ritchey, 2010). The triclosan greywater had a triclosan concentration of 2.0μg mL^{-1} (this concentration was selected to model triclosan amounts that would be present in greywater in a residential household). Each pot was irrigated weekly with 10mLs of the designated solution. Samples were taken on various sample dates, after watering.

Cultures and Primers

A *tet*A negative *Escherichia coli* strain B culture (Presque Isle Cultures) was obtained along with a *tet*A positive *Escherichia coli* culture (ATCC 47037). Both cultures were analyzed to assess tetracycline resistance and plated on 0.1X Trypticase Soy Agar (Difco) supplemented with 30μg mL^{-1} tetracycline (Sigma Chemical Corporation). Plasmids were prepared using Mo-Bio Plasmid Protocol for the Ultraclean Mini Plasmid Prep Kit. (Mo-Bio, 2009).

Primer sequences for *tet*A used EMBL accession number X00006, Forward 1432-1455 and Reverse 1989-2008 (Randall et al., 2004).

Methods

DNA in the soil was extracted using Mo-Bio Powersoil DNA Isolation Kit Protocol (Mo-Bio, 2010). DNA concentrations were found using NanoVue Plus Spectrophotometer. PCR was done in a Bio-Rad MyCycler Thermal Cycler using Guillaume's PCR procedure for *tet*A (Guillaume et al., 2000). Electrophoresis was done using a 1.5%-1.8% agarose gel, to detect PCR results.

CHAPTER 3

RESULTS

In this experiment there were 10 soil samples with *tet*A presence, which were irrigated with greywater only. There were 17 greywater only soil samples absent of the *tet*A gene. Greywater with triclosan irrigated soil had 20 soil samples with *tet*A present. Greywater with triclosan had 7 soil samples with *tet*A absent. (See Table 3.) A Chi Square analysis was done with a result of 6.075 with 1 degree of freedom. The two tailed p value equaled 0.0137. (See Figure 1.)

Table 3. Experimental tetA Results

Irrigation Type	***tet*A Results**	
	Present	**Absent**
Greywater	10	17
Greywater with Triclosan	20	7
Total	30	24

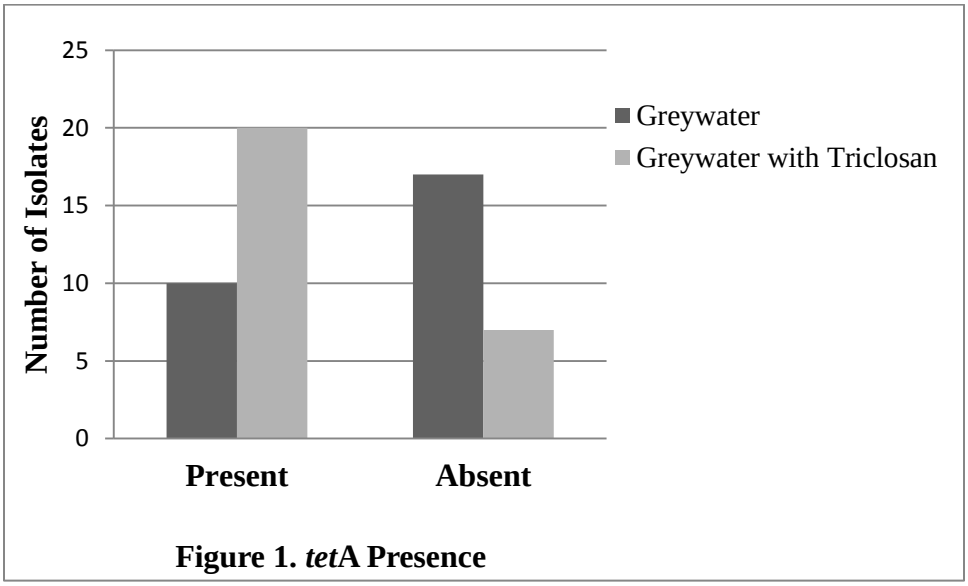


Figure 1. *tet*A Presence

Timeline data was analyzed for any variation in greywater and greywater with triclosan irrigated samples. Refer to Table 4 and Figure 2.

Table 4. Experimental Timeline *tet*A Present Data

Week Sampled	% *tet*A Present	% *tet*A Absent	∑ *tet*A Present
1	40	60	2/5
3	45	55	5/11
7	48	52	10/21
9	48	52	15/31
14	48	51	20/41
25	56	44	15/30

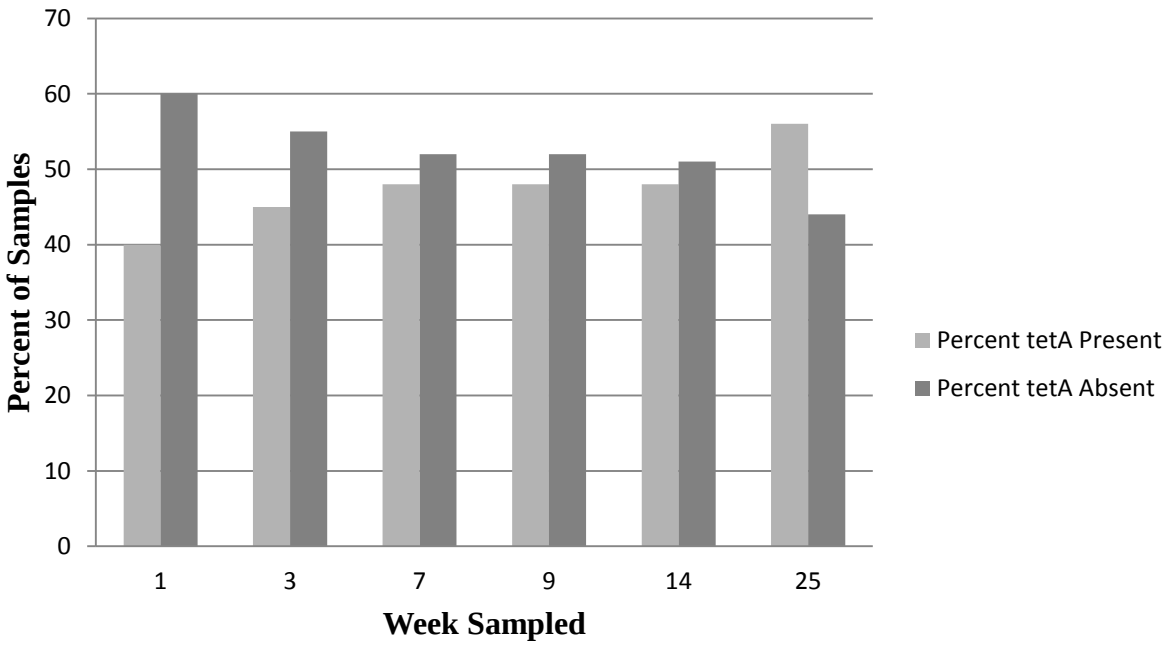


Figure 2. Timeline of Sample Results

A scatter plot of the percent *tet*A presence and absence was done to see if there was a trend in data. A line of best fit was also done. See Figure 3.

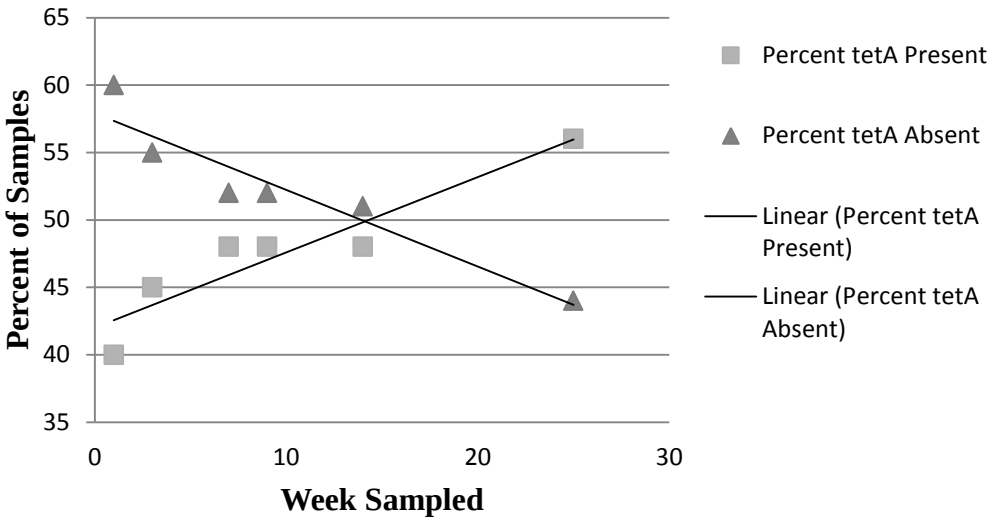


Figure 3: Timeline of Sample Results Line of Best Fit

CHAPTER 4

DISCUSSION

The data suggests that there is significance between greywater with triclosan and the greywater only soil samples. A p value of 0.0137 confirms significance. As Figure 1 shows, the *tet*A gene was found more often in the triclosan treated greywater samples. The timeline data suggests that, the presence of *tet*A significantly increases over time. Figure 2 shows an increase in *tet*A presence throughout the experiment, and significantly increased by week 25. The *tet*A absence is high in the beginning of the experiment and decreases throughout. Figure 3 shows a trend in data. The presence of *tet*A in soil samples increases through the experiment, and the absence of *tet*A decreases throughout the experiment. This is shown in Figure 3 with the line of best fit for both data sets. Further testing is needed to assess the specific timeline of the increase in *tet*A presence. These results suggest that triclosan may be able to select for tetracycline resistant bacteria in environmental conditions, even at low concentrations. Multiple types of antibiotic resistance might also be selected for. This could be catastrophic by reducing effective antibiotic treatments for bacterial caused illnesses. It would be advisable to stop using triclosan products, in healthy household settings, and retain triclosan products for only clinical purposes. Alternately, greywater with triclosan should not be used for irrigation. Wastewater treatment plants would also need to be engineered to minimize the amount of triclosan being discharged into the environment. Additional research focusing on triclosan effects on soil microbes is needed. Future testing, to add to this study, should involve a larger sample size and using additional *tet* family primers. Further studies should concentrate on timeline data, isolating specific bacteria containing the *tet*A gene, and quantifying the amount of *tet*A in the samples. These studies would increase our understanding of the effects triclosan irrigation has on soil

bacteria and terrestrial ecosystems.

CHAPTER 5

CONCLUTIONS

Triclosan has been integrated into common household products and is becoming a growing environmental concern. It has been determined that triclosan may be a significant selective pressure, in real environmental conditions, for tetracycline resistance in soil microorganisms. The use of triclosan, in healthy households, may be pressuring tetracycline resistant bacteria to become plentiful. Commonly used antibiotic's efficiencies can decrease with the population rise of antibiotic resistant bacteria. Additional testing should be done using a larger sample size and additional *tet* family primers. Timeline data collection, isolating, identifying, and quantifying bacteria with the *tet*A gene would add greatly to our understanding of using triclosan containing greywater.

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