GREYWATER REUSE: IMPACT OF TRICLOSAN ON SOIL MICROORGANISMS

A Thesis

by

Danielle I. Harrow

Program Advisor: Katherine Baker
Associate Professor of Environmental Microbiology
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School of Science, Engineering and Technology

GREYWATER REUSE: IMPACT OF TRICLOSAN ON SOIL MICROORGANISMS

A Thesis in
Environmental Pollution Control

by
Danielle I. Harrow

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ABSTRACT

The use of greywater for irrigation results in the direct discharge of trace quantities of personal care products and antimicrobial agents to the environment. The presence of antibacterial compounds (e.g. triclosan) in greywater raises concerns regarding potential impacts of these materials on the environment and human health. Our research examined the impact of triclosan on soil microbial communities using soil filled pots irrigated with greywater (synthetic) only or greywater with triclosan. Functional diversity of the heterotrophic microbial community was assayed. Soil samples were cultured for viable heterotrophic bacteria and triclosan-resistant heterotrophic bacteria in the two treatments. Isolates were evaluated for resistance to multiple antibiotics and used to quantify tetracycline resistance genes. Under constant exposure, the community structure, showed two very distinct heterotrophic assemblages between soils treated with triclosan and the control soil. There were statistically significant increases in the number of heterotrophic organisms resistant to triclosan when the two soils were compared. The frequency of the tet a gene increased significantly in subsamples irrigated with greywater with triclosan as well as the proportion of bacterial isolates resistant to multiple antibiotics in these soil samples. Our results indicate that triclosan in greywater can have significant impacts on soil microbes. This in turn can affect the types of available nutrients within the soil. While antibacterial products may be present in trace concentrations in greywater, repeated exposure to soil organisms may be selecting for bacteria resistant to multiple types of antibiotics.
Therefore, our results indicate that greywater should be treated to remove antibacterial agents before its use in lawn irrigation. Alternatively, the use of antibacterial containing products should be significantly reduced.
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Chapter 1

Introduction

The term greywater is defined as all the wastewater from a household including the laundry, bathtub, and bathroom sink discharge, but excluding any toilet water or, in most places, water from kitchen sinks (Nelson et al, 2011; Wiel-Shafian et al, 2006; Travis et al, 2010; Gross et al, 2007). Because of its non-potable nature, but relatively high quality, greywater has potential for reuse in many different areas. These areas include, but are not limited to, irrigation and laundry (Hocaoglu et al, 2010; Al-Jayyousi, 2003; Revitt et al, 2011; Hernández-Leal et al, 2011). Reuse of non-potable water is very promising when considering the amount of water generated by one household and the amount of available nutrients that can be found in greywater.

The ranges of nutrient concentrations like those found by Hocaoglu et al (2010) demonstrate that greywater composition is dependent upon the household and the products used. In the parts of the world where greywater is not being utilized, the greywater is combined with sewage and kitchen water (Blackwater), sent to the local wastewater treatment facility and treated as domestic sewage (Al-Jayyousi, 2003; Revitt et al, 2011; Hernández-Leal et al, 2011). Greywater reuse also would allow for a lower treatment load for the treatment plant, possibly less piping and a decrease in the use of fresh potable water, limiting its use to only when necessary.
Given the heavy surfactant concentrations in GW, there is some concern as to how this will affect soil properties. Weil-Shafman et al (2006) explored this further and found that the straight application of surfactant rich greywater can result in a water repelling soil. Nutrient and surfactant concentrations can be accounted for by simply treating greywater, but a new issue arises when dealing with compounds that are not easily removed, for instance any pharmaceuticals and personal care products (PPCPs).

The PPCP focused on in this paper is the antimicrobial compound triclosan. Known by many aliases, 5-cloro-2- (2,4 dichlorophenoxy) phenol, 2-4-4’ trichloro-2’ hydroxy diphenyl ether or Irgasan (EPA Red Report, Kookana et al, 2011; Bhagara and Leonard, 1996), triclosan is its most commonly used name. Triclosan, once dissolved, is one of the most versatile compounds around. On the market since the late 1960s, triclosan has been used in countless products for two reasons. The first and main one would be for antimicrobial purposes. The second use of triclosan relates back to the first, as a preservative. Triclosan is an antibacterial agent that is added into over 2300 registered products including detergents, dish soap, shampoos, lotions, any plastics used for
containers and such. Triclosan works by inhibiting fatty acid production as well as other macromolecules, making the microorganism incapable of carrying out basic processes (Dann et al., 2011; Fang et al., 2010; Bhargava and Leonard, 1996; McMurphy et al., 1998; Levy 1999). In some instances, triclosan is used as a fungicide or preservative, for instance in adhesives, fabric, rubber, latex paints and plastics. But for most cases triclosan is used as an antimicrobial agent in products such as hand soaps detergents and shampoos at concentrations ranging from 0.1- 0.3 % total weight (Fang et al., 2010; Allmyr et al., 2009). In the instances of triclosan being used as a preservative a possible explanation concerning the mechanism would be protecting the product from bacterial degradation. When used against gram positive bacteria triclosan has a minimum inhibitory concentration (MIC) ranging from 0.1-100 ppm and 0.01-1000 ppm for gram-negative bacteria (Bharagava et al., 1996).

This paper’s concern with triclosan is its affects on soil microbes. Soil microbial communities are essential to all ecosystems. Any damage to this structure would be detrimental to all life. Soil microbes are responsible for the fixation of nitrogen gas into a usable form for plant life and in turn animals. Without soil microbes there are no available minerals or metals like phosphorus or iron. All of the inorganic stores would remain contained within rocks and dead organisms. Some concerns with soil microbes are the development of antibiotic resistance and the potential change in the soil chemistry. Antibiotic resistance is the ability of a microorganism to survive in the
presence of a killing agent (Levy et al, 1999; Levy, 2001; Schweizer, 2001; Dann and Hontela, 2011; McMurry et al, 1998).

Through constant exposure due to overuse, many antibiotics and antimicrobial agents have lost their effectiveness by killing the microbes that are intolerant and leaving behind the tolerant microbes. As the tolerant microorganisms survive and multiply the probability of becoming infected with a resistant organism increases. This increased probability causes the most concern; limiting the number of defenses our health care system possesses to fight off infections. In addition, these resistant microbes can come into contact with non-resistant microbes and undergo horizontal gene transfer, passing on plasmids that may code for antibiotic resistance, for example an efflux pump, to another microorganism. An efflux pump is a transmembrane protein that protects by pumping out the unwanted materials, namely antibiotics, which have entered the cell. It has been proposed that efflux pumps are involved in triclosan resistivity (Levy, 2001; McMurry et al, 1998).

If triclosan resistance is achieved by efflux pump a connection between triclosan and antibiotic resistance could easily be drawn; a constant exposure to triclosan via greywater irrigation would cause a selection in soil microbes for only those that can survive in the presence of triclosan. The constant exposure to triclosan will also put additional stress on the microbes to produce more pumps that can pump out the unwanted materials. This
results in the over expression of efflux pumps which were already capable of pumping out antimicrobial substances.

Due to the high usage in a wide variety of personal care products, triclosan has been found in effluent sewage water from wastewater treatment plant at concentrations ranging from 0.01-0.65 µg L\(^{-1}\) and 0.05-2.3 µg L\(^{-1}\) in US streams (Veldhoen et al., 2006; Kolpin et al., 2002). The entrance and persistence within the environment is another source of concern in regards to triclosan. With triclosan’s structure and shape, it is comparable with naturally occurring hormones, for instance triiodothyronine, a thyroid regulating hormone and other steroidal hormones (Veldhoen et al., 2006; Fang et al. 2010; Ishibashi et al., 2004; Yu et al., 2011). The similarities with naturally occurring hormones put triclosan within the category of endocrine disrupters. Its lipophilic characteristics give it the potential to bioaccumulate within different species (Clayborn et al., 2010; Coogan et al., 2007; Fang et al., 2010; Kookana et al., 2011; Krishnan et al., 2010). The toxicity towards bacteria and the endocrine disrupting capabilities makes triclosan a compound with a potential to do physical damage towards large animals and humans.

In this experiment, we looked at the effects of irrigation with greywater on microbial diversity and heterotrophic populations. Is there a distinct shift in the types of food
sources being metabolized? This shift would signal a shift in the microbial communities. We also looked at antibiotic sensitivity with isolated colonies from both a control group (greywater only) and an experimental group (greywater with triclosan). Does exposure to triclosan, in concentrations mimicking effluent wastewater, have any effects on isolated soil colonies' survivorship with commonly used antibiotics?
Chapter 2

Methods and Materials

All methods used are as described in Harrow et al. (2010), Harrow and Baker (2010) and Harrow et al. (2011).
Chapter 3

Impact of Triclosan in Greywater on Antibiotic-Resistance in Soil Microbial Communities

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The utilization of greywater (GW) for irrigation and other non-potable uses has been proposed as a method to conserve water resources. These uses would result in the direct discharge of greywater to the environment with minimal treatment. The presence of antibacterial compounds in greywater raises concerns regarding potential impacts of these materials on the environment and human health. Our research examined the possible modification of microbial communities within the soil due to the presence of a commonly used antibacterial agent, triclosan in greywater. Along with community structure, we also looked at any selection for antibiotic resistance in bacteria associated with constant exposure to triclosan.

We examined the impact of triclosan on soil microbial communities using soil filled columns irrigated with solutions of known composition. Two types of solutions (treatments) were examined greywater (synthetic) only and greywater with triclosan. The solutions were applied to the soil columns on a weekly basis. And the effluent was collected from each column and cultured onto plates to determine the number of heterotrophic bacteria present. Isolates were taken from the plates to determine antibiotic sensitivity. Finally, functional diversity was measured by assessing substrate utilization using BIOLOG EcoPlates. Our findings show that under constant exposure, the community structure did, in fact, change showing two very distinct heterotrophic populations between those that were treated with triclosan and those that were not. It was also seen that resistance to the four tested antibiotics (ampicillin, chloramphenicol, streptomycin and tetracycline) increased with higher numbers of resistant isolates associated with the columns receiving triclosan.

Our results indicate that triclosan in greywater can have significant impacts on soil microbes. The change in microbial community structure could influence microbial soil processes such as nutrient cycling. Furthermore, while the antibacterial products may be present in very minute concentrations, their constant presence may be selecting for bacteria that are resistant to multiple types of antibiotics, thus making it harder to treat infections. Therefore, our results indicate that greywater should be treated to remove antibacterial agents before its use in lawn irrigation. Alternatively, the use of antibacterial containing products should be significantly reduced in the US.
INTRODUCTION

The use of greywater (GW) 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 (Christova-Boal et al, 1996; AlJayyous, 2004). With increasing awareness of the need for water conservation and reuse, GW irrigation is being used in other areas of the U.S. as well. Data collected by the Soap and Detergent Association (NPD Group, 1999) indicated that 7% of U.S. households were reusing GW. This number has no doubt increased since the time of the NPD study. While GW irrigation is practiced primarily in arid regions of the US, changing climate patterns and increased water demand associated with urbanization will likely make water reuse more important in temperate regions.

Domestic GW differs in composition from typical domestic wastewater. Specifically, GW is notable for the high concentration of soaps, detergents and antimicrobials found in it compared to domestic wastewater. While not of high enough quality for direct use, its better quality makes this water amenable for on-site treatment and non-potable reuse such as irrigation and toilet flushing.

GW irrigation has the potential to influence soil chemical and biological composition with subsequent impacts on plant growth and groundwater quality. Elevated salinity, resulting from the presence of cleaning salts (i.e. NaEDTA) in GW has the potential to limit irrigation to salt tolerant plants as well as to adversely impact soil properties (i.e. soil dispersion) (Bachmann et al., 2000). Oils and grease can alter the microbial community present while surfactants can change the soil permeability influencing the availability of water to plants. (Gross et al., 2005; ANZECC, 1992; Gross et al., 2007). Inorganic substances such as phosphorous and nitrogen provide essential macronutrients to plants; however excessive concentrations of these compounds or alterations in soil properties may allow them to leach into groundwater where some of these compounds (e.g. nitrite) may pose a health hazard. The introduction of particulate and organic matter such as surfactants can alter soil permeability (Wallach, 2005; WielShafran, et al., 2005). Detergents and soaps, the main sources of boron and surfactants found in domestic effluents, are more concentrated in GW because the toilet stream is excluded. While boron is an essential micronutrient for plants, excessive amounts are phytotoxic.

In addition to the possible introduction of chemical contaminants into the environment, one of the largest obstacles to the reuse of GW is concern about the possible impact of GW on microorganisms. Activities such as hand washing and washing of diaper may introduce bacteria into GW (Gross et al., 2007). Observations regarding bacteria in GW vary greatly between different studies. While some of these differences can be attributed to different detection methods, a major source is the variability found in the composition of GW which reflects the number and age of residents, frequency of bathing and laundering, and, in cases where kitchen waste is included, the eating habits of those living in the household (Gross et al., 2005).
The focus of most microbiological analysis of GW has been on fecal pollution and enteric pathogens. Reports of total coliforms in GW range from as low as $10^1$ to $10^7$ CFU (100 mL)$^{-1}$ (Rose et al., 1991; Casanova et al., 2001). In addition to total coliform organisms, enteric pathogens such as Salmonella, Shigella, and Poliovirus Type 1 have all been reported to be present in GW and concerns about the potential for re-growth or persistence of pathogenic microorganisms have been raised (Rose et al., 1991). Environmental strains of Legionella were observed in three raw GW samples analyzed for pathogens, as were Cryptosporidium, Giardia, and fecal enterococci (Birks et al., 2004). Since wash water is a major component of GW, several opportunistic pathogens associated with human skin—Staphylococcus aureus and Pseudomonas aeruginosa—also may be present in addition to enteric organisms.

While the bulk of the concern about microorganisms in greywater has focused on the presence of pathogenic microorganisms, much less consideration has been given to the possible impacts of GW on naturally occurring microorganisms. Several recent studies have proposed a link between triclosan resistance in bacteria and resistance to common antibiotics (McMurry et al., 1998; Levy, 2001; White and McDermott, 2001; Levy, 2002), although other studies have questioned the existence of such a linkage (Russell, 2003; Gilbert and McBain, 2001; Russell, 2004, Ledder et al. 2006). Triclosan (2,3,3-trichloro-2-hydroxyeiphenylether; TCS) is the most commonly used antibacterial agent in the United States with an estimate of 170,000—970,000 kg yr$^{-1}$ of this compound used (Halden and Paull, 2005). The presence of triclosan, and the related biocide triclocarb in wastewater and biosolids is well documented (Bester 2005) The use of GW for irrigation results in the release of significant quantities of triclosan into the soil environment and therefore, may result in an increase in the presence of antibiotic resistant microorganisms. Furthermore, the addition of biocides to the environment has the potential to influence both the size and composition of the normal microbial community present. The purpose of this research is to determine the short-term impacts of triclosan in GW on the microbial community in soils irrigated with GW.

METHODS

Soil Columns

Replicate soil columns were made by packing plexiglass tubes with a mixture of sand and commercial potting soil (1:2). The columns were divided into treatment groups on the basis of the solution used for routine irrigation as summarized in Table 1. On a weekly basis, each column was watered with 250 mL of the appropriate irrigation solution. The leachate water that flowed through the columns was collected in sterile 1 L bottles and used for microbiological analysis.
Table 1: Composition of Solutions Used for Irrigation of Soil Columns

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Greywater (GW) (per L tap water)</th>
<th>Greywater plus Triclosan GW Triclosan (per L tap water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shampoo</td>
<td>Johnson’s Baby Shampoo; Johnson and Johnson</td>
<td>0.80 mL</td>
<td>0.80 mL</td>
</tr>
<tr>
<td>Laundry Detergent</td>
<td>Seventh Generation: free and clear of perfumes and dyes</td>
<td>0.064 mL</td>
<td>0.064 mL</td>
</tr>
<tr>
<td>Cooking Oil</td>
<td>Crisco All Natural Pure Vegetable Oil</td>
<td>0.01 mL</td>
<td>0.01 mL</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Sigma 72779</td>
<td>10 mL ethanol with no triclosan was added</td>
<td>20 mg (dissolved in 10 mL ethanol)</td>
</tr>
</tbody>
</table>

Microbiological Analysis

Each sample was analyzed for two different populations of microorganisms (Table 2). Heterotrophic microorganisms, all bacteria capable of growth on complex organic substrates, were enumerated using spread plating onto 0.1X Trypticase Soy Agar (TSA; Difco). In addition, triclosan-resistant heterotrophic microorganisms were enumerated by spread plating onto 0.1X Trypticase Soy Agar supplemented with 20 mg L\(^{-1}\) (20 \(\mu\)g mL\(^{-1}\)) triclosan (Sigma).

Microbial numbers were log transformed before statistical analysis (Barnett, 2004). Comparisons of microbial numbers were made using ANOVA with post-hoc comparisons of the means using the Bonferroni test. All statistical analysis were performed using Prism 6.0 (GraphPad Software).

Table 2: Microbial Populations in this Study

<table>
<thead>
<tr>
<th>Population</th>
<th>Microorganism</th>
<th>Treatment</th>
<th>Culture and Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>Heterotrophic</td>
<td>Greywater</td>
<td>0.1X TSA</td>
</tr>
<tr>
<td>HT</td>
<td>Heterotrophic</td>
<td>Greywater with Triclosan</td>
<td>0.1X TSA</td>
</tr>
<tr>
<td>TRH</td>
<td>Triclosan-resistant</td>
<td>Greywater</td>
<td>0.1X TSA supplemented with 20(\mu)g mL(^{-1}) triclosan</td>
</tr>
<tr>
<td>TRHT</td>
<td>Triclosan-resistant</td>
<td>Greywater with Triclosan</td>
<td>0.1X TSA supplemented with 20(\mu)g mL(^{-1}) triclosan</td>
</tr>
</tbody>
</table>

Antibiotic-resistance was screened using individual isolates. Isolates were picked for each of the types of organisms using sterile toothpicks. The isolates were subcultured in 0.1X Trypticase Soy Broth and stored (under glycerol) at -80°C until they could be
characterized for antibiotic sensitivity. Antibiotic sensitivity was determined by inoculating individual isolates, (up to 96 isolates per type of organism per treatment) into Biolog® MT-2 plates containing 0.1 TSB supplemented with the appropriate antibiotic. The antibiotics used were ampicillin (10 μg mL⁻¹), streptomycin (10 μg mL⁻¹), chloramphenicol (30μg mL⁻¹), and tetracycline (30μg mL⁻¹). All antibiotics were obtained from Sigma Chemical Corporation.

In addition to examining specific groups of microorganisms, microbial community diversity was evaluated using Biolog EcoPlates (Bochner, 1989; Insam and Gobern, 2004). These are microtiter plates containing a suite of known substrates with a redox sensitive compound to indicate bacterial growth. Each plate contains a total of 96 separate wells divided into three repeating groups of 32 wells each. Leachate water samples were inoculated into each of the wells to ascertain the presence of microorganisms capable of growing on individual substrates. The pattern of growth on different substrates was used to compare the microbial communities in columns receiving different treatments of greywater (with and without triclosan).

RESULTS

Total Heterotrophic Microorganisms

Figure 1 summarizes the numbers of heterotrophic microorganisms and triclosan-resistant heterotrophic microorganisms found in columns irrigated with greywater and greywater plus triclosan over the 10-week study. There were significantly more heterotrophic microorganisms in the columns irrigated with greywater (H) compared to columns irrigated with greywater with added triclosan (HT) (p<0.001). In the columns irrigated with greywater alone, only a small fraction (<1%) of the heterotrophic microorganisms were resistant to triclosan (compare H to TRH). In the case of the columns irrigated with greywater containing triclosan, however, there were no significant differences (p>0.05) between the number of total heterotrophs (HT) and the number of triclosan-resistant heterotrophs (TRHT). Essentially 100% of the heterotrophic microorganism in these columns were resistant to triclosan indicating strong selection for these organisms.

Antibiotic-Resistant Microorganisms

Figure 2 summarizes the proportion of the organisms (heterotrophs and triclosan-resistant heterotrophs) isolated from each of the treatments (greywater alone and greywater plus triclosan) that were resistant to each of the antibiotics tested. Antibiotic-resistant microorganisms, both total heterotrophs and triclosan-resistant heterotrophs, were isolated from all of the columns.
Microbial Community Diversity

There were obvious differences in the diversity of substrates used by the heterotrophic microbial communities in columns irrigated with greywater and those irrigated with greywater plus triclosan (Figure 3). The responses of the microbial communities in the two treatment groups are complex and idiosyncratic. Thus there is no clear-cut pattern in which the use of any category of substrates by the microorganisms in the greywater only irrigated communities was increased exclusively compared to the triclosan irrigated communities. For example, among the eight (8) carbohydrates tested as substrates, use by members of the microbial community irrigated with greywater (compared to the community irrigated with greywater and triclosan) was increased for four (4) of the compounds tested, decreased for three (3) of the compounds, and unchanged for 1 of the compounds.
Figure 2: Microbial Isolates Resistant to Antibiotics
Figure 3: Substrate Utilization by Microbial Communities

DISCUSSION

Modern urban living results in the introduction of exotic, and potentially environmentally harmful, chemicals into greywater. In particular, antibacterial agents and biocides are found in a wide variety of household products. This research has focused on the impact of triclosan on the structure and function of soil microbial communities as a result of exposure to triclosan in GW. Our research indicates that the presence of triclosan in greywater significantly impacts the numbers, resistance to antibiotics and metabolic diversity of soil microbial communities.

Triclosan in GW had two opposing impacts on the numbers of microorganisms in soil communities. First, the substantial decrease in the number of heterotrophs indicate that
triclosan in GW is toxic to a significant portion of the naturally occurring microbial community. In addition this research indicates that irrigation with GW containing triclosan results in an increase in the proportion of the microbial population that carries resistance to this biocide.

This shift in microbial populations is reflected in the striking change in the proportion of the isolates in the soil microbial community that are resistant to antibiotics after exposure to triclosan. Essentially all of the increased antibiotic-resistance in the triclosan irrigated soil microorganisms was associated with triclosan-resistance. Since resistance to many microorganisms is mediated by efflux pumps which are also involved in antibiotic-resistance, these results are not surprising.

Microorganisms are capable of transferring genetic information for antibiotic-resistance not only between individual cells within the same species, but also across species lines. This means that any increase in antibiotic-resistance in natural soil microorganisms, even in non-pathogenic microorganisms increases the pool of antibiotic-resistance genes in the environment and thus increases the likelihood that these genes will be transferred to pathogens. Given that prior studies have indicated the presence of pathogenic microorganisms, albeit in low numbers, in GW, the continued presence of triclosan in household products and its discharge into the environment in GW could have significant public health impacts.

Heterotrophic microorganisms in soil are the dominant organisms involved in the processes of nutrient cycling and litter decomposition essential to soil fertility. Several authors have argued that diversity at a functional level, as is measured using the Biolog system, is essential for long-term stability and proper nutrient cycling in an ecosystem (Garland and Mills, 1996; Pankhurst et al. 1998) Thus, the altered functional diversity associated with the presence of triclosan in GW indicates that continued release of triclosan into the environment may have severe impacts on normal ecosystem processes.

CONCLUSIONS

The results of this study indicate that the presence of triclosan in greywater has a significant negative impact on the microbial community in soil irrigated with greywater. While the full extent of the negative impact has not been established, the possible increase in infections caused by antibiotic-resistant microorganisms as well as the disruption of essential soil processes underscores the need to either severely restrict the domestic use of triclosan and similar biocides or to develop treatment processes to remove these compounds from greywater before it is released into the environment.
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Chapter 4

XVIIth World Congress of the International Commission of Agricultural Engineering (CIGR)
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GREYWATER IRRIGATION: ANTIBACTERIAL AGENTS AS BARRIERS TO GREYWATER REUSE

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ABSTRACT The development and use of marginal water for non-potable uses (e.g. irrigation) is critical in light of global water shortages. Greywater (GW), household wastewater containing all used water except sewage, accounts for between 50 and 80% of the wastewater produced in households. The use of GW for irrigation could therefore result in substantial savings in potable water in arid regions. Because greywater reuse results in direct discharge to the environment, concerns have been raised about possible environmental and public health impacts. For example, the presence of antibacterial compounds in greywater raises concerns regarding the potential selection for and spread of antibiotic resistant microorganisms in the environment. Our findings show that microbial populations resistant to tetracycline increased in soil irrigated with GW containing triclosan. Furthermore, the structure of the soil microbial community changed showing two very distinct patterns of substrate utilization. The microbial community in the soil irrigated with GW plus triclosan was significantly less diverse that that irrigated with GW only. This difference could influence microbial soil processes such as nutrient cycling and ultimately impact plant growth and ecosystem health. Therefore, our results indicate that greywater should be treated to remove antibacterial agents before its use in lawn irrigation. Alternatively, the use of antibacterial containing products should be significantly reduced.

Keywords: greywater, water reuse, triclosan, pharmaceuticals and personal care products, soil, diversity, microorganisms.
INTRODUCTION The use of greywater (GW) 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 (Christova-Boal et al. 1996, Al-Jayyous 2004). With increasing awareness of the need for water conservation and reuse, GW irrigation is being used in other areas of the U.S. as well. Data collected by the Soap and Detergent Association (NPD Group, 1999) indicated that 7% of U.S. households were reusing GW. This number has no doubt increased since the time of the NPD study. While GW irrigation is practiced primarily in arid regions, changing climate patterns and increased water demand associated with urbanization will likely make water reuse more important in temperate regions.

Domestic GW differs in composition from typical domestic wastewater (Gross et al. 2005). Specifically, GW is notable for the high concentration of soaps, detergents as well as pharmaceuticals and personal care products (PPCPs), including antimicrobial agents, found in it compared to domestic wastewater. Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol; TCS) is the most commonly used antibacterial agent in the United States with an estimated use of 0.6 – 10 million kg yr$^{-1}$ (Halden and Paull 2005, Adolfsson-Erici et al. 2002, Chau, et al. 2008, Miller et al. 2008, TSCA 2003). Given the high volumes of this material used, concerns have been raised about its fate and transport, bioaccumulation potential, toxicity and other possible environmental impacts including the development of resistant microorganisms (Chu and Metcalfe 2007, Coogan et al. 2007, Ahn et al. 2008, Liu et al. 2009, Aranami and Readman 2007, Harrow et al. 2010).

Because of its widespread use, low $K_{ow}$ and apparent low degradability triclosan is among the most commonly detected emerging pollutants in surface water (Heidler and Halden 2009, Singh et al. 2010, Nishi et al. 2008). The presence of triclosan, and the related biocide triclocarban in wastewater and biosolids also is well documented (Heidler and Halden 2009, Sabourin et al. 2009). Less data is available concerning the presence of triclosan in GW discharge; however, indications are that triclosan is present in GW (Almqvist and Hanaeus 2006, Palmquist and Hanaeus 2005, Eriksson et al. 2003) The use of GW for irrigation, therefore, may result in the release of significant quantities of triclosan into the soil environment.

While the bulk of the concern about microorganisms in greywater has focused on the possible presence of pathogenic microorganisms (Rose et al. 1991, Casanova et al. 2001, Birks et al. 2004, Gross et al. 2007) much less consideration has been given to the possible impacts of GW on naturally occurring microorganisms. Several recent studies have proposed a link between triclosan resistance in bacteria and resistance to common antibiotics (McMurry et al. 1998, Levy 2001, Levy 2002, Birovsova and Mikulášová 2009, Chen et al. 2009), although other studies have questioned the existence of such a linkage (Russell 2003, Russell 2004, Ledder et al. 2006, Cotell et al. 2009). Thus, whether the presence in GW of biocides such as triclosan may result in an increase in the presence of antibiotic resistant among
indigenous soil microorganisms is an unresolved issue. Antibiotic resistant microorganisms may subsequently transfer antibiotic resistance to pathogenic microorganisms through horizontal gene transfer mechanisms.

In addition to the possible increase in antibiotic resistance among indigenous microorganisms, the addition of biocides to the environment has the potential to influence higher level characteristics of the soil microbiota including the composition, diversity and functioning of microbial populations and communities. Microorganisms are essential to the biogeochemical cycling and trophic relationships of all terrestrial ecosystems. As the primary organisms involved in the decomposition and recycling of organic materials, microbial communities are the basis of soil fertility providing nutrients both directly and indirectly to higher organisms. Thus, changes in the structure or function of either the entire heterotrophic microbial community or of component microbial assemblages has the potential to profoundly impact the rest of the terrestrial ecosystem (Cookson et al. 2008, Wang et al. 2009, Ndaw et al. 2009, Slabbert et al. 2010).

The purpose of this research, therefore, is twofold. First we examined the impact of triclosan on the numbers of several functional groups of soil bacteria to determine if exposure to this compound during irrigation with greywater increased the number of resistant bacteria in the soil microbiota. In addition, we examined the diversity of two microbial assemblages – culturable heterotrophs and triclosan-resistant heterotrophs - to determine if the addition of triclosan to the soil environment impacts functional diversity.

**MATERIALS AND METHODS** Replicate soil microcosms were made using plastic pots filled with a clayey-loam soil (2 parts clay soil obtained from a horse farm in Central Pennsylvania, 2 parts sand, and 1 part commercial potting soil). Each pot contained approximately 100 grams of the soil mixture. Pots were incubated in the dark to prevent the growth of plants. The pots were divided into two groups on the basis of the solution used for routine irrigation – control pots were irrigated with triclosan-free synthetic greywater (GW) while treatment pots were irrigated with synthetic greywater supplemented with 2.0 µg mL⁻¹ (final concentration) triclosan (GWT) (Gross et al. 2007, Harrow et al. 2010). On a weekly basis, each pot was watered with 15 mL of the appropriate irrigation solution. Approximately 1 hour after watering, one pot from each treatment group was sampled for the determination of microbial population size and diversity. Each sample was analyzed for four different populations of microorganisms (Table 1).
Table 1: Microbial Communities/Guilds/Populations in this Study

<table>
<thead>
<tr>
<th>Community</th>
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<th>Culture Method</th>
</tr>
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</tr>
<tr>
<td>Tetracycline-resistant</td>
<td>Subset of the heterotrophic community resistant to tetracycline</td>
<td>Growth on 0.1 X TSB supplemented with 30 μg mL⁻¹ (final concentration) tetracycline</td>
</tr>
<tr>
<td>Both-resistant to tetracycline and triclosan</td>
<td>Subset of the heterotrophic community resistant to triclosan and tetracycline</td>
<td>Growth on 0.1 X TSB supplemented with both triclosan and tetracycline</td>
</tr>
</tbody>
</table>

The number of each type of microorganism in soil from each of the pots sampled was determined using a modified MPN procedure in BIOLOG MT-2 plates. One gram of soil initially was transferred to 10 mL of sterile dilution water and shaken vigorously for 30-60 seconds to dislodge the bacteria from the soil particles. The resulting suspension was used to prepare serial dilutions of the sample which were then inoculated into MT-2 plates containing the appropriate medium. MT-2 plates are 96 well microtiter plates containing a redox dye that is reduced when microorganisms respire. This results in a change in the color of wells with respiring microorganisms and allows for rapid monitoring of microbial growth using a microtiter plate reader. MT-2 plates were incubated for 5 days at 25°C before they were read. This allowed sufficient time for slow growing microorganisms to be detected (data not shown). The number of wells for each dilution in the MT-2 plates showing microbial growth was used to calculate the size of the microbial population using a standard Most Probable Number Procedure.

In addition to enumerating specific groups of microorganisms, microbial diversity was evaluated using Biolog EcoPlates (Insam and Goberna, 2004). These are microtiter plates containing a suite of known substrates with a redox sensitive compound to indicate bacterial growth. Each plate contains a total of 96 separate wells divided into three repeating groups of 32 wells each. Diluted soil samples were inoculated into each of the wells to ascertain the presence of microorganisms.
capable of growing on individual substrates. The pattern of growth on different substrates was used to compare the microbial communities in columns receiving different treatments of greywater (with and without triclosan). The microorganisms in the soil were divided into two operationally defined groups. Thus, for each sample, the diversity of the culturable heterotrophic microbial community was evaluated as well as the diversity of the subset of the community resistant to triclosan. Diversity was calculated using the Shannon Index as described by Zak et al. (1994). Triplicate soil samples were evaluated for each diversity measurement. Diversity indices were compared using ANOVA with post-hoc comparisons of the means using the Bonferroni test. All statistical analysis were performed using Prism 6.0 (GraphPad Software).

RESULTS There were no significant changes in the numbers of culturable heterotrophic microorganisms between the pots irrigated with GW and those irrigated with GWT over the course of the first eleven weeks of this ongoing study (Figure 1). The number of microorganisms resistant to triclosan increased slightly in the pots receiving greywater alone; however, given the large variation associated with the MPN technique, it is unlikely that these differences are significant.

In the case of the soils irrigated with greywater containing triclosan, on the other hand, there was a significant increase in the number of triclosan resistant organisms. After exposure to greywater with triclosan, essentially all of the culturable soil microorganisms appear to have resistance to triclosan. In both of the treatments (GW and GWT), the number of tetracycline resistant microorganisms increased slightly. This pattern was seen also in terms of the number of microorganisms resistant to both tetracycline and triclosan. While the number of organisms resistant to both substances appears to be slightly higher in the triclosan pots, there is not enough data to determine if these differences are significant. Studies are continuing on this soil system to see if this preliminary data indicates a long-term trend of enrichment in antibacterial and antibiotic resistant microorganisms in soil receiving triclosan.
Figure 1. Concentrations of selected populations of soil microorganisms at the beginning and end (11 weeks) of the study in soils irrigated with greywater alone (Greywater) and greywater supplemented with triclosan (Triclosan) as determined using the MPN technique.

Microbial community structure was compared between the greywater only and the greywater and triclosan irrigated pots at the beginning, mid-point (week 6) and end of the study (week 11). There were no differences in the overall diversity of either the total heterotrophic community or the triclosan-resistant guilds when the irrigation treatments were compared at the beginning of the study (Figure 2). Comparisons of soil samples from the greywater alone and the triclosan irrigated later in the study revealed significant differences between the diversity, and hence the composition, of the microbial assemblages present (Figure 2). Microbial diversity (Shannon Index) was significantly higher (p<0.001) in the heterotrophic community from pots irrigated with greywater only (GW) compared to the structure of the heterotrophic community in the soils irrigated with greywater and triclosan (GWT) at either 6 or 11 weeks. There were marginal differences (p>0.05) between the community structure of the triclosan resistant guild in the soils irrigated with GW only and either the culturable heterotrophic community or the triclosan resistant guild in the soils irrigated with GWT indicating that exposure to triclosan may have reduced the diversity of both of these microbial groupings.
Figure 2. Diversity (Shannon index) of heterotrophic and triclosan resistant microbial communities/guilds in soils irrigated with greywater alone (GW) and with greywater plus triclosan

**DISCUSSION**  Short-term irrigation of soils with greywater supplemented with triclosan was shown to have impacts on both the presence of triclosan and antibiotic (tetracycline) resistant microorganisms in soil as well as on the structure of microbial communities and guilds present in the soil.

Several recent studies have shown a correlation between biocide use and an increased incidence of antibiotic resistant microorganisms in stream water and sediments (Stachowiak et al. 2009) and in leachate from soil columns (Harrow et al., 2010). Our data supports the possible enrichment of antibiotic resistant microorganisms in soil microbial communities after exposure to triclosan enriched greywater for less than three months. Given the widespread public health problems now associated with antibiotic resistant microorganisms as well as the ability of microorganisms to rapidly and easily spread resistance genes between different bacterial groups, even the slight increases found in our research have troubling implications and underscore the need for additional research in this area.

There is an increasing recognition of essential functions fulfilled by microorganisms involved with biogeochemical cycling, and decomposition of organic matter and pollutants soil environments. Generally, researchers have found that the presence of pollutants and other types of biological stress are associated with reduced microbial diversity. For example, Derry et al. (1998) found significant differences between the microbial communities in contaminated soil compared to soils with no
history of chemical contamination. Lewis et al. (2009) found microbial diversity in bauxite-mined soils was significantly lower than diversity in control soils that had not been mined and Anderson et al. (2009) reported that microbial diversity in smelter-impacted soils was lower than in non-impacted soils.

Our results indicate that short-term exposure to triclosan has a negative impact on the culturable heterotrophic microbial community in soil. The reduced microbial diversity found in GWT irrigated soils is likely to be the result of toxic effects of triclosan on specific microbial populations. The similarity of diversity seen in the culturable heterotrophic community in the GWT irrigated soils to diversity of the triclosan-resistant microbial groups may reflect a convergence of microbial population structures in response to the toxicity of triclosan. This reduced diversity may be associated with impairment or loss of microbially mediated processes essential to soil fertility.

**CONCLUSION** Irrigation of soil with triclosan-containing greywater (GWT) results in both an increase in resistant bacteria and a concomitant decrease in overall microbial community diversity. These changes in the soil microbiota raise public health and environmental concerns about the release of untreated household waste streams into terrestrial ecosystems. Before irrigation with greywater can become a useful water reuse alternative, additional research focusing on the long-term impacts of triclosan and other pharmaceuticals and personal care products are needed.

**REFERENCES**


Chapter 5

Impacts of Triclosan in Greywater on Soil Microorganisms

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Abstract
The use of greywater for irrigation is becoming a common practice in arid regions such as the Southwestern US, the Middle East, Australia, and China. While greywater supplies nutrients to soil ecosystems, the possible impact of trace contaminants, particularly pharmaceuticals and personal care products, has not been determined. This research examined the impact of triclosan, an antibacterial agent commonly added to consumer products, on microbial populations and microbial diversity in soil irrigated with greywater. While there was no change in the total number of heterotrophic microorganisms in the soil, both the types and the antibiotic resistance of the microorganisms was significantly influenced by triclosan. The proportion of the microbial isolates resistant to antibiotics increased while at the same time, overall diversity of the microbial community decreased.

1. Introduction
Greywater (GW) is the used water from households, excluding sewage from toilets and, in some countries, waste materials from food preparation [1-3]. Greywater accounts for between 50 to 80% of wastewater coming from individual households [3-5]. The use of GW for the irrigation of lawns, ornamental plants, and other landscape vegetation has become an accepted practice in the Southwest US, the Middle East, and the Australian dry lands [4-6]. While GW irrigation is practiced primarily in arid regions, changing climate patterns, increased water demand associated with urbanization, and increased awareness of the need for water conservation will likely make water reuse more important in temperate regions [7-10].

Domestic GW differs in composition from typical domestic wastewater [11-13]. Greywater is highly variable in composition depending on the number and lifestyle of the residents in a household [11,14,15]. Greywater is notable for the high concentration of soaps, detergents and oils it contains [12, 16]. In addition, GW has pharmaceuticals and personal care products (PPCPs), including antimicrobial agents such as triclosan, at concentrations equal to or higher than those in domestic wastewater [2,17].

Triclosan (TCS; 5-chloro-2-(2,4-dichlorophenoxy) phenol; CAS No. 9012-63-9) is the most commonly used antibacterial agent in the US. Current estimates are that
the discharge of this compound into the US environment is in the range of 300,000 - 500,000 kg yr\(^{-1}\) and use is increasing rapidly [18-21]. Triclosan is found in numerous products including clothing, toys, toothbrushes, rubber, hand soaps, toothpaste, deodorants, and laundry detergents [20]. A concentration of 0.1-0.3\% of triclosan can typically be found in consumer products [21].

Triclosan is active against a wide range of both Gram-positive and Gram-negative bacteria. Although triclosan may inhibit a variety of sites within the bacterial cell, it is generally agreed that the antimicrobial activity principally is a result of inhibition of the enoyl reductase enzyme (Fab1) involved in the synthesis of fatty acids [22-24]. Several recent studies have proposed a link between triclosan resistance in bacteria and resistance to common antibiotics [22, 25-30], although other studies have questioned the existence of such a linkage [31-34]. The mechanism responsible for the association between triclosan and antibiotic resistance is most often up-regulation of microbial efflux pumps, which effectively allow the bacteria to pump antibacterial agents or antibiotics outside of the cell [29,30].

Triclosan in GW has been found in the range of 0.075\(\mu\)g L\(^{-1}\)–16.6 \(\mu\)g L\(^{-1}\) [35]. Conventional municipal wastewater treatment processes such as activated sludge and trickling filters, are known to have relatively low (generally >95\%) removal efficiency for triclosan [36-38]. Several researchers [39-42] have developed small-scale, decentralized systems for the treatment of household GW. These systems typically are evaluated on their ability to remove conventional chemical and microbiological contaminants such as BOD and coliforms and there is no compelling reason to assume that any of the decentralized systems have superior removal of triclosan compared to municipal treatment plant performance. Even with treatment systems, final disposition of GW involves its application to soil as irrigation water. Thus, any contaminants remaining in GW may have an impact on the chemical and biological characteristics of the soil to which it is applied.

Triclosan can enter the terrestrial environment via a number of sources and recent studies have begun to examine the impact of triclosan in soils. Inputs of triclosan to soils are primarily associated with the secondary disposal of domestic wastewater and biosolids and with the reuse of marginal water such as GW, although agricultural wastes, such as manure application cannot be ignored. During wastewater treatment, triclosan partitions to biosolids. In the US, biosolids are frequently applied to agricultural lands for disposal with estimates of yearly amounts applied throughout the US in excess of 3 million dry tons [43]. Concentrations of triclosan in wastewater sludge in the mg kg\(^{-1}\) range have been reported [44,45]. This can serve to introduce triclosan (and associated pharmaceuticals and personal care products) into the soil environment with subsequent further dispersal into additional environmental compartments such as aquatic ecosystems and biota possible [46]. Cha and Cupples [47] reported a very low leaching potential for triclosan or triclocarban, a related biocide, indicating that these contaminants are likely to remain in terrestrial environments after they are applied in irrigation water. Thus, the introduction of triclosan-containing materials
into soil ecosystems is likely to result in selective partitioning of the triclosan onto soil particles and its possible accumulation in these systems.

Ying et al [48] found significant differences in the half-life of triclosan in soil when incubated under aerobic and anaerobic conditions with degradation of the compound under aerobic conditions much faster than under anaerobic conditions. Furthermore, they found no change in the concentration of triclosan in sterile soils (70 day incubation) and concluded that biological processes were responsible for the loss of material associated with non-sterile soils. Chen et al. [49] examined the fate of triclosan and other PPCPs in a reed bed sludge treatment system. Triclosan was reduced to 60%, 45%, and 32% of its original concentration in the top, middle, and bottom layer in these systems, but significant quantities of triclosan accumulated within soil over the duration of the study.

Research in our laboratory has focused on the impacts of triclosan in GW on soil microorganisms and microbial communities. Specifically, our work has focused on the diversity of microbial assemblages to determine if the addition of triclosan to the soil environment impacts their functional diversity. In addition, since several laboratory studies have indicated a correlation between exposure of specific microorganisms to triclosan and the possible development of bacterial resistance to TCS and/or to several commonly used antibiotics [23, 26,27]; we have monitored changes in the patterns of antibiotic resistance in microorganisms irrigated with GW containing triclosan.

2. Methods
2.1 Microcosm systems

Replicate soil microcosms were made using plastic pots filled with a clayey-loam soil (2 parts clay soil obtained from a horse farm in Central Pennsylvania, 2 parts sand, and 1 part commercial potting soil). Each pot contained approximately 100 grams of the soil mixture. Pots were incubated in the dark to prevent the growth of plants. The pots were divided into two groups on the basis of the solution used for routine irrigation – control pots were irrigated with triclosan-free synthetic greywater (GW) while treatment pots were irrigated with synthetic greywater supplemented with 2.0-µg mL⁻¹ (final concentration) triclosan (GWT) (Table 1) [3,12]. On a weekly basis, each pot was watered with 15 mL of the appropriate irrigation solution. Since the focus of this study was on acute, short-term changes to microbial systems resulting from exposure to low-levels of triclosan, irrigation was for a total of 10 weeks only. Approximately 1 hour after watering, triplicate pots from each treatment group were sampled for the determination of microbial population size and diversity.
Table 1: Composition of Synthetic Greywater Used in this Research

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Greywater (GW) (per L tap water)</th>
<th>Greywater plus Triclosan (GWT) (per L tap water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shampoo</td>
<td>Johnson’s Baby Shampoo; Johnson and Johnson</td>
<td>0.80 mL</td>
<td>0.80 mL</td>
</tr>
<tr>
<td>Laundry Detergent</td>
<td>Seventh Generation: free and clear of perfumes and dyes</td>
<td>0.064 mL</td>
<td>0.064 mL</td>
</tr>
<tr>
<td>Cooking Oil</td>
<td>Crisco All Natural Pure Vegetable Oil</td>
<td>0.01 mL</td>
<td>0.01 mL</td>
</tr>
<tr>
<td>Triclosan (CAS 9012-63-9)</td>
<td>Sigma 72779</td>
<td>10 mL ethanol with no triclosan was added</td>
<td>20 mg (dissolved in 10 mL ethanol)</td>
</tr>
</tbody>
</table>

The number of selected types of microorganism in soil (Table 2) from each of the pots sampled was determined using spread plates for viable counts. One gram of soil was transferred to 10 mL of sterile dilution water and shaken vigorously for 30-60 seconds to dislodge the bacteria from the soil particles. The resulting suspension was used to prepare serial dilutions of the sample, which were then spread onto the appropriate medium. Plates were incubated for 5 days at 25°C before counting. This allowed sufficient time for slow growing microorganisms to be detected (data not shown).

Table 2: Microbial Populations in this Study

<table>
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<td>Growth on 0.1 X TSA supplemented with 2.0μg mL⁻¹ (final concentration) triclosan</td>
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</tbody>
</table>

2.2 Antibiotic Resistance

Antibiotic resistance was screened using individual isolates. Antibiotic sensitivity was determined by inoculating individual isolates, (up to 96 isolates per type of organism per treatment) into Biolog® MT-2 plates containing 0.1 TSB supplemented with the appropriate antibiotic. The antibiotics used were (1) ampicillin (CAS 98520-55-9: 10 μg mL⁻¹), streptomycin (CAS 57-92-1: 10 μg mL⁻¹), chloramphenicol (CAS 85666-84-8: 30μg mL⁻¹), and tetracycline (CAS 6591-49-7: 30μg mL⁻¹). All antibiotics were obtained from Sigma Chemical Corporation.

DNA in the soil was extracted using a Mo-Bio PowerSoil DNA Isolation Kit following the manufacturer’s instructions. DNA concentrations were found using a
NanoVue Plus Spectrophotometer. DNA amplification was done in a Bio-Rad MyCycler Thermal Cycler using Guillaume’s PCR procedure for tet A [50]. For each set of samples, the appropriate negative (no template DNA) and positive (tet A plasmid) were included. Electrophoresis was done using a 1.5% agarose gel to detect PCR results. The number of positive samples for each treatment was summed over time to provide a semi-quantitative measure of the prevalence of the tet A gene in the microbial community.

2.3 Microbial Community Diversity

In addition to enumerating specific groups of microorganisms, microbial diversity was evaluated using Biolog EcoPlates [51]. The pattern of growth on different substrates was used to compare the microbial communities in soil receiving different treatments of greywater (with and without triclosan). The microorganisms in the soil were divided into two operationally defined groups. Thus, for each sample, the diversity of the culturable heterotrophic microbial community was evaluated as well as the diversity of the subset of the community resistant to triclosan. Diversity was calculated using the Shannon Index and the Substrate Utilization Index as described by Zak et al. [52]. Triplicate soil samples were evaluated for each diversity measurement.

2.4 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 6.0 at a significance level of p<0.01. Microbial numbers and community diversity were analyzed using ANOVA. Cumulative presence of the tet A gene and the proportion of isolates resistant to particular antibiotics were evaluated using the Chi-square test.

3. Results

3.1 Microbial Populations

There were significant differences in the numbers of culturable heterotrophic microorganisms between the pots irrigated with GW and those irrigated with GWT over the course of the study (Figure 1A) despite there being no statistically significant difference between the treatments initially. Generally, viable counts were higher in the soil irrigated with greywater than in the soil irrigated with greywater supplemented with triclosan. This is consistent with possible toxicity of triclosan towards soil heterotrophic microorganisms and is not unexpected since triclosan is a biocide. There were no clear temporal trends associated with the heterotrophic populations of microorganisms in either of the two treatments.

There also were statistically significant differences between the treatments when only the triclosan-resistant viable heterotrophic microorganisms were compared (Figure 1B). Initially, there were no statistically significant differences between the soils, however, the numbers of microorganisms quickly diverged when triclosan was present in the irrigation water. After one week, the number of triclosan-resistant viable organisms was higher in the soil irrigated with greywater supplemented with triclosan and the
number of resistant organisms remained higher in this treatment over the remainder of the study.
Figure 1: Number of selected microbial populations. Microbial numbers were determined as described in the methods. Data points are mean +/- standard error of the mean (SEM). Note mean and SEM were calculated using log transformed data.
3.2 Antibiotic resistance:

Figure 2 summarizes the proportion of the microorganisms resistant to each of four separate antibiotics at the conclusion of the study. A higher proportion of the viable microbial populations were resistant to the antibiotics in soil irrigated with greywater plus triclosan compared to soil irrigated with greywater alone. This pattern was particularly pronounced with triclosan-resistant viable heterotrophs from each of the soils were screened. In general, a higher percentage of the triclosan-resistant isolates were also resistant to at least one of the antibiotics regardless of the type of water used in irrigation possibly reflecting a common mechanism of resistance for both the biocide and antibiotics. Comparison of the soil irrigated with greywater alone to the soil irrigated with greywater plus triclosan shows a significantly higher proportion of the isolates from the soils exposed to triclosan also were resistant to antibiotics. PCR analysis (Figure 3) demonstrated that the cumulative number of soil samples positive for the presence of the \textit{tet} A gene was significantly higher in the soil exposed to triclosan.

3.3 Microbial Community Structure

Microbial community structure was compared between the greywater only and the greywater plus triclosan irrigated pots. There were no significant differences between the treatment groups initially (data not shown). By the end of the study (week 10), diversity as measured using the Shannon index, was significantly lower in the viable heterotrophic community in soil irrigated with greywater plus triclosan compared to the community in soil irrigated with greywater alone (Figure 4A). The reverse pattern was observed when the triclosan-resistant heterotrophs were compared for the two types of irrigation. The use of greywater plus triclosan for irrigation significantly increased the diversity of the triclosan-resistant heterotrophs in soil compared to triclosan-resistant organisms from soil irrigated with greywater alone (Figure 4B), reflecting selection for an adapted community as a result of exposure to triclosan.
Figure 2: Proportion of isolates resistant to selected antibiotics. Individual isolates of selected populations of microorganisms were screened for resistance to antibiotics. Values reported are the mean of all isolates after 10 weeks irrigation.
Figure 3: Cumulative prevalence of tet A gene in soils irrigated with greywater and greywater plus triclosan.

There were highly significant differences (p<0.0001) in overall substrate utilization diversity between the heterotrophic microbial communities associated with columns irrigated with greywater and those irrigated with greywater plus triclosan. The heterotrophic community in the columns irrigated with greywater alone were much more diverse in terms of substrate utilization (SI = 32.48) than was the community in columns with triclosan (SI = 0.52).

4. Discussion

Short-term irrigation of soils with greywater supplemented with triclosan was shown to have impacts on both the presence of triclosan and antibiotic-resistant microorganisms in soil as well as on the structure of microbial communities present in the soil. Several recent studies have shown a correlation between biocide use and an increased incidence of antibiotic resistant microorganisms in stream water and sediments [53] and in leachate from soil columns [54]; however these studies usually have involved long-term exposure to biocides. Our research underscores the acute impacts of biocide exposure with demonstrable changes found in microbial numbers and community structure occurring after less than a single typical irrigation season.

Our data supports the possible enrichment of antibiotic-resistant microorganisms in soil microbial communities after exposure to triclosan-enriched greywater for less than three months. Given the widespread public health problems associated with antibiotic-resistant microorganisms as well as the ability of microorganisms to rapidly and easily spread resistance genes between different bacterial groups, even the slight increases found in our research have troubling implications and underscore the need for additional research in this area.
The addition of biocides to the environment has the potential to influence higher-level characteristics of the soil microbiota including the composition, diversity and functioning of microbial populations and communities. Microorganisms are essential to the biogeochemical cycling and trophic relationships of all terrestrial ecosystems. As the primary organisms involved in the decomposition and recycling of organic materials, microbial communities are the basis of soil fertility providing nutrients both directly and indirectly to higher organisms. Thus, changes in the structure or function of either the entire heterotrophic microbial community or of component microbial assemblages has the potential to profoundly impact the rest of the terrestrial ecosystem [55-58].
There is an increasing recognition of essential functions fulfilled by microorganisms involved with biogeochemical cycling, and decomposition of organic matter and pollutants soil environments. Generally, researchers have found that the presence of pollutants and other types of biological stress are associated with reduced microbial diversity. For example, Derry et al. [59] found significant differences between the microbial communities in contaminated soil compared to soils with no history of chemical contamination. Lewis et al. [60] found microbial diversity in bauxite-mined soils was significantly lower than diversity in control soils that had not been mined and Anderson et al. [61] reported that microbial diversity in smelter-impacted soils was lower than in non-impacted soils. Certainly the reduced numbers and diversity of viable heterotrophs in soil irrigated with greywater plus triclosan indicates inhibitory and toxic effects of this compound on soil microorganisms. This is not surprising since triclosan has been reported to be toxic to a range of organisms [62–68] and, as an antimicrobial, is specifically targeted against microorganisms.

Our results indicate that short-term exposure to triclosan has a negative impact on the culturable heterotrophic microbial community in soil. The reduced microbial diversity found in GWT irrigated soils is likely to be the result of toxic effects of triclosan on specific microbial populations although the exact mechanism responsible for the observed change has not been determined. In addition to the possible direct impacts of triclosan as a biocide, interactions such as sorption, change in pH, and exchange of materials within the soil organic fraction, as well as interactions between microbial populations may have contributed to the observed inhibition and should be further explored. The similarity of diversity seen in the culturable heterotrophic community in the GWT irrigated soils to diversity of the triclosan-resistant microbial groups may reflect a convergence of microbial population structures in response to the toxicity of triclosan. This reduced diversity may be associated with impairment or loss of microbially mediated processes essential to soil fertility.

Irrigation of soil with triclosan-containing greywater (GWT) results in both an increase in resistant bacteria and a concomitant decrease in overall microbial community diversity. These changes in the soil microbiota raise public health and environmental concerns about the release of untreated household waste streams into terrestrial ecosystems. Before irrigation with greywater can become useful water reuse alternative, additional research focusing on the long-term impacts of triclosan and other pharmaceuticals and personal care products are needed.

References


Chapter 6

Conclusion

Triclosan exposure decreased the number of microorganisms within the soil. Within this group of triclosan resistant microbes, the frequency of the tet A gene increased in comparison to isolates that were not previously exposed to triclosan. This suggests that triclosan resistance is closely related to antibiotic resistance and that if a microbe is resistant to one, the likelihood of it being resistant to the other increases. Carbon type metabolism and heterotrophic populations were significantly decreased by the presence of triclosan within the synthetic greywater. The type of substrates used by the microbes can have a direct effect on the types of available nutrients found within the soil. This may have an effect the plant life that is dependent on these nutrients. With all these outcomes serious consideration towards the use of triclosan needs to be done especially regarding the reuse of greywater. Due to triclosan’s lipophilic nature and other chemical properties, treatment has many challenges. According to Aiello et al (2007) there are no significant differences between washing hands with antibacterial soap and soap without triclosan or other antibacterial agents. With this said, another possible option to deal with the potential threat triclosan poses is to decrease the overall use of triclosan.
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