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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 phamaceuticals 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 triclocarb 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 Hanæus 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, Birosova and Mikulásová 2009, Chen *et al.* 2009.), although other studies have questioned the existence of such a linkage (Russell 2003, Russell 2004, Ledder *et al.* 2006, Cottell *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 micoorganisms 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

Community	Microorganisms Included	Culture Method
Culturable Heterotrophs	All bacteria capable of growth and colony formation on a mixture of complex organic carbon compounds	Growth on 0.1 X Trypticase Soy Broth (TSB) after incubation for 5 days at 25^{o}C
Triclosan-resistant	Subset of the heterotrophic community resistant to the biocide triclosan	Growth on 0.1 X TSB supplemented with 2.0µg mL^{-1}(final concentration) triclosan
Tetracycline-resistant	Subset of the heterotrophic community resistant to tetracycline	Growth on 0.1 X TSB supplemented with 30 µg mL^{-1} (final concentration) tetracycline
Both- resistant to tetracycline and triclosan	Subset of the heterotrophic community resistant to triclosan and tetracycline	Growth on 0.1 X TSB supplemented with both triclosan and tetracycine

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^{o}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.

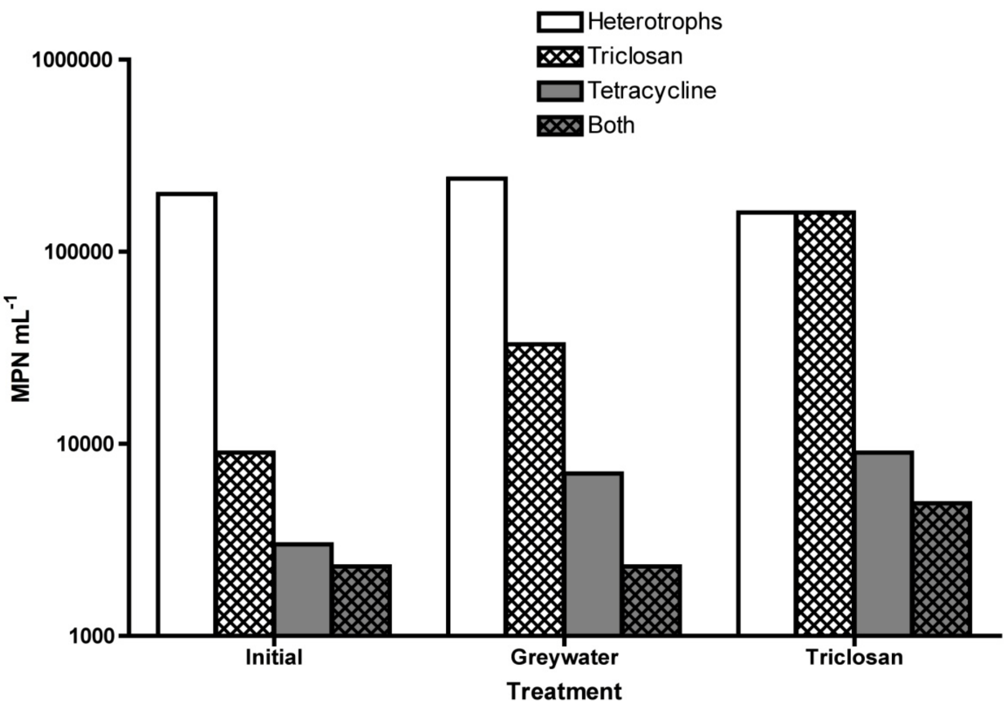

Figure 1. Concentrations of selected populations of soil microorganisms at the beginning and end (11 weeks) of the study in soils irrigated with greywater alone (Greywater) and greywater supplemented with triclosan (Triclosan) as determined using the MPN technique.

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.

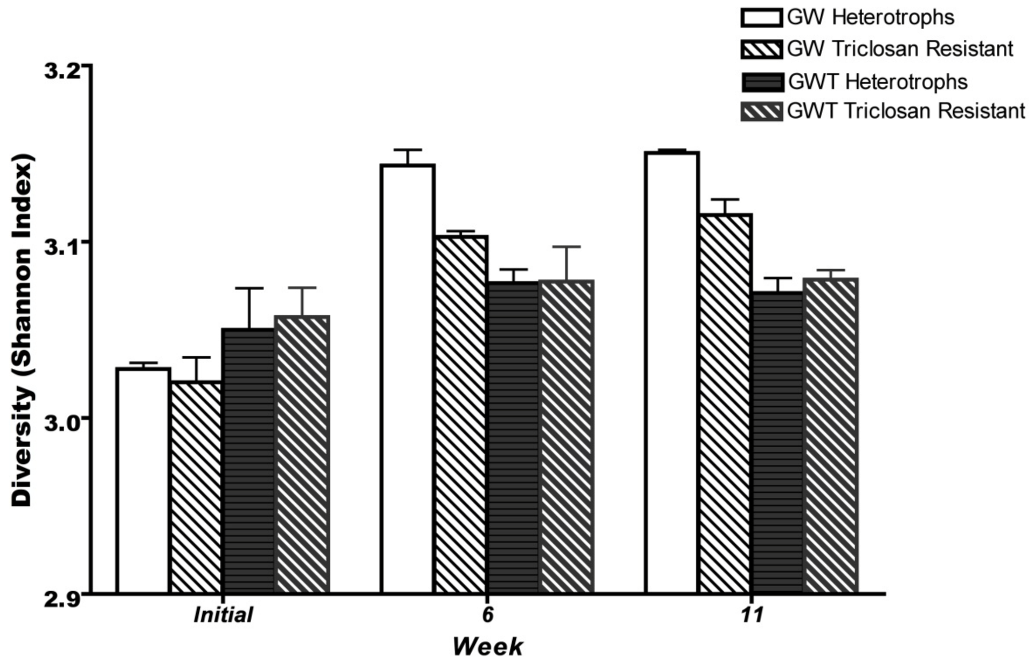

Figure 2. Diversity (Shannon index) of heterotrophic and triclosan resistant microbial communities/guilds in soils irrigated with greywater alone (GW) and with greywater plus triclosan

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 antiboitic 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.

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