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Research Article

Impacts of Triclosan in Greywater on Soil Microorganisms

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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 paper 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 were 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 upregulation 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 μgL^{-1}–16.6 μgL^{-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 has 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 nonsterile 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.

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).

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: 30 μg mL^{-1}), and tetracycline (CAS 6591-49-7: 30 μg 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

Table 1: Composition of Synthetic Greywater Used in this Research.

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

Table 2: Microbial 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 Agar (TSA) after incubation for 5 days at 25°C
Triclosan-resistant	Subset of the heterotrophic community resistant to the biocide triclosan	Growth on 0.1 X TSA supplemented with 2.0 μg mL^{-1}(final concentration) triclosan

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 semiquantitative 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 1(a)) 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 this 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 1(b)). 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.

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

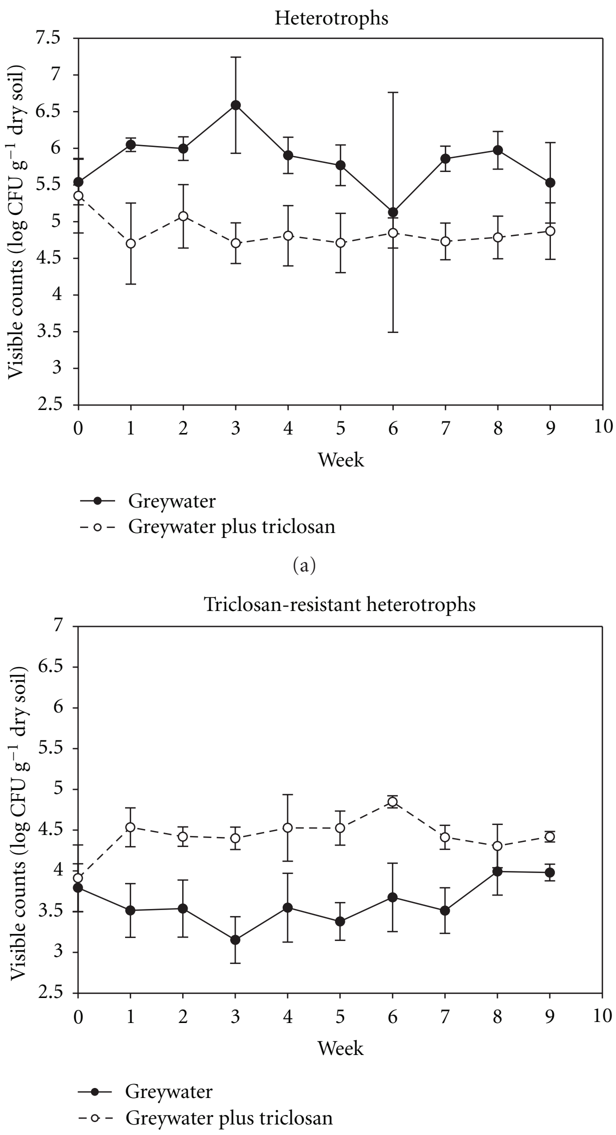

Figure 1: Number of selected microbial populations. Microbial numbers were determined as described in the methods. Data points are mean +/− standard error of the mean (SEM). Note: mean and SEM were calculated using log transformed data.

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 *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 4(a)). 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 4(b)), reflecting selection for an adapted community as a result of exposure to 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].

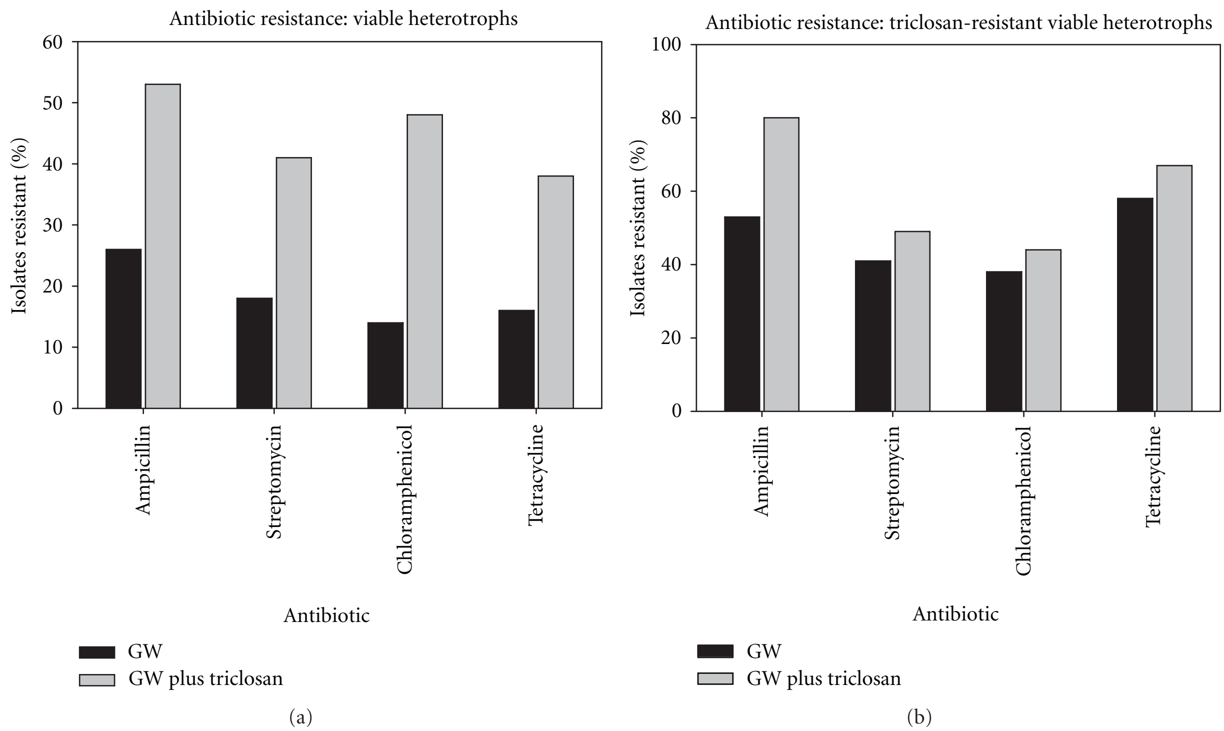

Figure 2: Proportion of isolates resistant to selected antibiotics. Individual isolates of selected populations of microorganisms were screened for resistance to antibiotics. Values reported are the mean of all isolates after 10 weeks irrigation.

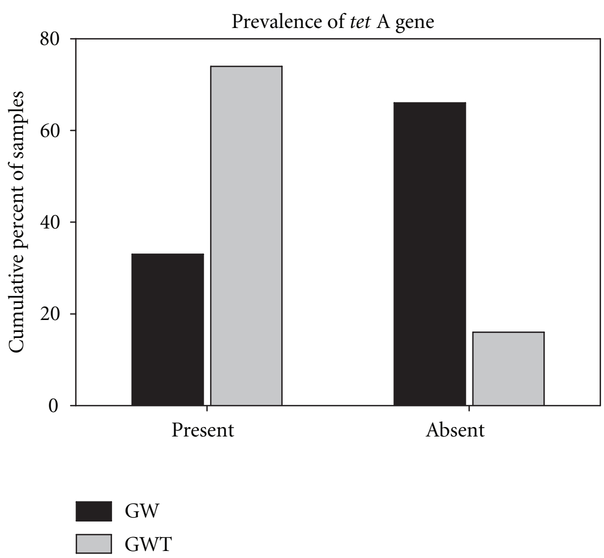

Figure 3: Cumulative prevalence of *tet* A gene in soils irrigated with greywater and greywater plus triclosan.

There 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 nonimpacted 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

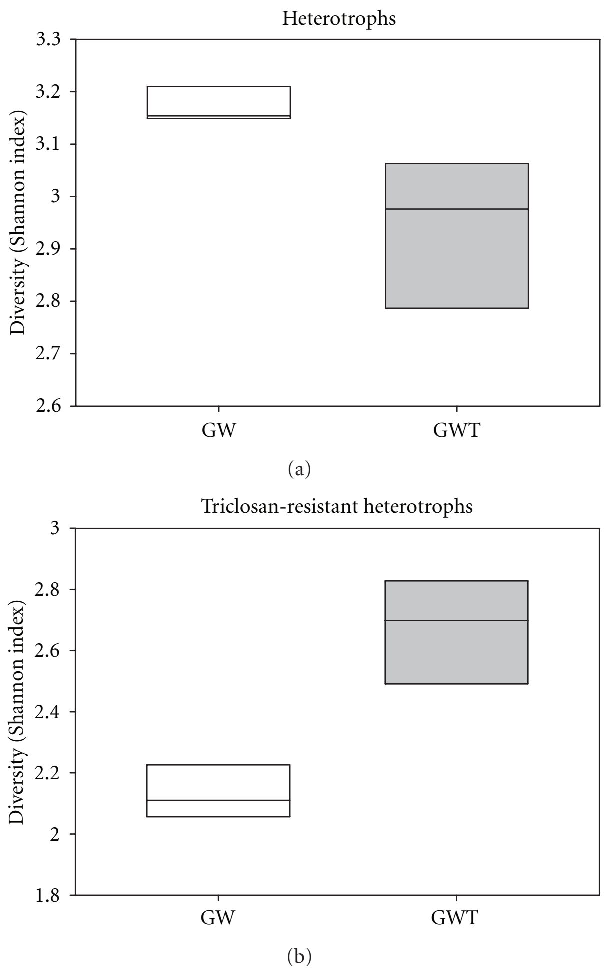

FIGURE 4: Diversity (Shannon index) of soil microbial communities after 10 weeks of irrigation.

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 a useful water reuse alternative, additional research focusing on the long-term impacts of triclosan and other pharmaceuticals and personal care products is needed.

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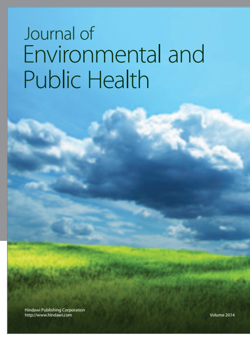
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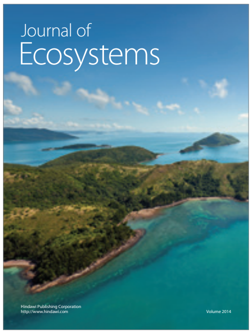
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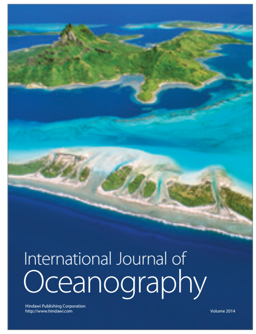
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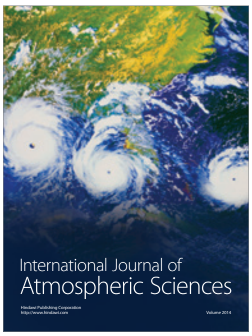
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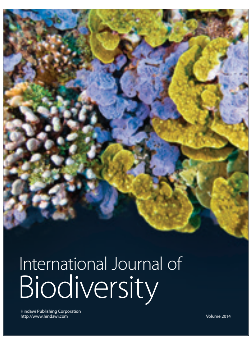
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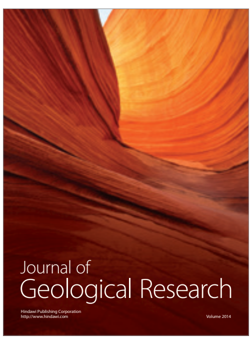
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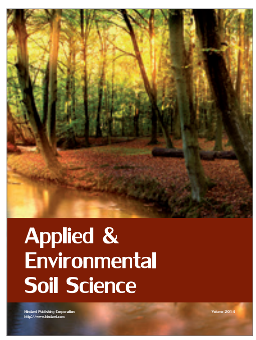
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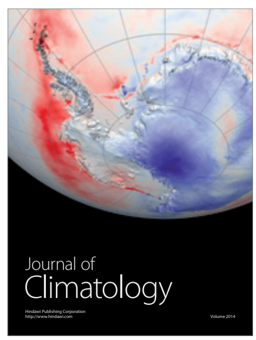
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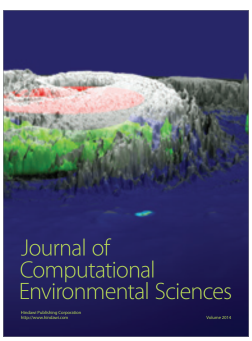
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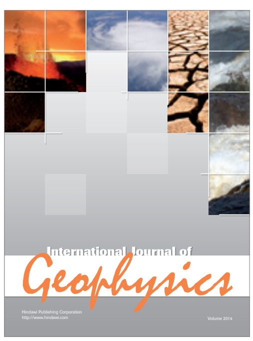
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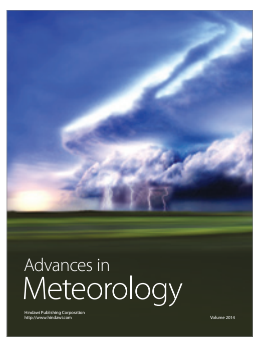
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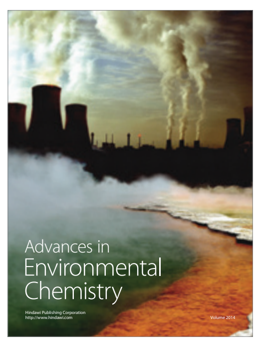
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