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Journal of Environmental Science and Health Part A (2007) **42**, 1–8

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934520701513498

Effects of tetracycline on antibiotic resistance and removal of fecal indicator bacteria in aerated and unaerated leachfield mesocosms

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Antibiotics can be present in low concentrations in domestic wastewater, but little is known about their effect on bacteria in onsite wastewater treatment systems. Mesocosms, consisting of soil-filled lysimeters representing the leachfield of a septic system under aerated (AIR) and unaerated (LEACH) conditions, were used to study the effects of tetracycline addition (5 mg L^{-1}) to septic tank effluent on tetracycline resistance in the fecal indicator bacteria *Escherichia coli* and fecal streptococci, and on their removal. The mesocosms were dosed with antibiotic for 10 days, and effects monitored for 52 days. The fraction of resistant bacteria in mesocosm drainage water relative to that in septic tank effluent, Γ_{Res}, for *E. coli* ranged from 0 to 0.66 in the AIR treatment and from 0 to 3.32 in the LEACH treatment. For fecal streptococci, Γ_{Res} ranged from 0 to 0.41 and from 0.63 to 1.06 in the AIR and LEACH treatments, respectively. No significant differences in antibiotic resistance of fecal indicator bacteria were observed among sampling dates in soil or water from either treatment. Tetracycline had no significant effect on removal of fecal indicator bacteria, which ranged from 99.9% to 100% for *E. coli* and from 95.9 to 100% for fecal streptococci. Our results suggest that short-term addition of tetracycline at environmentally-relevant concentrations is likely to have minimal consequences on pathogen removal from wastewater and development of antibiotic resistance among pathogenic bacteria in leachfield soil.

Keywords: tetracycline, leachfield, antibiotic resistance, fecal indicator bacteria.

Introduction

Approximately 23% of households in the United States rely on onsite wastewater treatment systems (OWTS) for disposal of wastewater.[1] In conventional septic systems this is accomplished by passage of wastewater through a septic tank—where settling of solids and some anaerobic digestion occurs—and dispersion of septic tank effluent (STE) in a soil absorption trench, or leachfield, where effluent percolates through the soil before reaching the groundwater. As this happens, contaminants in STE are removed or transformed by various physical, biochemical, and microbial processes. Pathogens, including bacteria, viruses, and eukaryotic parasites, are partially removed from the effluent via filtration, predation, and exposure to adverse conditions.[2] Much of this is thought to take place mainly in the biomat, a restrictive layer of microbial growth, slime, and trapped sediment that develops at the wastewater–soil interface.[2,3]

When site conditions do not allow for the installation of a conventional septic system, or an existing system fails, an alternative system may be used. One emerging technology—the alternative system used in this study—involves aeration of the leachfield soil, which improves hydraulic function.[4] In a study of aerated vs. unaerated leachfield mesocosms, Potts et al.[5] found that aeration also increases the removal of nitrogen, fecal coliform bacteria, and *Escherichia coli* compared to unaerated leachfield soil.

Wastewater can contain antibiotics, many of which pass through the human body and are excreted largely unaltered.[6] For example, Yang et al.[7] found tetracycline concentrations in the influent of a wastewater treatment plant in the range of 0.05–0.27 μg L^{-1}, and Costanzo et al.[8] found concentrations of cephalexin in wastewater influent averaging 2.0 μg L^{-1}. The presence of antibiotics in environmental matrices may lead to the development of reservoirs of antibiotic resistant bacteria. It may also disrupt microbial processes, such as the removal of nitrogen and organic carbon from wastewater, and the ecological interactions involved in pathogen removal. In the case of a septic system

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Received March 19, 2007.

leachfield, where high levels of human pathogenic bacteria may be present, the development of antibiotic resistance is of concern because illnesses caused by resistant bacteria may be difficult to treat.[9]

Antibiotic resistance can develop through several different mechanisms, including transformation, conjugation, and transduction, but is thought to be most easily developed when a bacterial population is exposed to low concentrations of the antibiotic.[10] This is likely to occur when antibiotics are used domestically and excreted and diluted with the household wastewater. In this situation, bacterial populations are exposed to low levels of an antibiotic continuously over the time the antibiotic is being taken—generally for 1 to 2 weeks—which can lead to selection for resistant bacteria. Resistance can be transferred to other surviving bacteria.[6,11] These bacteria, some of which may be human pathogens, may then be released to the environment, especially in the case of improperly functioning OWTS, increasing public health risks.

We conducted a mesocosm-scale study using lysimeters filled with soil with headspace that was vented to a leachfield (LEACH) or aerated (AIR). Septic tank effluent amended with tetracycline was used to dose the lysimeters regularly for a period of 10 days. Tetracycline was chosen as the antibiotic for evaluation because (i) it has been shown to persist in the environment by adsorbing to soils,[12,13] (ii) it continues to exhibit antibacterial activity while bound to the soil,[14] and (iii) it is a broad-spectrum antibiotic, effective against both Gram-negative (e.g. *E. coli*) and Gram-positive (e.g., fecal streptococci) bacteria.[15]

The objectives of this work were to (i) evaluate the development of tetracycline resistance in *E. coli* and fecal streptococci (*Enterococcus faecalis* and *Enterococcus faecium*) in leachfield soil and drainage water from this soil during the 10-day tetracycline-addition period and for a subsequent recovery period, (ii) determine whether the addition of tetracycline to septic tank effluent affects the removal of *E. coli* and fecal streptococci as this effluent passes through soil, and (iii) assess whether tetracycline resistance in, and removal of, fecal indicator bacteria differed among aerated and unaerated mesocosms.

Materials and methods

Experimental facility

The study was conducted at a research facility in Westbrook, CT, USA adjacent to a two-family home fitted with a conventional septic system. The home was inhabited continuously by 3 to 6 people during the study. Details of the experimental facility can be found in Potts et al.[5] Briefly, STE was diverted to an air-tight storage tank (1,325 L capacity; residence time ∼2 d) situated in a climate-controlled (17–19°C) room above the laboratory and mixed at regular intervals using a pump. Every 6 h effluent from the storage tank was pumped to dosing tanks in the laboratory, which was also climate-controlled (18–20°C). The wastewater dose (12 cm d^{-1}) flowed by gravity into a series of stainless steel cylindrical lysimeters (35.6 cm i.d., 61.0 cm height). The bottom of the lysimeters was filled with 7.5 cm of No. 4 synthetic silica sand (dia. = 0.71 to 0.21 mm, uniformity coefficient < 1.8). A total of 30 cm of soil (a mixture of B and C horizon soil (sandy-skeletal, mixed, mesic, Typic Udorthent; 92% sand, 8% silt; pH of 7.6 from Kingston, RI)) was placed over the sand. This soil is typical of that used in leachfield construction in Rhode Island. The remaining space in the lysimeters (∼22 cm) constituted the headspace.

The mesocosms began receiving wastewater on 13 August, 2003 at a rate of 4 cm d^{-1}. On 22 June, 2004, this rate was increased to 12 cm d^{-1}, remaining constant for the duration of the experiment.

Aeration treatments

The headspace of mesocosms was either vented to the leachfield trench of the adjacent house to simulate a conventional leachfield atmosphere (LEACH treatment) or was supplied with air at regular intervals to maintain an O_2level of ∼0.21 mol mol^{-1} (AIR treatment) using a blower. This resulted in a slight (2.5–6.7 kPa) positive pressure. Each treatment was replicated 3 times.

Antibiotic addition

Mesocosms were dosed with STE amended with tetracycline (final concentration ∼5 mg L^{-1}) every 6 h for 10 days, beginning on 13 June, 2005 at 3 p.m. (Day 0). The level of tetracycline in STE was chosen to represent the approximate concentration resulting from a household with two people each taking 1,000 mg tetracycline d^{-1}, assuming 2.57 persons per household[1] each generating 192 L of STE d^{-1}[2] and assuming no degradation occurs in the septic tank. A peristaltic pump was actuated by a solenoid valve every 6 h to deliver ∼28 mL of tetracycline stock solution (500 mg tetracycline·HCl L^{-1} (CAS 64-75-5, Sigma Aldrich, USA)) to a horizontal distribution pipe within the mesocosms, coincident with STE dosing. This resulted in mixing of the antibiotic stock solution with STE.

Water sampling

Collection of drainage water from the mesocosms coincided with the 9 a.m. effluent dose. Sampling took place on 3, 15, 17, 21, 23, 28 June, 7 and 21 July, and 3 August 2005, corresponding to 0, 2, 4, 8, 10, 15, 24, 38 and 51 days after initial addition of antibiotic, respectively. Samples were collected in autoclaved (121°C, 15 min), 1-L polypropylene screw-cap bottles that were purged with N_2 gas prior to sample collection to preclude reactions involving O_2. To ensure that the water sample in the bottle continued to be exposed to the same atmosphere as that in the lysimeter, a connection was made to the headspace of the lysimeter. STE was collected from a sampling port in the supply line prior to entering the mesocosms.[5]

Soil sampling

On Days 0, 11, and 52, approximately 2 h after the 9 a.m. dosing event, ponded water on the soil surface of the LEACH mesocosms was removed by siphoning, and five soil cores (2.75 cm dia., 4 cm deep) were taken from each lysimeter using surface-sterilized (70% methanol), cut-off, 60 mL plastic syringes. Soil cores were placed in sterile Whirl-Pak® bags and kept on ice for transport to the laboratory in Kingston, RI (∼1 h).

Analysis of water for fecal indicator bacteria

Drainage water from mesocosms and STE samples were analyzed for *E. coli* and fecal streptococci using a standard membrane filtration method.[16] *E. coli* were selected for using mTEC agar (BD Difco) and incubation in a water bath at 44.5°C for 24 h. Colonies were confirmed to be *E. coli* using a urea test. Fecal streptococci were selected using KF Streptococcus agar (BD Difco) and incubation at 35°C for 48 h. Development of tetracycline resistance was monitored using mTEC and KF Streptococcus plates containing tetracycline (10 mg L^{-1}). A detection limit of 4 colony counts per plate (either unamended or tetracycline-amended) was employed, and plates with colony counts below the limit were assigned a value of zero. Given the relatively low numbers of fecal indicator bacteria generally found in mesocosm drainage water,[5] this approach was less likely to overestimate both the number of *E. coli*/fecal streptococci and the level of resistance.

Because the number of fecal indicator bacteria in STE inputs varied over time, resistance of bacteria in drainage water samples was expressed as a ratio, Γ_{Res}, calculated using Eq. 1:

$$\Gamma_{\text{Res}} = \frac{F_{Drainage}}{F_{STE}} \tag{1}$$

where F_{Drainage} and F_{STE} correspond to the fraction of antibiotic resistant bacteria in lysimeter drainage water and in septic tank effluent, respectively. Removal rates for *E. coli* and fecal streptococci were determined by comparing numbers of bacteria in drainage water with those in STE.

Analysis of soil for fecal indicator bacteria

E. coli and fecal streptococci were extracted from 10 g soil (fresh weight) using a 1:10 soil:saline buffer ratio.[17] The extract was analyzed for *E. coli* and fecal streptococci using the membrane filtration method.[16]

Effects of tetracycline concentration on E. coli *in soil*

Soil cores were taken aseptically (as described previously) from LEACH and AIR mesocosms that had not been dosed with tetracycline. The cores were divided into 10 g (fresh weight) aliquots and placed in autoclaved, 38-mL amber glass bottles. Each bottle was dosed with 5 mL of aqueous solution containing 0, 10, 40, 400, or 4,000 mg of tetracycline L^{-1} (corresponding to 0, 0.006, 0.03, 0.3, or 3.0 mg tetracycline g^{-1} dry soil, respectively). Bottles containing soil from the AIR treatment were loosely capped with foil, whereas those with soil from the LEACH treatment were sealed with rubber septa and the headspace gases exchanged twice with gases collected from the headspace of a LEACH lysimeter. The contents of the bottles were mixed by shaking and incubated for 24 h at room temperature. All treatments were replicated 3 times. After incubation, *E. coli* were extracted and enumerated as described previously.

Statistical analyses

The Kolmogorov-Smirnov test was used to determine whether populations were normal. To identify differences in tetracycline resistance among sampling dates, a one-way repeated measures ANOVA was performed on from AIR and LEACH treatments. A Z-test was employed to determine whether the value of Γ_{Res} was significantly different from 1. A Friedman repeated measures ANOVA on ranks was performed on removal rates of *E. coli* and fecal streptococci because these data were not normally distributed. Multiple comparisons were performed using Tukey's test. The effects of tetracycline concentration on numbers of *E. coli* were determined on log transformed data using Student's *t*-test with comparison to a control. SigmaStat® software (SPSS Inc., Chicago, IL) was used for all statistical analyses. The *P*-value for all analyses was <0.05.

Results and discussion

Water quality parameters and composition of headspace gases

Typical water quality data and headspace gas composition for AIR and LEACH treatments can be found in Potts et al.[5] and Patenaude.[18] Briefly, the headspace of LEACH mesocosms had elevated levels of CH_4, H_2S, and CO_2, whereas levels of O_2were considerably below ambient, conditions typical of leachfield soil environments.[19] Dissolved oxygen (DO) in drainage water was significantly lower in LEACH than in AIR mesocosms, with high levels of Fe^{2+} observed in drainage water from the former. In contrast, aerobic conditions were maintained in AIR mesocosms throughout the course of the experiment, as evidenced by ambient levels of O_2 in the headspace, near-saturation levels of DO in drainage water, and the absence of Fe^{2+} in drainage water.

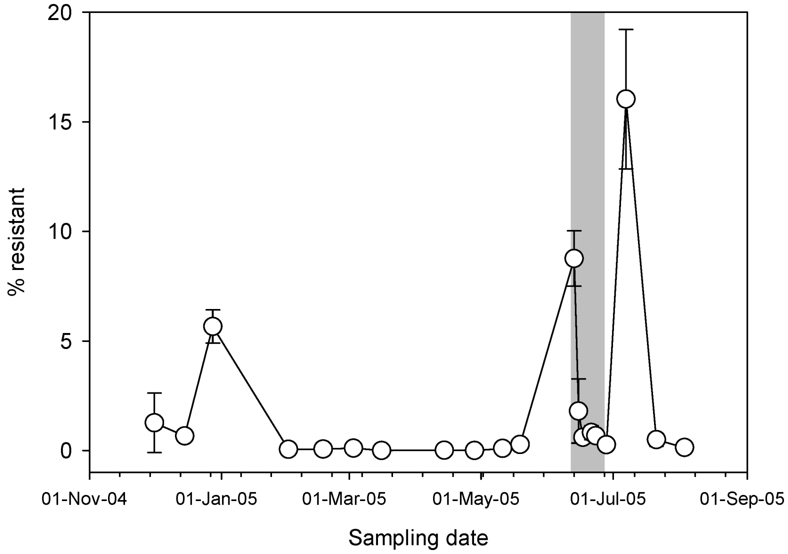

Fig. 1. Tetracycline resistance in *E. coli* in septic tank effluent prior to, during, and after tetracycline addition experiment. Bars represent on standard deviation (n = 3). Shaded area indicates tetracycline dosing period.

Antibiotic resistance of E. coli *in STE*

Monitoring of STE for tetracycline resistance prior to, during, and after tetracycline dosing indicated that the fraction of resistant *E. coli* present varied, ranging from <1% to greater than 16% over the course of approximately 8 months, with maxima of 6, 8 and 16% resistance observed (Fig. 1). Although the highest resistance value was observed after the experimental addition of tetracycline, this value could not be related to antibiotic addition because STE was sampled from a point in the system before it was mixed with antibiotic in the mesocosms. Reinthaler et al.[20] found that resistance rates of *E. coli* to tetracycline in the influent of three separate wastewater treatment plants ranged from 6 to 29%. Harwood et al.[21] found the fraction of fecal coliforms from wastewater treatment plant influent that were resistant to 20 mg L^{-1} chlortetracycline to be >35%. These

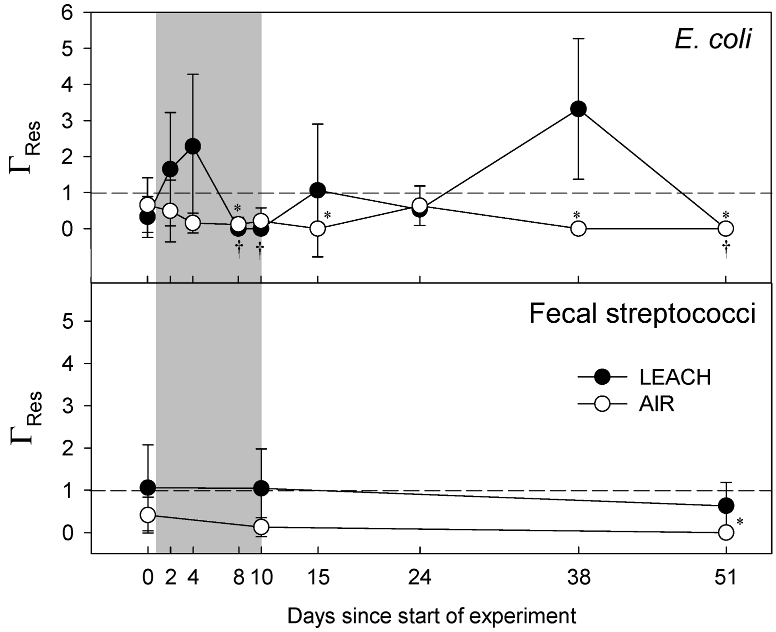

Fig. 2. Effects of tetracycline addition (5 mg L^{-1}) on Γ_{Res} in aerated (AIR) and unaerated (LEACH) mesocosms. Shaded area indicates tetracycline dosing period. Bars represent one standard deviation (n = 3). Dashed line indicates equal antibiotic resistance in inputs and outputs ($\Gamma_{Res} = 1$). Values of Γ_{Res} that are significantly different from 1 are indicated with a (*) for the AIR treatment and with a (†) for the LEACH treatment.

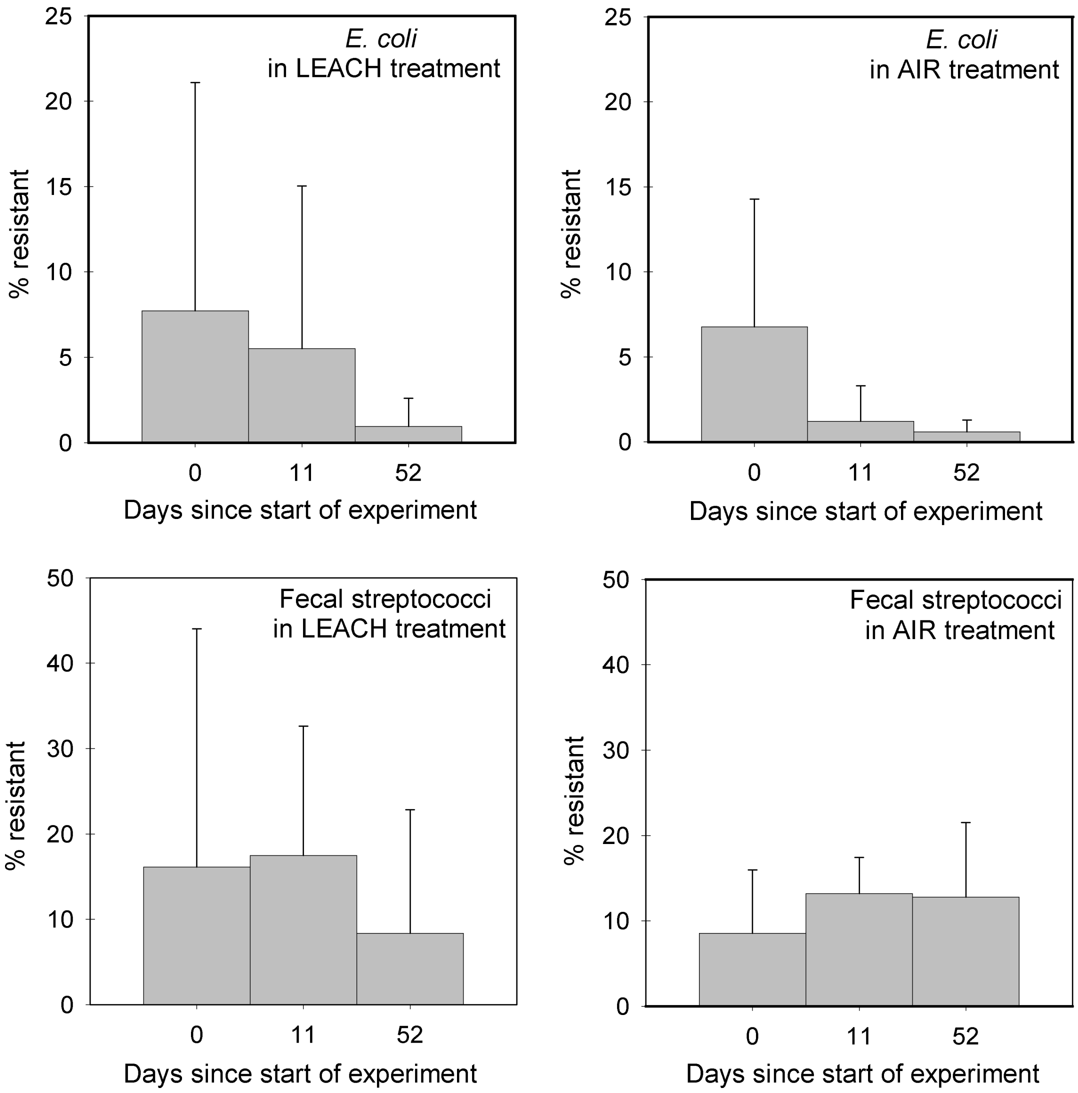

Fig. 3. Effects of tetracycline addition (5 mg L^{-1}) on fraction of tetracycline-resistant *E. coli* and fecal streptococci in soil from aerated (AIR) and unaerated (LEACH) mesocosms. Bars represent one standard deviation (n = 3).

levels of resistance are considerably higher than those we observed in our system, which served two households. The difference likely results from the large number and diversity of wastewater sources that contribute to wastewater treatment plant influent, which increases the probability of finding resistant bacteria. In addition, exposure of bacteria to antibiotics is likely to be more prolonged and sustained in plant influent, increasing the probability for development of resistance.

Effects of tetracycline on antibiotic resistance

To account for the temporal variation observed in the total number and fraction of resistant indicator bacteria in STE (Fig. 1), a resistance ratio, Γ_{Res}, was calculated according to Eq. 1. This ratio represents the fraction of tetracycline-resistant bacteria mesocosms drainage water relative to the fraction of resistant bacteria in STE. There were no significant differences in the value of Γ_{Res} among sampling dates in either the AIR or the LEACH treatments (Fig. 2). If the fraction of resistant bacteria in STE is equal to that in mesocosm drainage water, Γ_{Res} should equal 1.

A value greater than 1, observed on four of nine sampling dates in the LEACH treatment for *E. coli* and one of three dates for fecal streptococci (Fig. 2), suggests that the bacterial population in drainage water had a higher fraction of resistant bacteria than those in STE inputs. However, Γ_{Res} was not significantly different from 1 on those dates. In most instances, Γ_{Res} was significantly lower than 1, indicating that bacterial populations in drainage water had a lower fraction of resistant bacteria than those in STE inputs. The low number of fecal indicator bacteria normally found in drainage water (after removal during passage through the soil[5]) may have contributed to an apparent decrease in resistance by

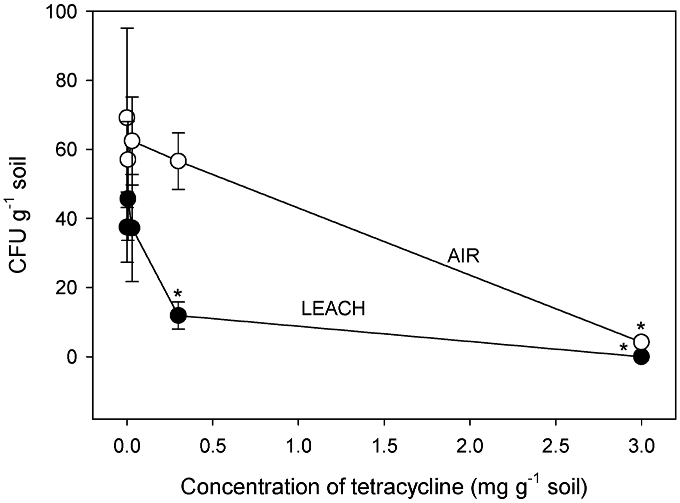

Fig. 4. Effects of tetracycline concentration on *E. coli* in soil from aerated (AIR) and unaerated (LEACH) mesocosms. Bars represent one standard deviation (n = 3). Asterisks indicate significant difference from control treatment (tetracycline concentration = 0 mg g^{-1} soil).

lowering the probability of detecting resistant bacteria. Our results are in contrast to those of Mezrioui and Baleux[22] who found that passage of bacteria through an aerobic wastewater treatment lagoon significantly increased the percentage of antibiotic resistant fecal coliforms. They hypothesized that this effect may be due both to acquisition of resistance plasmids during residence time in the lagoon and to selection for antibiotic resistant bacteria when they are exposed to adverse environmental factors in the lagoon (i.e., possession of a resistance plasmid confers fitness when exposed to stresses other than antibiotics).

The effect of tetracycline addition on tetracycline resistance among *E. coli* and fecal streptococci in soil from the AIR and LEACH mesocosms was also determined (Fig. 3). No significant differences in resistance of either indicator bacteria were among sampling dates in either treatment. However, the fraction of resistant *E. coli* found in soil did decrease with time in both treatments. There was no significant correlation between the fraction of resistant *E. coli* in STE and that found in soil, suggesting that the population of resistant *E. coli* in soil was not affected by resistant *E. coli* in STE.

Our results do not support the hypothesis that antibiotic resistance will develop in soil lysimeters following a 10-day exposure to low concentrations of tetracycline. The concentration of antibiotic used in this experiment may not have been high enough to inhibit the growth of non-resistant (susceptible) bacteria, a condition required to select for resistant bacteria. A selective antibiotic concentration is one that exceeds the MIC of the susceptible population, but not that of the antibiotic-resistant population.[11] For a bacterium to be considered sensitive to tetracycline—according to the guidelines of the Clinical and Laboratory Standards Institute[23]—the MIC is ≤ 4 mg L^{-1}.

We conducted an experiment to determine the concentration at which tetracycline affected the number of *E. coli*

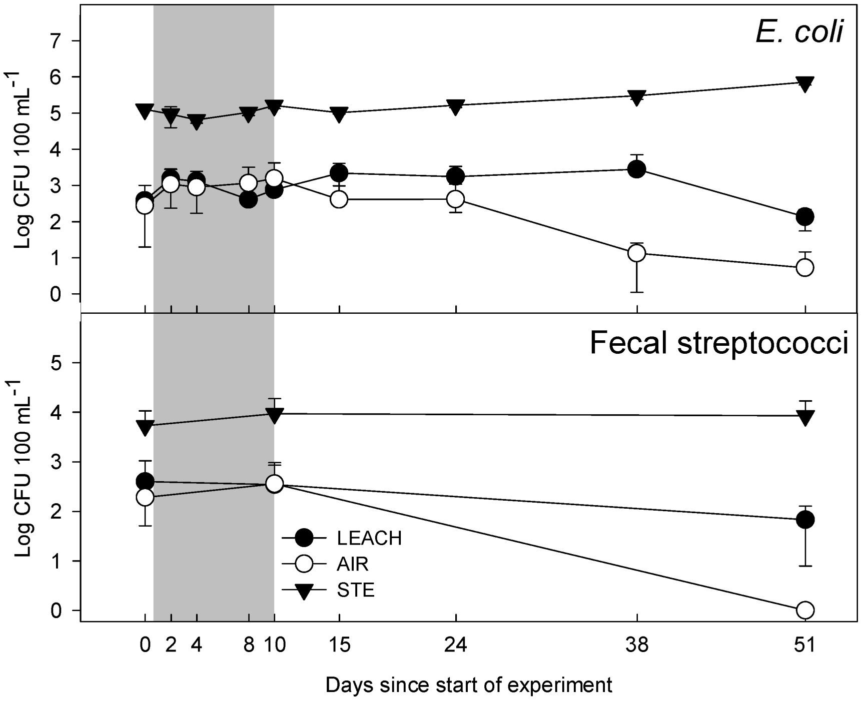

Fig. 5. Numbers of *E. coli* and fecal streptococci in septic tank effluent and drainage water from aerated (AIR) and unaerated (LEACH) mesocosms as a function of time. Shaded area indicates tetracycline dosing period. Bars represent one standard deviation (n = 3).

using soil from unamended mesocosms. Tetracycline had to be added to both AIR and LEACH soil at a rate of 0.3 mg g^{-1} soil (equivalent to 400 mg L^{-1}) for inhibition to be apparent, and at 3.0 mg g^{-1} soil (equivalent to 4,000 mg L^{-1}) to completely inhibit growth (Fig. 4). If we assume, based on the strong tendency of tetracycline to adsorb to soil particles,[24] that all tetracycline added to the soil in the mesocosms was retained in the top 10 cm, the addition of tetracycline at a concentration of 5 mg L^{-1} for 10 days would have resulted in a final concentration of 0.0381 mg tetracycline g^{-1} soil. This is an order of magnitude lower than the concentration necessary to partially inhibit growth of *E. coli* in our experiment. If growth of tetracycline-susceptible bacteria was not limited in our mesocosms, resistant bacteria would not be selected for, explaining the absence of an effect of tetracycline on resistance in soil or drainage water.

Effects of tetracycline on removal of E. coli *and fecal streptococci*

Average removal rates for *E. coli* ranged from 99.92 to 99.99% in the LEACH treatment, a reduction of 2–3 log units (Fig. 5), and 99.94 to 100% in the AIR treatment, a reduction of 2–5 log units. Although not significantly different, the rate of removal was generally lower in LEACH than in AIR mesocosms, resulting in levels of *E. coli* in LEACH drainage water that were often 1 to 2 orders of magnitude higher than those in AIR (Fig. 5). Average removal rates for fecal streptococci ranged from 95.88 to 99.89% in the LEACH treatment, a reduction of 1–2 log units (Fig. 5), and 96.28 to 100% in the AIR treatment, a reduction of 1–3 log units. There were no significant differences in rate of removal among sampling dates for fecal streptococci or *E. coli* in either the AIR or LEACH treatment. It appears that the mechanisms by which fecal indicator bacteria are removed in leachfield soil, e.g., filtration, predation, and exposure to adverse conditions, are unaffected by the presence of low levels of tetracycline in STE.

Conclusion

Our data suggest that the transient presence of an environmentally relevant concentration of tetracycline (5 mg L^{-1}) in domestic wastewater is unlikely to have a significant impact on receiving leachfield soil with respect to either pathogen removal or development of antibiotic resistance. In addition, the absence of an effect does not appear to be a function of aeration.

Acknowledgments

This research was supported by funds from the Rhode Island Agricultural Experiment Station (Contribution No. 5107) and by a grant from the University of Rhode Island's Office of the Provost to J. Atoyan.

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