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Article

Microbial Community Structure of a Leachfield Soil: Response to Intermittent Aeration and Tetracycline Addition

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Abstract: Soil-based wastewater treatment systems, or leachfields, rely on microbial processes for improving the quality of wastewater before it reaches the groundwater. These processes are affected by physicochemical system properties, such as O_2 availability, and disturbances, such as the presence of antimicrobial compounds in wastewater. We examined the microbial community structure of leachfield mesocosms containing native soil and receiving domestic wastewater under intermittently-aerated (AIR) and unaerated (LEACH) conditions before and after dosing with tetracycline (TET). Community structure was assessed using phospholipid fatty acid analysis (PLFA), analysis of dominant phylotypes using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR–DGGE), and cloning and sequencing of 16S rRNA genes. Prior to dosing, the same PLFA biomarkers were found in soil from AIR and LEACH treatments, although AIR soil had a larger active microbial population and higher concentrations for nine of 32 PLFA markers found. AIR soil also had a larger number of dominant phylotypes, most of them unique to this treatment. Dosing of mesocosms with TET had a more marked effect on AIR than LEACH soil, reducing the size of the microbial population and the number and concentration of PLFA markers. Dominant phylotypes decreased by ~15% in response to TET in both treatments, although the AIR treatment retained a higher number of phylotypes than the LEACH treatment. Fewer than 10% of clones were common to both

AIR and LEACH soil, and fewer than 25% of the clones from either treatment were homologous with isolates of known genus and species. These included human pathogens, as well as bacteria involved in biogeochemical transformations of C, N, S and metals, and biodegradation of various organic contaminants. Our results show that intermittent aeration has a marked effect on the size and structure of the microbial community that develops in a native leachfield soil. In addition, there is a differential response of the microbial communities of AIR and LEACH soil to tetracycline addition which may be linked to changes in function.

Keywords: PLFA; PCR-DGGE; domestic wastewater; intermittent aeration; tetracycline

1. Introduction

An understanding of how microbial communities respond to changes in physicochemical conditions and disturbances is necessary for effective development and management of innovative soil-based wastewater treatment systems. Although microorganisms are universally acknowledged as key components in the treatment of septic tank effluent (STE) in soil-based systems, information about the size, structure and function of these microbial communities—and their response to changes in environmental conditions—is scant. This is in contrast with biological processes in centralized wastewater treatment plants, to which state-of-the-art molecular techniques have been applied to elucidate the structure and function of the microbial communities involved in wastewater renovation for some time [1].

Early studies examining microbial populations of soil absorption systems employed culture-based methods [2,3]. Culture-based analyses of the microbial community, although a useful first step, provide limited information, since only a fraction of the community—that amenable to growth under the conditions provided – can be analyzed using this approach [4]. Culture-based analyses of microbial communities can lead to erroneous conclusions regarding the importance of particular organisms in treatment processes and thus ineffective or counterproductive recommendations for their optimization.

Amador *et al.* [5] employed molecular techniques to examine the microbial community structure of soil-based treatment systems using mesocosms filled with synthetic sand. Phospholipid fatty acid (PLFA) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analyses indicated that intermittent aeration affected the size and structure of the microbial community. Proteobacteria and actinomycetes/sulfate-reducing bacteria constituted a higher proportion of the community in the aerated treatment, whereas anaerobic Gram-negative bacteria/firmicutes were more prominent in the unaerated treatment. In addition, higher species richness was found in the aerated treatment. The marked effects of intermittent aeration on community structure of soil-based treatment systems are likely linked with improvements in water quality (e.g., BOD, nutrient and pathogen removal) resulting from aeration [6]. More recently Tomaras *et al.* [7] used 16S rDNA gene sequence analysis to assess microbial community diversity in onsite wastewater treatment systems (OWTS). They reported strong differences in community composition among septic tank effluent, the biomat at the infiltrative surface, and soil that had not received STE. Furthermore, there was no overlap of

sequences between STE and biomat communities, with considerably less phylogenetic diversity in the latter.

In the present study we describe the results of a mesocosm-scale study at an OWTS research facility using mesocosms filled with native soil to simulate conventional and intermittently aerated soil treatment areas. STE amended with tetracycline (TET) was used to regularly dose the lysimeters 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 [8,9]; (ii) it is a broad-spectrum antibiotic used in human medicine that is effective against both Gram-negative and Gram-positive bacteria [10]; and (iii) several of its degradation products also have antibiotic activity [11]. The soil microbial community was characterized using PLFA analysis, PCR-DGGE, and cloning followed by 16S rDNA gene sequence analysis. Differences in community structure were examined between aerated and unaerated soil before the addition of TET, and in response to TET addition for each treatment.

2. Materials and Methods

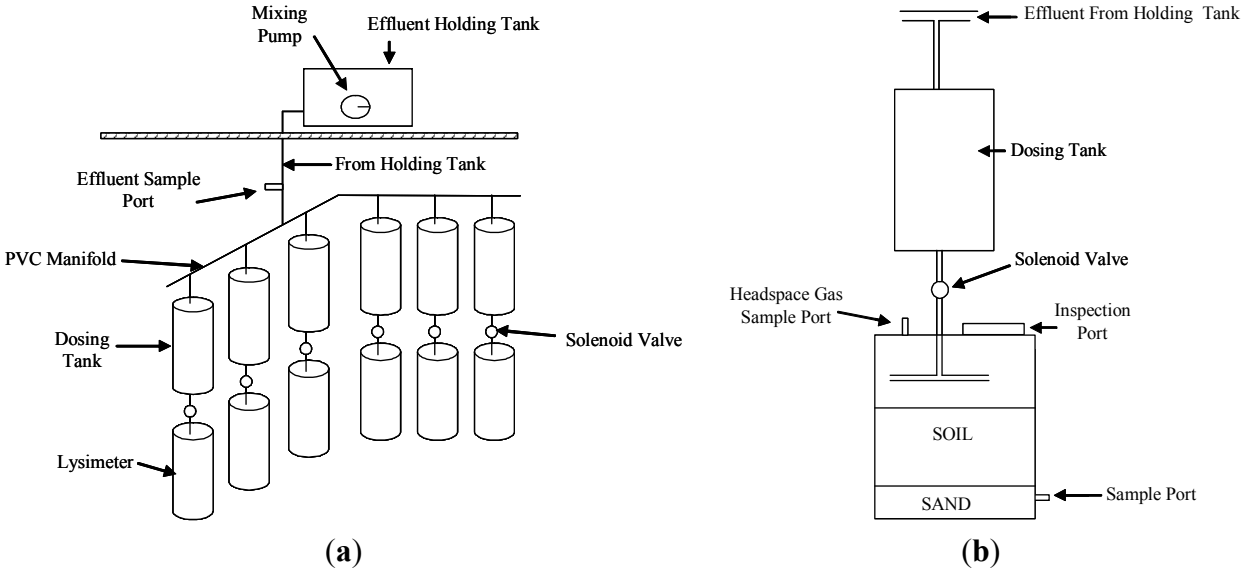
2.1. Experimental Facility

The study was conducted at a research facility in southeastern Connecticut, USA built adjacent to a two-family home fitted with a conventional septic system. Three to six people inhabited the home continuously during the study. A detailed description of the facility can be found in Potts *et al.* [6]. To the best of our knowledge, none of the residents was taking antibiotics during the course of our study. Septic tank effluent was diverted to a high-density polyethylene (HDPE) storage tank (1325 L) above the laboratory in a climate-controlled room (17–19 °C) (Figure 1). STE from the storage tank was pumped every 6 h (3:00 a.m., 9:00 a.m., 3:00 p.m. and 9:00 p.m.) to dosing tanks in the laboratory. Levels of dissolved organic carbon in STE ranged from 71 to 121 mg C L^{-1}. The dose flowed by gravity from these tanks into mesocosms consisting of stainless steel lysimeters (35.6 cm i.d., 61 cm height) filled with a mixture of B and C horizon soil from a sandy-skeletal, mixed, mesic Typic Udorthent (particle size distribution: 92% sand, 8% silt), representative of soil used in OWTS construction in the southern New England, USA region. The soil was homogenized using a cement mixer prior to use. The remaining space constituted the headspace. The dose was delivered to the soil surface through a horizontal PVC pipe in which holes were drilled. The bottom of the mesocosms was filled with 7.5 cm of No. 4 silica sand overlaid with 30 cm of native soil. The mesocosms began receiving wastewater on 13 August 2003 at a rate of 4 cm day^{-1}. On 22 June 2004, this rate was increased to 12 cm day^{-1}, remaining constant for the duration of the experiment.

2.2. Aeration

The headspace of mesocosms was either vented to the septic system leachfield of the house to simulate a conventional leachfield atmosphere (LEACH treatment) or was aerated intermittently with ambient air (AIR treatment) using a process that has been employed successfully to rejuvenate hydraulically-failed septic systems [12]. Each treatment was replicated three times. Air was pumped at regular intervals into the headspace of the AIR mesocosms to maintain O_2 levels close to atmospheric (~0.21 mol mol^{-1}) (Figure 1).

Figure 1. (**a**) Schematic diagram of laboratory facility and (**b**) leachfield mesocosms employed in this study. Drawings are not to scale (after Patenaude *et al.* [13])



2.3. Antibiotic Dosing

Mesocosms were dosed with STE amended with tetracycline (final conc. = 5 mg L^{-1}) every 6 h for 10 days, beginning on 13 June 2005 at 3 p.m. (Day 0). The rationale for antibiotic dosing along with wastewater properties, are described in Patenaude *et al.* [13] and Atoyan *et al.* [14]. To amend the wastewater with TET, an aqueous stock solution (500 mg tetracycline HCl L^{-1}; CAS 64-75-5, Sigma Aldrich, Saint Louis, MO, USA) was prepared and kept at ~8 °C in an insulated container packed with ice and equipped with an IceProbe® thermoelectric water chiller (Coolworks®, San Rafael, CA, USA). A peristaltic pump (Thomas Scientific, Swedesboro, NJ, USA) was actuated by a solenoid valve to deliver ~28 mL of TET stock solution to the horizontal PVC pipe within the lysimeters (Figure 1) every 6 h, coincident with wastewater dosing. This mixed the antibiotic stock solution with the wastewater as it flowed into the lysimeters.

2.4. Soil Sampling

Soil samples (4-cm deep) were collected on Days 0 and 11. Approximately 4 h prior to the 3 p.m. dosing event the access port was opened, and STE on the soil surface of the LEACH mesocosms was removed by siphoning and stored. No STE had accumulated on the soil surface of AIR mesocosms, thus there was no need for removal. Five soil cores (2.75-cm dia., 4-cm height) were taken aseptically from each mesocosm using cut-off, 60-mL plastic syringes. STE was returned to the mesocosms after soil sampling. Soil cores were placed in sterile Whirl-Pak® bags and kept on ice during transport to the laboratory. Immediately upon returning to the laboratory, 50 g of homogenized soil from each mesocosm was shipped on ice by overnight courier to Microbial Insights, Inc. (Rockford, TN, USA) for PLFA analysis. The remaining soil was stored at −80 °C for subsequent analysis.

2.5. Phospholipid Fatty Acid Analysis

PLFAs were extracted using a modification [15] of the method of Bligh and Dyer [16], with one soil sample analyzed per mesocosm. Fatty acid methyl esters were separated by gas chromatography and identified by retention time and mass spectrometry as described by Tunlid *et al.* [17]. The detection limit was 7 pmoles of PLFA. For the purpose of community structure analysis, PLFAs were divided into markers for six different microbial groups [18–21]: (i) firmicutes/anaerobic Gram-negative bacteria, (ii) proteobacteria, (iii) anaerobic metal reducers, (iv) sulfate-reducing bacteria (SRB)/actinomycetes, (v) general bacteria, and (vi) eukaryotes.

2.6. DNA Extraction from Soil

DNA was extracted from ~1 g homogenized soil from each mesocosm using the bead-beating UltraClean Soil DNA Isolation kit (MoBio, Carlsbad, CA, USA) per manufacturer's instructions. DNA was further purified by spin-column chromatography following the protocol for BD Chroma Spin + TE-100 columns (Clontech, Mountain View, CA, USA), and concentrated by ethanol precipitation and resuspension in 20 μL EB buffer.

2.7. PCR-DGGE

Extracted DNA was amplified by polymerase chain reaction (PCR) with the primers 518R (5'-ATT ACC GCG GCT GCT GG-3') and 357F-GC (5'-CCT ACG GGA GGC AGC AGC GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG-3') specific for the 16S rDNA gene of bacteria, modified from Marchesi *et al.* [22] by the addition of a GC clamp [23]. Four PCR reactions were performed for each replicate mesocosm. PCR was performed using the Taq PCR Master Mix kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol with 10 ng of template DNA per 50 μL reaction. PCR was performed in a GeneAmp thermocycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s, and a final extension at 72 °C for 7 min. PCR products were purified and concentrated using the Qiaquick PCR Purification kit (Qiagen). The products from all four PCR reactions from a mesocosm were applied to one column and quantified using an Ultrospec 4000 spectrophotometer (Pharmacia Biotech, Piscataway, NJ, USA).

Approximately 200 ng of PCR product per lane was loaded onto a polyacrylamide gel for generation of community profiles. Electrophoresis was run as described by Muyzer *et al.* [24] using a CBS Scientific DGGE system (Del Mar, CA, USA) on a 0.75-mm thick, 8% (*w*/*v*) polyacrylamide gel with a gradient from 60% to 40% denaturant, where 100% denaturant had a concentration of 7 M urea and 40% (*v*/*v*) formamide. The gel was run in 0.5 × TAE buffer for 16 h at 200 V and 60 °C and stained for 30 min in SYBR Green dye. The gel was visualized using a Typhoon 9410 variable mode imager. Bands were identified using ImageJ software [25] with rolling ball subtraction (r = 10).

2.8. Clone Libraries

Extracted DNA was amplified by PCR with primers B27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3'), specific for the 16S rDNA of bacteria [22]. Four

PCR reactions were performed for each replicate mesocosm. PCR, amplicon purification, and quantification were performed as for PCR-DGGE analysis. Four clone libraries were constructed: one per treatment—AIR and LEACH—for Day 0 and Day 11. Cloning reactions were performed following the standard protocol for the TOPO TA Cloning Kit for Sequencing (Invitrogen, Chicago, IL, USA) using mixed PCR product from each of the three replicates per treatment weighted by the concentration of DNA in each replicate. Approximately 100 colonies were then chosen randomly for sequencing on a Beckman Coulter CEQ 8000 using the primer B27f. Clone library sequences were aligned and chimeric sequences were removed using the NAST alignment tool and Bellerophon [26]. Clones were analyzed for phylogenetic similarity using the Greengenes DNA maximum likelihood (DNAML) classification tool.

2.9. Data Analysis

The Dice similarity coefficient, Cs, was calculated as described by Amador *et al.* [5]. Indices of richness (S) were calculated based on Staddon *et al.* [27]. Paired *t*-tests were used to compare the responses of this variable to TET addition (Day 0 *vs.* Day 11) within a particular treatment. The *p* value for all analyses was <0.05. Principal component analysis was performed on PLFA concentration (expressed as nmoles g^{-1} soil) and the DGGE presence/absence matrix using XLSTAT (Version 2008.1; Addinsoft, New York, NY, USA).

3. Results

3.1. Effects of Intermittent Aeration

3.1.1. PLFA Analysis

A total of 37 different PLFAs were detected on Day 0 from all AIR and LEACH treatments, of which 32 were common to all six mesocosms (data not shown). The active microbial biomass—represented by the total concentration of PLFA in a sample—prior to the addition of tetracycline was approximately twice as high in AIR as in LEACH soil (Table 1) and was significantly different. The main group contributing to total PLFA in both treatments was Proteobacteria, which accounted for a significantly larger proportion of the community in AIR (64%) than in LEACH soil (54%). In addition, the contribution of anaerobic metal reducers to total PLFA was significantly higher in the AIR treatment. General markers for bacteria, SRB/Actinomycetes and Firmicutes/anaerobic Gram-negative bacteria made up a significantly higher fraction of total PLFA in soil from the LEACH treatment. Eukaryotes constituted approximately 3% of the total PLFA in both treatments.

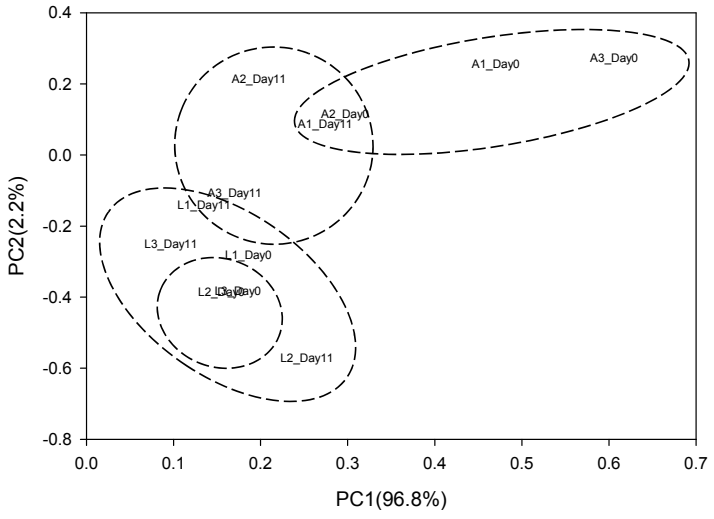
The Dice similarity coefficient (Cs)—computed from a presence/absence matrix of individual PLFAs—was 0.97, indicating a high degree of similarity between AIR and LEACH treatments. When principal component analysis was performed based on the concentration of individual PLFAs, there was clear separation between AIR and LEACH treatments along PC1 and PC2, which explained 96.8% and 2.2% of the variability, respectively (Figure 2).

Table 1. Active microbial biomass and relative amounts of PLFA for different microbial groups in AIR and LEACH soils before (Day 0) and after (Day 11) the tetracycline dosing period. Values are means (n = 3).

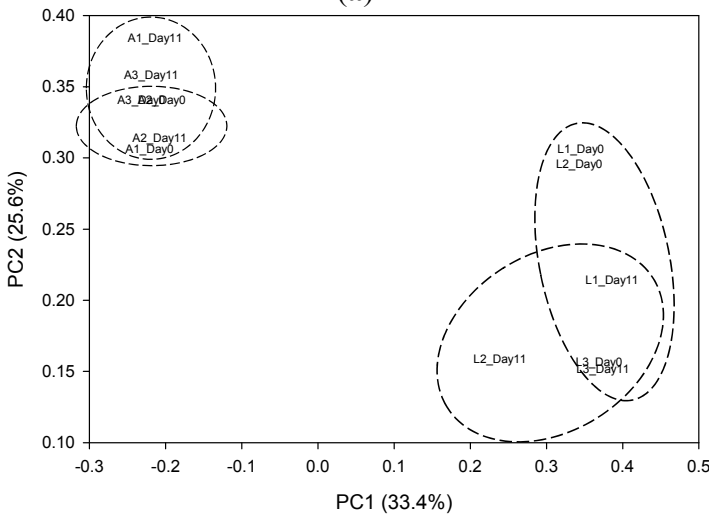
Tmt	Day	Total PLFA concentration [a] (nmol g^{-1} soil)	Community structure					
			Firmicutes/ Anaerobic G^- bacteria	Proteobacteria	Anaerobic metal reducers	Actinomycetes /SRB	General	Eukaryotes
			% of total PLFA					
AIR	0	**117,673**	**9.3**	**63.5**	**2.4**	**0.6**	**21.1**	3.1
	11	55,305	8.5	61.2	2.4	0.8	21.5	5.5
LEACH	0	58,599	13.3	54.2	1.9	1.3	26.8	2.6
	11	53,819	11.9	55.6	1.9	1.3	26.5	2.9

Note: [a] Significant differences between AIR and LEACH treatments on Day 0 are indicated in **bold.**

Figure 2. (**a**) Principal component analysis based on PLFA concentration and (**b**) dominant phylotypes in soil from replicates of intermittently-aerated (AIR; A1, A2, A3) and unaerated (LEACH; L1, L2, L3) leachfield mesocosms before (Day0) and after (Day11) dosing with tetracycline.



(**a**)



(**b**)

3.1.2. PCR-DGGE Analysis

A total of 10 DGGE bands—or dominant phylotypes—were common to all six mesocosms from both treatments (data not shown). An average of 51 bands was detected in AIR soil, of which 49 were common to all three replicates in the AIR treatment. Soil from the LEACH treatment had an average of 27 bands, of which only 16 were common to all three replicates, indicating greater variability in the composition of the microbial community among replicate LEACH mesocosms. Of all the bands detected in all replicates within a treatment, 20 were unique to the AIR treatment and four were unique to the LEACH treatment. Species richness—based on the number of bands detected—was significantly higher in AIR soil (Table 2). The Dice similarity coefficient computed from the PCR-DGGE presence/absence data showed clear differences between soil from the LEACH and AIR treatments, with a Cs of 0.78. Similarly, principal component analysis based on DGGE data clearly separated AIR and LEACH treatments along PC1 and PC2, which explained 33.4 and 24.5% of the variation between treatments, respectively (Figure 2).

Table 2. Richness (S) index based on PCR-DGGE data for intermittently aerated (AIR) and unaerated (LEACH) soil from leachfield mesocosms before (Day 0) and after (Day 11) dosing with tetracycline.

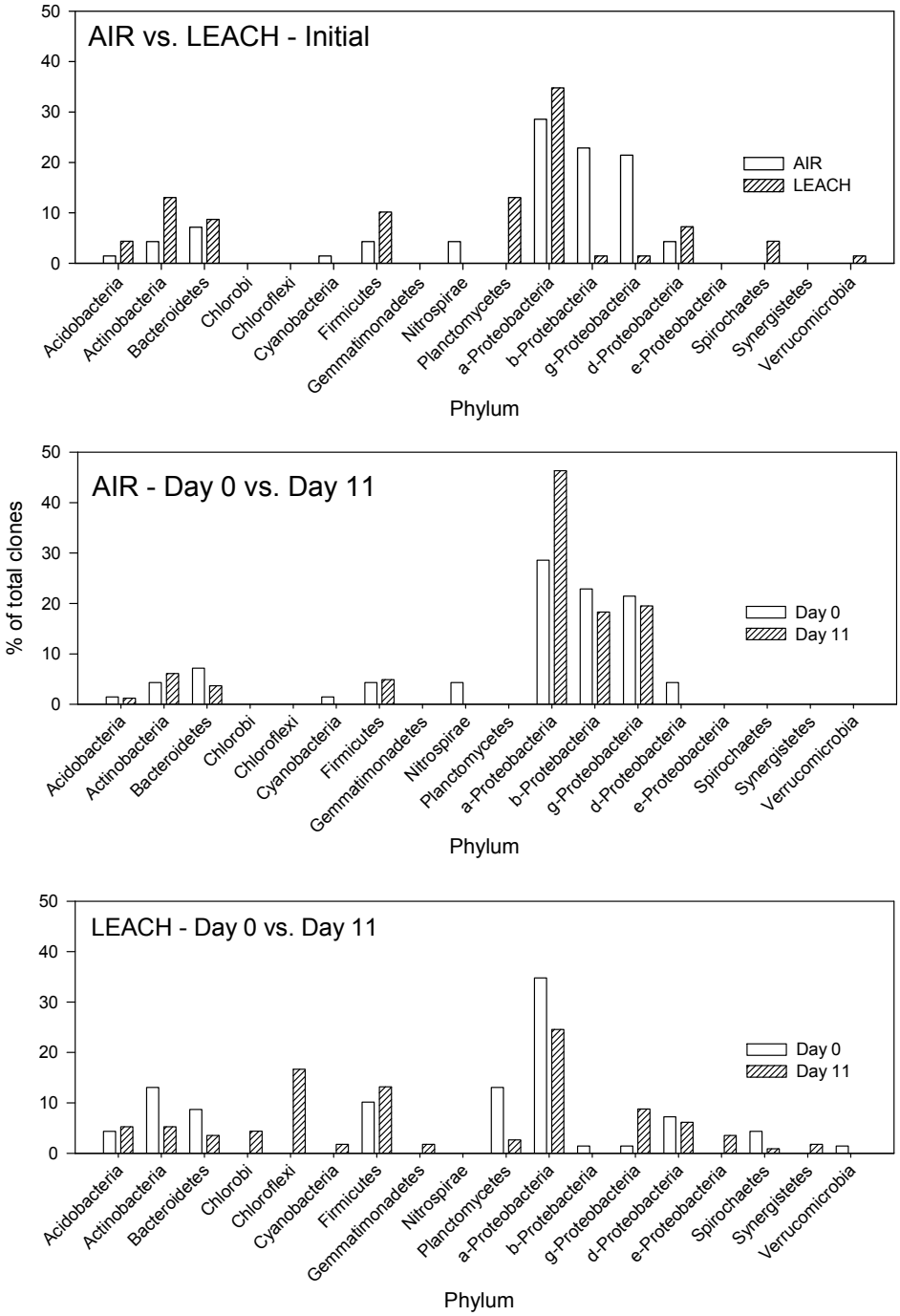
Treatment	Day 0	Day 11
AIR	**50.7**	44.0
LEACH	27.0	23.0

Notes: Significant differences between AIR and LEACH treatments on Day 0 are indicated in **bold**; significant differences between Day 0 and Day 11 within a treatment are indicated by underlining.

3.1.3. Clone Libraries

Analysis of clone libraries also indicated that there were differences in community composition between treatments. Of all the clones obtained, a total of 87 and 82 were sequenced from AIR and LEACH soil, of which 70 and 69 were free of chimeras and subjected to matching. Within these sequences, there were 42 and 48 unique operational taxonomic units (OTUs) in the AIR and LEACH soil, respectively. Bacteria from 10 different phyla were detected in both treatments (7 in AIR and 8 in LEACH soil) (Figure 3). Of these, five were common to both treatments (Acidobacteria, Actinobacteria, Bacterioidetes, Firmicutes, Proteobacteria), with two phyla unique to AIR soil (Cyanobacteria and Nitrospirae) and three unique to LEACH soil (Planctomycetes, Spirochaetes and Verrucomicrobia). As was the case for PLFA analysis, the soil microbial community from both treatments was dominated by Proteobacteria, which accounted for 77% and 45% of all clones in AIR and LEACH soil, respectively (Figure 3). Within this phylum, the class α-Proteobacteria accounted for 29% and 35% of all clones in AIR and LEACH soil, respectively.

Figure 3. Relative distribution of clones in different phyla in soil from intermittently aerated (AIR) and unaerated (LEACH) leachfield mesocosms before (Day 0) and after (Day 11) dosing with tetracycline.



Only four OTUs were common to both treatments, belonging to the β-Proteobacteria, γ-Proteobacteria, Firmicutes, and Acidobacteria. None of these common OTUs met the 97% similarity threshold for identification. Homology with an isolate of known genus and species was observed for 21% of the OTUs from the AIR treatment and 22% of those from the LEACH treatment. Of the clones analyzed from AIR soil, 17 were identified with a particular genus or genus and species (applying a 97% similarity threshold for identification), whereas nine clones from LEACH soil were identified with a genus or genus and species (Table 3).

Table 3. Phylum, genus and species (closest match; similarity ≥ 97%) and potential function for OTUs from intermittently aerated (AIR) and unaerated (LEACH) soil from leachfield mesocosms before (Day 0) and after (Day 11) dosing with tetracycline. Dark squares indicate the presence of an OTU in a treatment.

Phylum	Genus and species	Treatment				Potential function
		AIR		LEACH		
		Day 0	Day 11	Day 0	Day 11	
Acidobacteria	*Terriglobus roseus*		■		■	Extracellular polysaccharide production [28]
Actinobacteria	*Leucobacter komagatae*		■			Biosurfactant production [29]
	Mycobacterium arupense		■			Pathogen [30]
	Mycobacterium sp.	■		■	■	Pathogen; PAH degradation [30,31]
	Rhodococcus coprophilus		■			Phenol degradation [32]
Bacteroidetes	*Flavobacterium succinicans*	■				Cellulose & polysaccharide degradation [33]
Firmicutes	*Bacillus* sp.			■	■	Pathogen; various
	Clostridium sp.			■	■	Pathogen; various
Nitrospirae	*Nitrospira* sp.	■				NO_2^- oxidation [34]
α-Proteobacteria	*Caulobacter* sp.				■	Unknown [35]
	Phenylobacterium sp.	■	■		■	Degradation of chlorinated N-heterocyclics & linear alkylbenzenesulfonates [36]
	Beijerinckia sp.			■		Non-symbiotic N fixation; degradation of aromatic compounds [37]
	Afipia sp.		■	■	■	Pathogen [38]
	Bradyrhizobium elkanii		■	■	■	Symbiotic N fixation [39]
	Nitrobacter vulgaris		■	■	■	NO_2^- oxidation [40]
	Methylocystis parvus	■				CH_4 oxidation [41]
	Methylocystis sp.			■		CH_4 oxidation [41]
	Labrys sp.		■			Unknown
	Erythrobacter sp.	■	■			Aerobic phototrophic bacteria
	Sphingobium sp.		■			Degradation of phenolic compounds [42]
	Sphingopyxis sp.	■				Degradation of polyvinyl alcohols [42]
β-Proteobacteria	*Acidovorax defluvii*		■			Denitrification [43]
	Acidovorax facilis	■				Degradation of polyhydroxyalkanoates [44]
	Thiobacillus sp.		■			Fe, S & S^{2-} oxidation
	Dechloromonas sp.	■	■			Perchlorate reduction [45]
	Rhodocyclus tenuis	■	■			Purple, non-S photosynthetic bacteria; methanol & formate oxidation
	Zoogloea ramigera	■				Extracellular polysaccharide production
δ-Proteobacteria	*Desulfovibrio desulfuricans*			■		SO_4^{2-} & NO_3^- reduction
γ-Proteobacteria	*Legionella pneumophila*				■	Pathogen [46]
	Methylosarcina sp.				■	Methane oxidation [47]
	Pseudomonas stutzeri	■				Pathogen; denitrification; degradation of CCl_4 [48–50]
	Pseudomonas umsongensis	■				Various [51]
	Pseudomonas sp.	■				Various
	Luteibacter rhizovicinus	■			■	Chitin degradation [52]
	Lysobacter sp.	■	■		■	Glucan & chitin degradation [53]
	Thermomonas sp.	■	■			Fe^{2+} oxidation; NO_3^- reduction [54]

3.2. Effects of Tetracycline

3.2.1. PLFA Analysis

The mass of PLFA in the AIR soil declined to 55,305 nmol PLFA g^{-1} soil in response to TET addition, nearly 50% of the value on Day 0. By contrast, total mass of PLFA in LEACH soil declined by only 8% (Table 1). These effects were not statistically significant for either treatment. Total PLFA values were similar for AIR and LEACH treatments after TET dosing. The relative contribution of different microbial groups to total PLFA in soil from the LEACH treatment was not significantly affected by the addition of tetracycline (Table 1).

The total number of PLFAs detected in LEACH soil declined from 37 on Day 0 to 34 after TET dosing. Four previously present PLFA general bacteria markers were absent on Day 11. In addition, one previously absent marker for eukaryotes was present following TET dosing. TET dosing had no significant effect on the concentration the PLFA markers present in LEACH soil on both Day 0 and Day 11, nor did it affect the relative contribution of different microbial groups to total PLFA.

The total number of PLFAs detected in AIR soil declined from 36 on Day 0 to 32 after TET dosing (Table 3). Four previously present general markers for bacteria in AIR soil were absent following TET dosing—these were the same markers lost in response to TET dosing in soil from the LEACH treatment. TET dosing had no significant effect on species richness (Table 2) in the AIR treatment. The contribution of different microbial groups to total PLFA in AIR soil was minimally affected by TET dosing, with only the contribution of Proteobacteria decreasing significantly from 64% on Day 0 to 61% on Day 11 (Table 1).

Principal component analysis performed on individual PLFA concentrations showed separation between Day 0 and Day 11 for the AIR treatment along PC1, which accounted for 96.8% of the variability (Figure 2), but no separation was observed for the LEACH treatment. PC2, which explained 2.2% of the variability, did not separate Day 0 and Day 11 for either treatment.

3.2.2. PCR-DGGE Analysis

The number of dominant phylotypes common to all replicates in both treatments declined from 10 to 4 (data not shown) after TET dosing. An average of 44 bands was present in AIR mesocosms (a decline of ~13%), of which 35 were common to all three replicates. A total of 36 phylotypes persisted in soil from all AIR replicates following TET dosing. One phylotype absent on Day 0 was detected in soil from all three replicates in the AIR treatment on Day 11. The average number of DGGE bands in soil from the LEACH treatment decreased to 23 in response to TET (a decline of ~15%, from an average of 27 on Day 0). Of these, 10 were common to all replicates and no new phylotypes were detected. Species richness based on number of OTUs was significantly lower in both treatments following TET dosing (Table 2). Principal component analysis based on PCR-DGGE data did not separate pre- and post-TET dosing communities in either treatment (Figure 2).

3.2.3. Clone Libraries

The total number of clones sequenced from AIR and LEACH soil after TET addition was 82 and 84, respectively. Of these, 45 unique OTUs were identified in the AIR treatment and 62 in the LEACH treatment. The number of unique phyla in the AIR treatment declined from seven before TET dosing to five after, with Cyanobacteria and Nitrospirae absent following TET dosing (Figure 3). Proteobacteria continued to dominate the distribution of phyla after TET dosing, accounting for 85% and 46% of all clones in AIR and LEACH soil, respectively. Eight different OTUs persisted in soil from the AIR treatment after TET dosing: one Firmicute and seven Proteobacteria (Table 3).

Eight phyla were represented in LEACH soil before TET addition, whereas 12 phyla were present after dosing with antibiotic (Figure 3). Chlorobi, Chloroflexi, Cyanobacteria, Gemmatimonadetes, and Synergistetes were newly detected, whereas Verrucomicrobiales were lost from the community following TET addition. The microbial community of LEACH soil was dominated by Proteobacteria before and after TET dosing, accounting for 45% and 43% of total clones on Day 0 and Day 11, respectively. A total of 18 OTUs persisted after TET addition, belonging to six phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Planktomycetes and Proteobacteria. The persistent OTUs included *Mycobacterium* sp., *Bacillus* sp., *Clostridium* sp., *Afipia* sp., *Bradyrhizobium elkanii*, and *Nitrobacter vulgaris* (Table 3).

4. Discussion

4.1. Effects of Intermittent Aeration

LEACH mesocosms have elevated levels of CH_4, H_2S, and CO_2, and levels of O_2 that are considerably below ambient. In addition, dissolved oxygen (DO) levels in drainage water are low and levels of Fe^{2+} are high [6,13]. By contrast, aerobic conditions prevail in AIR mesocosms, 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 [6,13]. The pH of soil and drainage water of LEACH mesocosms is near-neutral, whereas in AIR mesocosms it is acidic [13]. In addition, levels of dissolved organic carbon in drainage water are consistently higher in LEACH (65 to 105 mg C L^{-1}) than in AIR (6 to 20 mg C L^{-1}) mesocosms [13]. These differences in physicochemical properties and carbon availability within LEACH and AIR mesocosms argue for divergence in community composition, which we observed and discuss below. However, the presence of PLFA markers for the same groups of organisms, as well as shared phylotypes and OTUs found in both treatments, indicates that there is a fraction of the microbial community that is present under both sets of environmental conditions. The disparate conditions under which these organisms are found suggest that many of these are facultative anaerobes capable of tolerating a wide range of pH values and high levels of H_2S, and the presence and absence of O_2. Furthermore, the PLFA markers common to both treatments represent a wide range of active prokaryotic and eukaryotic organisms, and the common OTUs represent three different prokaryotic phyla, suggesting that this tolerance is present across a broad range of taxa.

Beyond the fraction of the microbial community shared by both treatments, there was considerable divergence among these communities in terms of size, richness and diversity. The size of the active microbial population in AIR soil was larger (Table 1), the relative amounts of PLFA contributed by

microbial groups were different (Table 1), and a number of individual PLFA markers were present at higher levels in the AIR treatment. The two communities were clearly separated based on the concentration of PLFA markers and presence/absence of dominant phylotypes by principal component analysis (Figure 2). The AIR soil had a larger number of dominant phylotypes and, of the phylotypes present in all replicates within a treatment, there were 5× more that were unique to the AIR soil community. In addition, only 4.8% of all unique OTUs were common to both treatments. The fact that the soil used in our mesocosms and the STE inputs were the same for both treatments, suggests that differences in microbial community structure are being driven by intermittent aeration.

The larger community size and greater species richness in AIR mesocosms are consistent with the expectations for ecosystems with few physicochemical constraints [55]. In a previous study at the same experimental facility on the effects of intermittent aeration in leachfield mesocosms filled with synthetic silica sand, Amador *et al.* [5] observed differences between AIR and LEACH treatments using PLFA and PCR-DGGE analysis similar to those observed in the present study using mesocosms filled with native soil. The similarities in response to aeration for mesocosms filled with media with such different physical, chemical and biological properties (synthetic sand *vs.* native soil) further suggest that intermittent aeration exerts an important control on the structure of the leachfield microbial communities that develop.

Species accumulation curves indicated that the full diversity of these soils was not covered by the number of clones sequenced (data not shown). Thus we are unable to quantitatively evaluate differences in species composition between AIR and LEACH treatments. Nevertheless, the genus (and in some instances, species) of bacteria found in soil from AIR and LEACH treatments prior to TET addition provide us with a qualitative picture of the presence of pathogens as well as bacteria that may be involved in biogeochemical transformations and metabolism of organic pollutants (Table 3). AIR soil had bacteria in the genus *Mycobacterium* and the species *Pseudomonas stutzeri*, and, in LEACH soil, bacteria in the genus *Mycobacterium*, *Bacillus*, *Clostridium* and *Afipia* were present. All of these genera include species known to be human pathogens. Among bacteria with the capacity to be involved in biogeochemical processes in AIR soil we found *Nitrospira* (nitrite oxidation), *Methylocystis parvus* (methane oxidation), *Flavobacterium succinicans* (cellulose, polysaccharide degradation), *Erythrobacter* (aerobic phototrophic bacteria); *Rhodocyclus tenuis* (purple non-sulfur photosynthetic bacterium; methanol, formate oxidation), *Zooglea ramigera* (extracellular polysaccharide production), *Pseudomonas stutzeri* (denitrification), *Luteibacter rhizvicinus* (chitin degradation), *Lysobacter* sp. (glucan, chitin degradation), and *Thermomonas* (iron oxidation, nitrate reduction). Bacteria involved in biogeochemical processes found in LEACH soil include *Beijerinckia* (non-symbiotic nitrogen fixation), *Bradyrhizobium elkanii* (symbiotic nitrogen fixation), *Nitrobacter vulgaris* (chemoautotrophic nitrite oxidation), *Methylocystis* (methane oxidation), and *Desulfovibrio desulfuricans* (sulfate, nitrate reduction). Bacteria with potential for metabolism of organic contaminants found in AIR soil include *Phenylobacterium* (degradation of N-heterocyclic chlorinated compounds), *Sphingopyxis* (degradation of polyvinyl alcohols), *Acidovorax facilis* (degradation of polyhydroxyalkanoates), *Dechloromonas* (perchlorate reduction), *P. stutzeri* (carbon tetrachloride degradation), as well as *Nitrospira*, *Nitrobacter*, and *Methylocystis*, known to oxidize a variety of aromatic and low-molecular weight halogenated alkanes.

4.2. Effects of Tetracycline

Dosing of mesocosms with TET for 10 days caused a decrease in microbial biomass in AIR mesocosms (to the level observed in the LEACH treatment), whereas TET had no effect on biomass in the latter. The differential effect of TET is likely associated with the physiological state of the microbial community in AIR mesocosms and the mode of action of the antibiotic. AIR soil has been shown to have population densities of bacteriovores (protozoa and nematodes) that are orders of magnitude larger than LEACH mesocosms, and their grazing activities are expected to keep the microbial community in a continuous state of growth [5]. Tetracycline is a bacteriostatic agent—it does not directly kill bacteria but rather prevents protein synthesis, thereby inhibiting their growth [10]. Grazing of bacteria by protozoa and nematodes in AIR soil likely lowers the biomass, and tetracycline prevents bacterial replication, resulting in a greater impact on the active microbial biomass in AIR. By contrast, there is less grazing pressure in the LEACH soil, where protozoa and nematode numbers are lower and bacteria are less likely to be in the growth phase, thus this treatment was less affected by TET dosing.

Species richness generally decreased in both treatments in response to antibiotic dosing, with some of the effects of tetracycline addition on community composition shared by both treatments. For instance, four PLFA biomarkers for general bacteria that were present in soil from both treatments prior to dosing were absent in both treatments following tetracycline addition. In addition, of the 10 dominant phylotypes shared by all replicates in both treatments, six were absent after dosing with tetracycline. Thus, there is a fraction of the microbial community present in both treatments that is susceptible to the effects of tetracycline. However, analysis of dominant phylotypes indicates that a large proportion of the microbial community persists following TET dosing, as indicated by the persistence of ~70% and ~90% of previously present bands in the AIR and LEACH treatments, respectively, following TET dosing.

Beyond the shared responses, there were a number of differences in community structure in response to TET dosing. Whereas dosing had little effect on the relative contribution of different microbial groups to total PLFA in LEACH soil, in the AIR soil it resulted in a significantly lower contribution of Proteobacteria. Furthermore, there were lower concentrations of biomarkers for anaerobic Gram-negative/Firmicutes, anaerobic metal reducers, and general bacteria. PLFA biomarkers whose concentration declined likely represent those organisms that were actively growing in soil. These results also suggest that TET affects most of the groups that make up this community, as expected for a broad spectrum antibiotic. The overall effects of TET on AIR soil communities—as measured by PLFA analysis—are likely the result of shared susceptibility to the antibiotic and/or indirect effects of TET, such as selection for resistant bacteria.

The detection of OTUs and PLFAs only after TET dosing in soil from both treatments suggests that some of the effects of the antibiotic on these microbial communities are indirect. For example, TET dosing may have suppressed competing organisms, allowing otherwise less competitive—but TET-resistant—organisms to grow in numbers. Alternatively, TET may be used as a carbon source by some bacteria, as has been shown for a number of other antibiotics in soil [56], selecting for organisms capable of this function. These interpretations must be tempered by the limitations of the PCR-based methods used, which tend to result in a picture of the bacteria community that is skewed towards the most numerous organisms. Thus, lack of detection of an OTU prior to TET addition may not be due to its absence from soil, but rather to its low population density. Independent of mechanism, the eleven

OTUs that were detected only after TET addition to AIR soil (Table 3) were associated with a variety of potential functions, including pathogens (*Mycobacterium arupense*, *Afipia* sp.), degradation of aromatic compounds (*Rhodococcus coprophilus*, *Sphingobium* sp.), production of surfactants and polysaccharides (*Terriglobus roseus*, *Leucobacter komagatae*), nitrogen cycling (*Bradyrhizobium elkanii*, *Nitrobacter vulgaris*, *Acidovorax defluvii*), and iron and sulfur transformations (*Thiobacillus* sp.). The seven OTUs found only in LEACH soil after TET dosing (Table 3) also represented a variety of potential functions, including pathogens (*Legionella pneumophila*), extracellular polysaccharide production (*Terriglobus roseus*), degradation of heterocyclic compounds (*Phenylobacterium*), methane oxidation (*Methylosarcina* sp.), and degradation of chitin and glucans (*Luteibacter rhizovicinus*, *Lysobacter* sp.).

The differential effects of TET dosing on the community structure of AIR and LEACH soil would be expected to affect the community function in these ecosystems. For example, our data for the AIR mesocosms—although limited in terms of genus and species identified with a particular function (Table 3)—suggest that a number of processes in this treatment may be unaffected by TET dosing (e.g., Fe oxidation, NO_3 reduction), whereas some may diminish (e.g., degradation of polyhydroxyalkanoates), and others may be enhanced (e.g. phenol degradation). In a companion study Patenaude *et al.* [13] reported lower concentrations of Fe^{2+} and SO_4^{2-} in drainage water and higher levels of H_2S and CH_4 in the headspace of LEACH mesocosms dosed with TET. Effects on iron and sulfate concentrations were apparent for at least six weeks after antibiotic additions ceased, whereas gas levels returned to pre-dosing conditions shortly after dosing stopped. Some of the organisms that disappeared in response to TET dosing in LEACH mesocosms may represent iron-reducing and/or sulfur-oxidizing bacteria susceptible to TET. Changes in H_2S and CH_4 levels suggest that some of the absent organisms were also associated with sulfide- and methane-oxidizing bacteria sensitive to TET, with the transient nature of the effect suggesting eventual recovery of these populations. Our results lend qualitative support to this interpretation, as suggested by the loss of *Methylocystis* sp. and *Desulfovibrio desulfuricans* from the LEACH soil following TET dosing (Table 3). Within AIR mesocosms, a transient decrease in N removal capacity was observed by Patenaude *et al.* [13] in response to TET dosing, which was ascribed to inhibitory effects on nitrification (Patenaude *et al.* [13]). We observed the disappearance of *Nitrospira* sp., which carries out nitrite oxidation, in response to TET dosing of AIR mesocosms (Table 3). In addition, diminished N removal may also be associated with effects on denitrifiers, which could be reflected in the lower concentrations of various PLFAs observed in response to antibiotic dosing, since the capacity to denitrify is associated with a wide range of bacteria [50]. The relatively small effect of TET dosing on the water quality functions of AIR mesocosms [13] is in contrast with the various negative effects of TET on microbial community structure observed in the present study. This disparity may be the result of greater functional redundancy and/or prevalence of TET resistance within the microbial community of AIR soil, which may make OWTS that incorporate this technology more resilient to environmental disturbances.

5. Conclusions

Our results suggest that the microbial communities of intermittently aerated and unaerated leachfield native soil can differ markedly with respect to size and structure. Leachfield soil under

intermittent aeration has a larger active microbial biomass and significantly higher richness and diversity of taxa, as indicated by data from PLFA and PCR-DGGE analysis. Qualitative analysis of community function based on sequencing of OTUs suggests that there may also be differences in the presence or absence of pathogenic bacteria and bacteria involved in elemental cycling and degradation of organic contaminants. Tetracycline dosing appears to have a differential effect on the leachfield communities, with intermittently aerated soil exhibiting greater loss of active microbial biomass and a higher proportional loss of richness and diversity relative to unaerated soil. These data provide evidence that the size, structure and function of the microbial community of leachfield soil can be manipulated by the introduction of air. Furthermore, the introduction of air can also affect the response of the community to disturbances such as short-term exposure to antibiotics relative to unaerated soil.

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