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Mesocosm-Scale Evaluation of Faunal and Microbial Communities of Aerated and Unaerated Leachfield Soil

José A. Amador,* David A. Potts, Mary C. Savin, Peter Tomlinson, Josef H. Görres, and Erika L. Nicosia

ABSTRACT

Aeration improves the capacity of leachfields to decontaminate and reduce the nutrient load of wastewater. To gain a better understanding of the effects of aeration, we examined the faunal and microbial communities of septic system leachfield soil (0–4 and 4–13 cm) using replicated (n = 3) mesocosms that were actively aerated (AIR) or unaerated (LEACH). Protozoa were 40 to 140 times more abundant in AIR than in LEACH soil. No nematodes were found in LEACH soil, whereas AIR soil contained 5 to 14 $\times$ 10^3 nematodes (all bacteriovores) kg^{-1}. Active microbial biomass was four to five times higher in AIR than LEACH soil. Proteobacteria and actinomycetes/sulfate-reducing bacteria constituted a higher proportion of the community in AIR soil, whereas anaerobic Gram-negative bacteria/firmicutes were more prominent in LEACH soil. Ratios of prokaryotic to eukaryotic phospholipid fatty acids (PLFAs) were higher in LEACH soil, as were membrane stress index values, whereas the starvation index was higher in AIR soil. Community-level physiological profiles showed that 29 and 30 different substrates were used for growth by LEACH and AIR soil microorganisms, respectively. The AIR soil had more microorganisms capable of growing on 10 substrates, whereas growth on two substrates was higher in LEACH soil. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) analysis of 16S rRNA gene fragments revealed greater diversity of dominant phylotypes in AIR than LEACH soil, with communities separated by treatment. Aerated leachfield soil had a larger and more diverse faunal and microbial community than unaerated soil, possibly due to differences in the type and availability of electron acceptors.

Almost a quarter of the households in the USA rely on OWTS (on-site water treatment systems) for treatment of domestic wastewater. Originally, OWTS were a means of disposing of large volumes of water and removing pathogens (Anonymous, 1889, 1904). Environmental and public health concerns have changed over the years, such that OWTS are currently expected to reduce nutrient loadings to ground water (USEPA, 2002). Recent concerns about ground water contamination with pharmaceutical and personal care products will probably demand further modifications of OWTS technology for removal of these compounds, requiring a better understanding of leachfield soil ecology.

The ability of soil absorption fields to improve water quality depends largely on the types of organisms present and their relative abundance (structure) and the processes they carry out (function). Organic C removal and nutrient transformations are biochemical processes, primarily performed by microorganisms, whereas soil fauna feeding on microorganisms are thought to control pathogens. The leachfield soil community develops its structure and function from: (i) the organisms originally present in the soil, (ii) colonization of the soil by organisms present in septic tank effluent, (iii) the type and amounts of electron donors and acceptors, (iv) the physical and chemical conditions (e.g., pore size, pH, temperature), and (v) the interactions among members of the food web.

Current understanding of the microbial ecology of leachfield soils, conventional or otherwise, is sparse. A number of studies have examined the presence of specific groups of microorganisms (Calaway et al., 1952; Pell et al., 1990) and of meso- and macrofauna (Brink, 1969) in absorption fields. In terms of function, the emphasis has been on ecological interactions that remove pathogens (Bomo et al., 2004) and affect microbial population dynamics (Woombs and Laybourn-Parry, 1986, 1987), and on nutrient transformations (Walker et al., 1973; Kristiansen, 1981a).

In a previous study we found that active aeration of model leachfields improved the removal of N, fecal coliforms, and BOD_5 (5-d biological oxygen demand) relative to conventional (unaerated) mesocosms (Potts et al., 2004). The intermittent introduction of air appeared to overcome limitations on nitrification, subsequent denitrification, and oxidation of biodegradable C, while enhancing pathogen removal. These improvements were observed in the absence of a clogging layer or biomat. Aeration appeared to cause a major shift in the function of the soil community that improved water quality and reduced the risk of hydraulic failure by clogging. This change in function is probably the result of differences in faunal and microbial community structure.

Because the leachfield aeration technology employed in our study (Potts et al., 2004) is a recent innovation (Potts, 2000), no data are available on its effects on the microbial ecology of leachfield soil. In this study, we characterized the microbivorous fauna (protozoa and nematodes) and microflora associated with soils in the model leachfields under aerated and unaerated (conventional) conditions employed in Potts et al. (2004). Microbial community structure was examined using both culture-independent (PLFA [phospholipid fatty acid] analysis [Guckert et al., 1985], PCR-DGGE [polymerase

J.A. Amador, J.H. Görres, and E.L. Nicosia, Laboratory of Soil Ecology and Microbiology, University of Rhode Island, Kingston, RI 02881. D.A. Potts, Geomatrix LLC, Killingworth, CT 06419. M.C. Savin and P. Tomlinson, Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR 72701. Received 14 Oct. 2005. *Corresponding author (jamador@uri.edu).

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677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: AIR, aerated lysimeters; BOD_5, 5-d biological oxygen demand; CLPP, community-level physiological profile; DGGE, denaturing gradient gel electrophoresis; LEACH, lysimeters vented to leachfield; MPN, most-probable number; PCR, polymerase chain reaction; PLFA, phospholipid fatty acid; SRB, sulfate-reducing bacteria.

chain reaction–denaturing gradient gel electrophoresis; Amann et al., 1995]) and culture-dependent (Biolog EcoPlates) methods, where the latter is more closely linked to function than the former because it measures substrate use. Protozoa and nematodes were characterized using MPN (most-probable number; Ingham, 1994a) and direct enumeration (Ingham, 1994b) techniques, respectively. Analyses were conducted on soil from different depths because soil communities are expected to stratify in systems with unidirectional inputs, such as leachfield soils. The analyses were conducted in aerated (AIR) and unaerated (LEACH) mesocosms to examine the differential effects of the presence of O_2 on the community ecology of leachfield soils. Silica sand was used in our experiment because it is relatively inert and presumed to be devoid of microorganisms, allowing evaluation of the effects of aeration on water quality under more stringent conditions than expected using native soil. Because aeration removes the energetic constraints imposed by the low levels of O_2 found in leachfield soil, we hypothesized that the faunal and microbial communities in aerated leachfield soil would be larger and more diverse than in unaerated soil.

MATERIALS AND METHODS

Leachfield Mesocosms

The study was conducted in a climate-controlled laboratory facility adjacent to a two-family home (3–6 people) in southeastern Connecticut, USA, that was built in 1983. The home was fitted with a new conventional septic system in 1996, which was not pumped during the course of the study. Details of the experimental setting can be found in Potts et al. (2004). Leachfield mesocosms consisted of lysimeters constructed using HDPE (high-density polyethylene) cylinders (43.2-cm i.d., 45.7-cm height) and filled with 7.5 cm of no. 4 silica sand (diameter = 4.75–1.40 mm; uniformity coefficient <1.8), above which was placed 30 cm of no. 00 silica sand (henceforth referred to as *soil*; diameter = 0.71–0.21 mm; uniformity coefficient <1.6;), with headspace constituting the volume above the soil. Lysimeters were dosed with septic tank effluent every 6 h at a rate of 12 cm d^{-1} for the first 24 mo of the experiment. After 24 mo, the dosing rate was changed to 4 cm d^{-1} for an additional 2 mo. Data for headspace gases and water quality before soil sampling are found in Potts et al. (2004).

Treatments

A piston pump was used to introduce ambient air at regular intervals into the headspace of AIR lysimeters to maintain an O_2 level of 0.20 to 0.21 mol mol^{-1}. This resulted in a positive pressure (2.5–6.7 kPa) within AIR lysimeters. To mimic the in situ composition of the atmosphere found in a conventional leachfield, the headspace and the gravel bed below the soil of LEACH lysimeters were vented to a septic system leachfield trench in which the O_2 concentration ranged from <0.021 to 0.16 mol mol^{-1} (Potts et al., 2004). Each treatment was replicated three times.

Soil Sampling and Processing

Soil cores were obtained on 25 June 2003 from the top 13 cm of each lysimeter using a cut-off, 60-mL plastic syringe (2.54 cm i.d., 13.4 cm long) that was surface sterilized with a 70% methanol solution before sampling. Wastewater remaining on the surface of the soil in the mesocosms was siphoned off before sampling. Three cores were removed from each mesocosm within a 5-cm radius. Cores were cut into 0- to 4- and 4- to 13-cm sections, placed in sterile plastic bags, and kept on ice during transport to the laboratory in Kingston, RI. Equal amounts of soil from a single lysimeter were mixed to produce one composite sample per lysimeter for each depth. One composite soil sample per lysimeter and depth was employed for determination of CLPPs (community-level physiological profiles), enumeration of protozoa and nematodes, enumeration of *Escherichia coli*, and analysis of community structure by PCR-DGGE. Analysis of community structure using PLFA was conducted on a single composite sample from all three lysimeters within a treatment. Samples for analysis by PLFA and PCR-DGGE were kept at −80°C and shipped by overnight courier within 24 h of collection to Knoxville, TN, and Fayetteville, AR, respectively. Samples for analysis of protozoa and nematodes were kept at 4°C and shipped to Corvallis, OR, by overnight courier within 24 h of collection.

Faunal Community Analysis

Enumeration and identification of protozoa and nematodes were conducted by Soil FoodWeb (Corvallis, OR). Protozoa were enumerated using a MPN technique (Ingham, 1994a), a method based on determination of the presence or absence of microorganisms in individual portions of several consecutive dilutions of soil (Alexander, 1982), and were grouped into flagellates, ciliates, and amoebae. Nematodes were extracted using the sugar flotation method (Ingham, 1994b) and enumerated and assigned to trophic groups based on microscopic examination. Analyses were conducted on one composite sample from each replicate lysimeter.

Phospholipid Fatty Acid Analysis

The PLFA analysis was performed by Microbial Insights (Rockford, TN). Composite samples from three replicate lysimeters per treatment and depth were used for analyses. The PLFAs were extracted using a modification (White et al., 1979) of the method of Bligh and Dyer (1959). Fatty acid methyl esters were separated by gas chromatography and identified by retention time and mass spectrometry as described by Tunlid et al. (1989). The detection limit was 7 pmol PLFA. For the purposes of community structure analyses, PLFAs were divided into markers for six different groups (Dowling et al., 1986; Edlund et al., 1985; White et al., 1996, 1997): anaerobic Gram-negative bacteria/firmicutes (i14:0, i15:0, α15:0, i16:0, i17:0, α17:0), proteobacteria (16:1ω9c, 16:1ω7c, 16:1ω7t, 16:1ω5c, 17:1ω6c, cy17:0, 18:1ω9c, 18:1ω7c, 18:1ω7t, 18:1ω5c, 19:1α, 19:1β, cy19:0), anaerobic metal reducers (i15:1α, i15:1β, i16:1α, i17:1ω7c, br19:1α), actinomycetes/sulfate-reducing bacteria (br16:0, 10me16:0, 12me16:0, 10me17:0, 11me17:0, 12me17:0/18:2, 10me18:0, 12me18:0), general (14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0), and eukaryotes (18:2α, 18:2β, 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3). The relative contribution of each group to the total PLFA pool was determined from the sum of the amount of all PLFA markers for a particular group divided by the total amount of PLFA in the sample. The biomass ratio of prokaryotes to eukaryotes was determined from the sum of PLFA markers for firmicutes, proteobacteria, anaerobic metal reducers, actinomycetes, and general bacteria divided by the eukaryotic PLFA. The starvation index was determined from the sum of the ratios of cy17:0 to 16:1ω7c and cy19:0 to18:1ω7c (Kieft et al., 1994). The membrane stress index was calculated from the sum of 16:1ω7t/16:1ω7c and 18:1ω7t/18:1ω7c (Guckert et al., 1985, 1986). A factor of 20000

cells pmol^{-1} of PLFA—developed by Microbial Insights—was used to calculate the number of viable organisms.

Community-Level Physiological Profiles

EcoPlates (Biolog, Hayward, CA) were used to determine CLPPs using the method of Gamo and Shoji (1999), which involves determining the MPN of bacteria capable of growing on 31 different substrates. Freshly sampled soil from both depths (1.0 g) was diluted 1:10 with 50 μM phosphate buffer (pH 7.2). The resulting suspension was shaken vigorously in a reciprocal shaker for 2 h (Balser et al., 2002) and the soil allowed to settle before sampling the supernatant liquid. Six 10-fold dilutions were used, with each dilution replicated three times. Well inoculation volume was 150 μL. After inoculation, plates were incubated at 22°C for 6 d. Optical density (OD) was measured at 595 nm with a Model EL311 Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT). The minimum OD for positive wells was 0.30. The MPNs were determined as described by Woomer (1994). Replicates from each treatment/depth combination were analyzed independently.

Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis Analysis

The DNA was extracted from soil following the protocol for the bead-beating FastDNA Spin kit for soil (Qbiogene, Carlsbad, CA) and quantified by labeling with Hoeschst 33258 dye and measuring fluorescence, using calf thymus DNA as a standard. Extracted DNA was used as a template and amplified by PCR with primers 338FGC and 518R (Övreås et al., 1997), which are specific for 16S rRNA gene fragments of eubacteria. The PCR conditions for each 100-μL reaction were: 10 mM Tris–HCl, 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM $MgCl_2$, 0.2 μM each primer, 200 μM each deoxynucleotide, 400 ng μL^{-1} bovine serum albumin, 2.5u of Taq DNA polymerase (Promega, Madison, WI), and 2 μL of template DNA. The PCR was performed in a MJ Research thermal cycler (Waltham, MA) with 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 30 s, and a final extension at 72°C for 20 min. The PCR products were resolved on 1.0% agarose gels stained with ethidium bromide, visualized using a Kodak EDAS 290 system, and analyzed using Kodak 1D software (Kodak, New Haven, CT). The DNA content in agarose gels was quantified by comparison with a precision molecular mass standard. Approximately 1800 ng of PCR product was loaded onto polyacrylamide DGGE gels for generation of community profiles. Electrophoresis was run in a D-Code system (Bio-Rad Laboratories, Hercules, CA) on a 1.5-mm-thick vertical gel containing 8% polyacrylamide (37.5:1 acrylamide/bisacrylamide) with a linear gradient of 55 to 70% denaturant solutions (where 100% is equivalent to 7 M urea and 40% deionized formamide). Gels were run in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA [ethylenediaminetetraacetic acid] at pH 7.6) for 16 h at 70 V and 60°C and stained for 30 min in SYBR Green I dye. Gels were pictured using a Kodak EDAS 290 system and analyzed using Quantity One software (Bio-Rad). All samples were run on a single DGGE gel to enable the software to detect and match bands based on migration distances.

Enumeration of *Escherichia coli*

The number of *E. coli* in freshly sampled soil was determined using the method described by Turco (1994).

Data Analysis

Differences between treatments in the numbers of protozoa, nematodes, and microorganisms growing in the CLPP assay, *E. coli*, and DNA concentration were determined using Student's t-test. A one-way analysis of variance and a pairwise multiple comparison procedure were used to determine differences in diversity indices.

The DGGE and PLFA data were analyzed by constructing a similarity matrix using binary data (presence and absence of bands only) and the Dice similarity coefficient (Cs) was calculated using the equation:

$$Cs = 2j/(a + b) \quad [1]$$

where j is the number of DGGE bands or PLFA markers in common between two samples, a is the number of bands or markers in one sample, and b is the number of bands or markers in the second sample. Dendrograms for DGGE data were created using Quantity One software (Bio-Rad) after calculating the distance, using neighbor joining as the cluster method. Indices of diversity (H) and evenness (E) were calculated based on Staddon et al. (1997).

RESULTS

Faunal Community Analysis

Nematodes and protozoa were present in significantly higher numbers in AIR than in LEACH treatment soil at both depths (Table 1). The total number of protozoa in surface AIR soil was more than 40 times that in LEACH soil, and more than 100 times higher in AIR than LEACH soil at 4 to 13 cm. Flagellates were the most abundant protozoa in AIR soil, followed by amoebae and ciliates. In contrast, amoebae were most abundant in LEACH soil, with ciliates absent at both depths, and flagellates absent at 4 to 13 cm. The wastewater contained (per 100 mL) a total of 1270 protozoa, including 400 flagellates, 800

Table 1. Mean (n = 3) numbers of flagellates, amoebae, ciliates, and total protozoa and nematodes in aerated (AIR) and unaerated (LEACH) leachfield soil at two depths.

Depth	Treatment	Protozoa				Nematodes
		Flagellates	Amoebae	Ciliates	Total	
cm		no. kg^{-1}				
0–4	AIR	13.646×10^6*	8.216×10^6*	187×10^3*	22.048×10^6*	14×10^3***
	LEACH	99×10^3	435×10^3	0	533×10^3	0
4–13	AIR	3.462×10^6*	2.342×10^6**	26×10^3*	5.830×10^6*	5×10^3***
	LEACH	0	42×10^3	0	42×10^3	0

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.
*** Significant at $P < 0.001$.

Table 2. Microbial biomass, community structure, and physiological status of the microbial community determined by PLFA (phospholipid fatty acid) analysis in aerated (AIR) and unaerated (LEACH) leachfield soil at two depths. Values are for composite samples.

			Community structure							Physiological status	
Depth	Treatment	Biomass	Anaerobic Gram-negative/ firmicutes	Proteo-bacteria	Anaerobic metal reducers	Actinomycetes/ S-reducing bacteria	General	Eukaryotes	Prokaryote to eukaryote ratio	Starved	Membrane stress
cm		cells $\times 10^{12}$ kg^{-1}	% of total PLFA								
0–4	AIR	2.16	8.7	60.7	3.6	3.4	18.7	5.0	19	0.80	0.16
	LEACH	0.44	21.6	43.5	4.0	1.0	26.1	3.8	25	0.19	0.30
4–13	AIR	1.29	10.6	55.9	4.0	3.9	19.0	6.5	14	0.66	0.13
	LEACH	0.32	23.8	40.4	4.1	0.9	27.4	3.4	28	0.18	0.35

amoebae, and 70 ciliates. No nematodes were present in LEACH soil at either depth, whereas 5 to 14 g^{-1} were found in AIR soil (Table 1), all of which were bacteriovores. Only bacteriovores (1 per 100 mL) were found in wastewater.

Microbial Community Analysis Using Phospholipid Fatty Acid Analysis

Analysis of the microbial community by PLFA showed that the active microbial population in surface soil was roughly five times larger in AIR than in LEACH soil (Table 2). The ratio of prokaryotes to eukaryotes was lower in AIR than in LEACH soil (Table 2). The starvation index was higher in AIR than LEACH soil, whereas membrane stress was higher in LEACH than in AIR soil.

Proteobacteria made up the majority of the microbial community in both AIR and LEACH soil (Table 2). Anaerobic Gram-negative bacteria/firmicutes made up a larger fraction of the community in LEACH than in AIR soil. Actinomycetes/SRB (sulfate-reducing bacteria) constituted a higher proportion of the community in AIR than in LEACH soil. The contribution of anaerobic metal reducers to the microbial community was similar in both treatments. Eukaryotes accounted for a slightly higher proportion of the microbial community in AIR than in LEACH soil.

A total of 45 different PLFAs were detected in all the soil samples, of which 30 were common to all samples. The total number of detectable PLFA markers—a measure of species richness—extracted from AIR soil was higher than for LEACH soil (Table 3). Other diversity indices (H and E) were similar for both treatments (Table 3). Values of Cs indicated that the microbial communities of soils in the LEACH treatment at 0 to 4 and 4 to 13 cm were most similar (0.94), followed by AIR and LEACH soil at 4 to 13 cm (0.90), AIR soil at 0 to 4

Table 3. Indices of richness, diversity (H), and evenness (E) for aerated (AIR) and unaerated (LEACH) leachfield soil based on different methods of microbial community analysis.

Method†	Treatment	Depth	Richness‡	H	E
		cm			
PLFA	AIR	0–4	43	2.7	0.7
		4–13	38	2.8	0.8
	LEACH	0–4	33	2.8	0.8
		4–13	31	2.9	0.8
CLPP	AIR	0–4	30 a§	0.8 a	0.5 a
		4–13	30 a	0.7 a	0.4 a
	LEACH	0–4	29 a	1.2 a	0.4 a
		4–13	29 a	1.2 a	0.4 a
PCR-DGGE	AIR	0–4	37.0 a	–¶	–
		4–13	28.0 a	–	–
	LEACH	0–4	19.7 b	–	–
		4–13	18.3 b	–	–

† PLFA = phospholipid fatty acid; CLPP = community-level physiological profile; PCR-DGGE = polymerase chain reaction–denaturing gradient gel electrophoresis.

‡ Richness is defined as the total number of markers (for PLFA), total number of different growth substrates (for CLPP), or total number of bands (for PCR-DGGE).

§ Values followed by the same letter within a column and method were not significantly different ($P < 0.05$).

¶ Not determined.

and 4 to 13 cm (0.89), with AIR and LEACH soil at 0 to 4 cm being least similar (0.87).

Two markers associated with proteobacteria (19:1α and 19:1β) were present exclusively in AIR soil, with a third marker, 17:1ω6c, present only in soil from 0 to 4 cm in AIR and LEACH soil (Table 4). Of the markers for anaerobic metal reducers, i15:1α was absent from LEACH surface soil, and i15:1β was absent from LEACH soil at both depths. Six markers for actinomycetes/SRB were absent from soil at both depths in the LEACH treatment, whereas one and three markers were absent at 0 to 4 and 4 to 13 cm in AIR soil, respectively. Of the general markers for microorganisms, 22:0 was present only in surface AIR soil. Of the six eukaryotic markers detected, 18:3ω3 was absent from AIR soil at both depths. In LEACH soil, 20:5ω3 was absent at both depths, and 18:2α was absent at 4 to 13 cm.

Microbial Community Analysis Using Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis

The concentration of extracted DNA was significantly higher in AIR (12.7 ± 4.7 mg kg^{-1} in surface soil; 10.3 ± 4.8 mg kg^{-1} at 4–13 cm) than in LEACH soil (1.8 ± 1.3 mg kg^{-1} in surface soil; 0.8 ± 0.7 mg kg^{-1} at 4–13 cm). The PCR-DGGE analysis revealed 61 bands among all the AIR and LEACH samples (Table 5). The diversity of dominant phylotypes was higher in surface AIR than in LEACH soil, as shown by differences in the number of bands—a measure of species richness—detected in gel analysis (Table 3). Of the 61 total bands, at least half were found in AIR soil at 0 to 4 cm, with fewer bands identified in the 4- to 13-cm depth. In contrast, the number of amplified bands in LEACH soil was similar at both depths. In addition to the amplification and separation of more bands in AIR soil, 18 bands were unique to AIR samples. Despite the variability observed within treatments, LEACH samples shared enough bands to form a separate cluster from the AIR samples using the neighbor-joining cluster dendrogram method (Fig. 1).

Table 4. Presence (+) or absence (−) of PLFA (phospholipid fatty acid) markers in aerated (AIR) and unaerated (LEACH) leachfield soil from two depths. Only markers for which differences were found among treatments are shown.

		0–4 cm		4–13 cm	
Group	**PLFA**	**AIR**	**LEACH**	**AIR**	**LEACH**
Proteobacteria	**17:1ω6c**	+	+	−	−
	19:1α	+	−	+	−
	19:1β	+	−	+	−
Anaerobic metal reducers	**i15:1α**	+	+	+	−
	i15:1β	+	−	+	−
Actinomycetes/ S-reducing bacteria	**br16:0**	+	−	−	−
	12me16:0	+	−	+	−
	10me17:0	+	−	+	−
	11me17:0	+	−	−	−
	12me17:0/18:2	−	−	+	−
	12me18:0	+	−	−	−
General	**22:0**	+	−	−	−
Eukaryotes	**18:2α**	+	+	−	−
	18:3ω3	−	−	+	+
	20:5ω3	+	−	−	−

Community-Level Physiological Profiles

A total of 30 of the substrates was utilized for growth in AIR soil and 29 in LEACH soil, with no organisms from either treatment able to grow on 2-hydroxybenzoic acid (Fig. 2). We were able to determine numbers of microorganisms (i.e., dilution to extinction was achieved) for 22 substrates. The average number of microorganisms that grew on these 22 substrates in surface AIR soil (4.562×10^7 MPN kg^{-1}) was significantly higher than in LEACH soil (3.16×10^6 MPN kg^{-1}). No significant differences in any of the diversity indices were observed among treatments (Table 3).

The number of microorganisms capable of growth was higher in AIR than in LEACH soil for cellobiose, β-methyl-D-glucoside, D-xylose, i-erythritol, glucose-1-phosphate, D,L-α-glycerol phosphate, L-phenylalanine, L-threonine, glycyl-L-glutamic acid, and phenylethylamine (Fig. 2). The LEACH soil had higher numbers of microorganisms that grew on α-D-lactose and D-malic acid. Soil at 4 to 13 cm had values similar to those for surface soil, although numbers of organisms were lower for both treatments (data not shown).

Numbers of *Escherichia coli*

Numbers of *E. coli* in AIR and LEACH in surface soil were 5×10^3 and 6.2×10^4 CFU kg^{-1}, respectively. At 4 to 13 cm, AIR and LEACH soil had 6×10^3 and 5.0×10^4 CFU *E. coli* kg^{-1}, respectively. Differences between treatments at a particular depth were statistically significant.

DISCUSSION

Structure and Function of the Faunal Community

The sand used to construct our mesocosms was synthetic, and thus an unlikely source for the microbivorous fauna found in our treatment. Rather, wastewater inputs were the likely source for the protozoa and nematodes that were found in the lysimeters and developed during the course of 26 mo into the faunal community we observed.

Conditions in the LEACH soil lysimeters had a negative effect on microbivorous fauna. Numbers of protozoa in the flagellate and amoebae groups were one to three orders of magnitude lower in LEACH soil, and ciliates were absent from this treatment. Furthermore, nematodes were absent from LEACH soil. The smaller contribution of eukaryotic PLFAs to the LEACH microbial community supports the data obtained by enumeration of protozoa and nematodes. Although some species of free-living nematodes are capable of surviving periods of anoxia (Wright, 1998), and some parasitic species remain viable after prolonged exposure to anaerobic conditions (Stott, 2003), most protozoa and bacterivorous nematodes use O_2 as a terminal electron acceptor, a condition favored in the AIR treatment.

Table 5. Presence (+) or absence (−) of bands detected in DGGE gel of aerated (AIR) and unaerated (LEACH) leachfield soil from two depths. Three replicates per treatment were analyzed. Letters correspond to depths of 0 to 4 cm (T) and 4 to 13 cm (B); numbers correspond to gel lanes.

	AIR						LEACH					
Band no.	**T-1**	**B-2**	**T-3**	**B-4**	**T-5**	**B-6**	**T-7**	**B-8**	**T-9**	**B-10**	**T-11**	**B-12**
1	−	−	−	−	−	−	−	−	+	−	−	−
2	−	−	+	−	−	−	+	−	−	+	−	−
3	−	−	−	−	−	−	−	−	−	+	−	+
4	+	+	+	+	+	−	−	−	−	−	−	−
5	−	−	−	−	−	−	+	+	+	+	+	+
6	+	+	−	+	+	+	−	−	−	−	−	−
7	−	−	−	−	−	−	+	+	+	+	+	+
8	+	−	−	−	+	+	−	+	−	+	−	+
9	+	+	−	+	+	+	−	−	−	−	−	−
10	+	+	−	+	+	+	+	−	+	+	+	+
11	+	+	+	−	−	−	+	+	+	−	−	−
12	−	−	−	−	−	+	−	−	−	−	−	−
13	+	−	−	−	+	−	−	−	−	−	−	−
14	+	+	+	−	+	+	−	+	−	−	−	−
15	−	−	−	−	−	−	+	+	+	−	−	−
16	+	+	+	+	+	+	−	−	−	−	−	−
17	+	+	+	−	−	+	+	+	−	−	−	−
18	−	−	−	+	+	+	−	−	+	+	+	+
19	+	+	+	+	+	+	+	+	−	−	−	−
20	+	−	−	−	+	−	−	−	+	−	−	−
21	−	−	−	−	−	+	+	+	+	+	+	+
22	+	−	−	−	−	−	−	−	−	+	−	−
23	+	+	+	+	+	+	+	−	−	+	−	−
24	+	+	+	+	+	+	−	+	+	−	−	−
25	−	−	+	−	−	+	−	−	−	−	−	−
26	+	+	+	+	+	+	+	+	+	+	+	−
27	−	−	+	−	+	−	−	+	−	−	−	−
28	+	−	−	+	+	−	−	−	−	−	−	−
29	+	+	+	+	+	+	+	+	+	+	+	−
30	+	−	−	−	+	+	−	−	−	−	−	−
31	+	+	−	−	+	−	−	−	−	−	−	−
32	−	−	+	−	−	−	−	−	−	−	−	−
33	−	−	−	−	+	−	−	+	−	−	−	−
34	+	−	+	+	+	−	+	−	−	−	−	−
35	+	+	+	−	+	−	+	+	−	−	−	−
36	+	−	−	−	−	−	−	−	−	−	−	−
37	+	+	+	+	+	+	+	+	−	−	−	−
38	+	+	+	−	+	+	+	+	−	−	−	−
39	−	−	−	+	+	+	−	−	−	−	−	−
40	+	+	+	−	+	−	−	−	−	+	−	−
41	−	−	+	+	+	−	−	−	+	−	−	−
42	+	+	+	+	+	+	+	+	+	−	−	−
43	+	−	+	+	+	−	+	+	+	+	−	−
44	+	+	+	−	+	−	−	−	−	−	−	−
45	+	+	+	+	+	+	−	−	−	−	−	−
46	−	−	−	−	−	−	−	+	+	+	+	+
47	−	+	−	−	+	+	+	−	+	−	−	−
48	+	−	−	+	+	−	+	−	+	−	−	−
49	+	+	+	+	+	+	−	+	+	−	−	−
50	+	+	+	+	+	+	−	−	−	−	−	−
51	+	+	+	+	+	+	−	+	−	−	−	−
52	+	+	−	−	+	+	+	+	+	+	+	+
53	−	−	−	−	−	−	+	−	+	+	−	−
54	−	−	−	−	−	+	+	−	+	+	−	−
55	−	−	−	−	+	−	−	−	−	−	−	−
56	+	−	+	−	+	−	+	+	+	−	−	−
57	−	−	−	−	−	−	−	+	+	+	+	+
58	+	+	+	+	+	+	−	−	−	−	−	−
59	−	−	−	−	−	−	+	+	−	−	−	−
60	+	+	+	+	+	+	−	−	−	−	−	−
61	+	−	−	−	+	+	−	−	−	−	−	−
Total	**40**	**28**	**30**	**25**	**42**	**31**	**25**	**26**	**24**	**19**	**10**	**10**

Grazing by micro- and mesofauna is often cited as a mechanism of removal for pathogenic organisms in soil infiltration systems (Weber-Shirk and Dick, 1997). If this is the case, this mechanism is more likely to be of significance in AIR soil. Enumeration of *E. coli* in soil showed that LEACH soil contained a significantly higher number of these bacteria than AIR soil. Previous results (Potts et al., 2004) showed that the number of fecal coliform bacteria in drainage water was also significantly lower in the AIR treatment. Bomo et al. (2004) observed that removal of *Aeromonas hydrophila*, a fish pathogenic bacterium, in sand filters was proportional to the number of protozoa present in sand.

Consumption of bacteria by protozoa and nematodes can enhance bacterial metabolism (Griffiths, 1994; Woombs and Laybourn-Parry, 1986), the release of mineral N from bacteria (Amador and Görres, 2004), and the activities of soil nitrifiers (Griffiths, 1989). As such, micro-

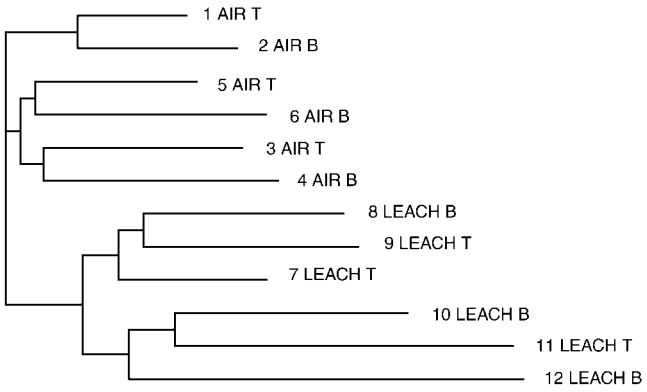

Fig. 1. Dendrogram showing the relationship among aerated (AIR) and unaerated (LEACH) leachfield soil from two different depths. Letters correspond to depths of 0 to 4 cm (T) and 4 to 13 cm (B). Numbers correspond to lane numbers on the DGGE (denaturing gradient gel electrophoresis) gel (Table 5).

bivorous grazing may have important consequences for C and N use in AIR and LEACH soil. Higher numbers of microbivorous fauna in AIR soil, and consequent grazing of microorganisms, requires additional C consumption by microorganisms to maintain the microbial community at steady state. This greater need for C may be reflected in the complete consumption of BOD_5 in AIR lysimeters (Potts et al., 2004) and would contribute to the high starvation index exhibited by the microbial community in AIR soil. With respect to N, grazing by microbivorous fauna results in the mineralization and excretion of a higher proportion of bacterial N because of the disparity in C/N ratios of the grazing fauna (5–6) and bacteria (4–5) (Amador and Görres, 2004). Nitrogen is excreted as NH_4 (Griffiths, 1994; Wright, 1998), which can be oxidized to NO_3. Enhanced N mineralization and subsequent nitrification would support higher rates of denitrification and greater N removal in AIR lysimeters, as observed previously (Potts et al., 2004).

Structure and Function of the Microbial Community

As was the case for the microbivorous fauna, the microbial community that developed during 26 mo in the mesocosms probably had its origins in wastewater inputs, inasmuch as the sand medium used was of synthetic origin.

The total number of microorganisms calculated based on PLFA in AIR soil is of the same order of magnitude (10^{12} cells kg^{-1}) as that observed by Kristiansen (1981b) in sand filter trenches at comparable temperatures and wastewater loads. Differences in microbial biomass as a result of aeration and depth are supported by differ-

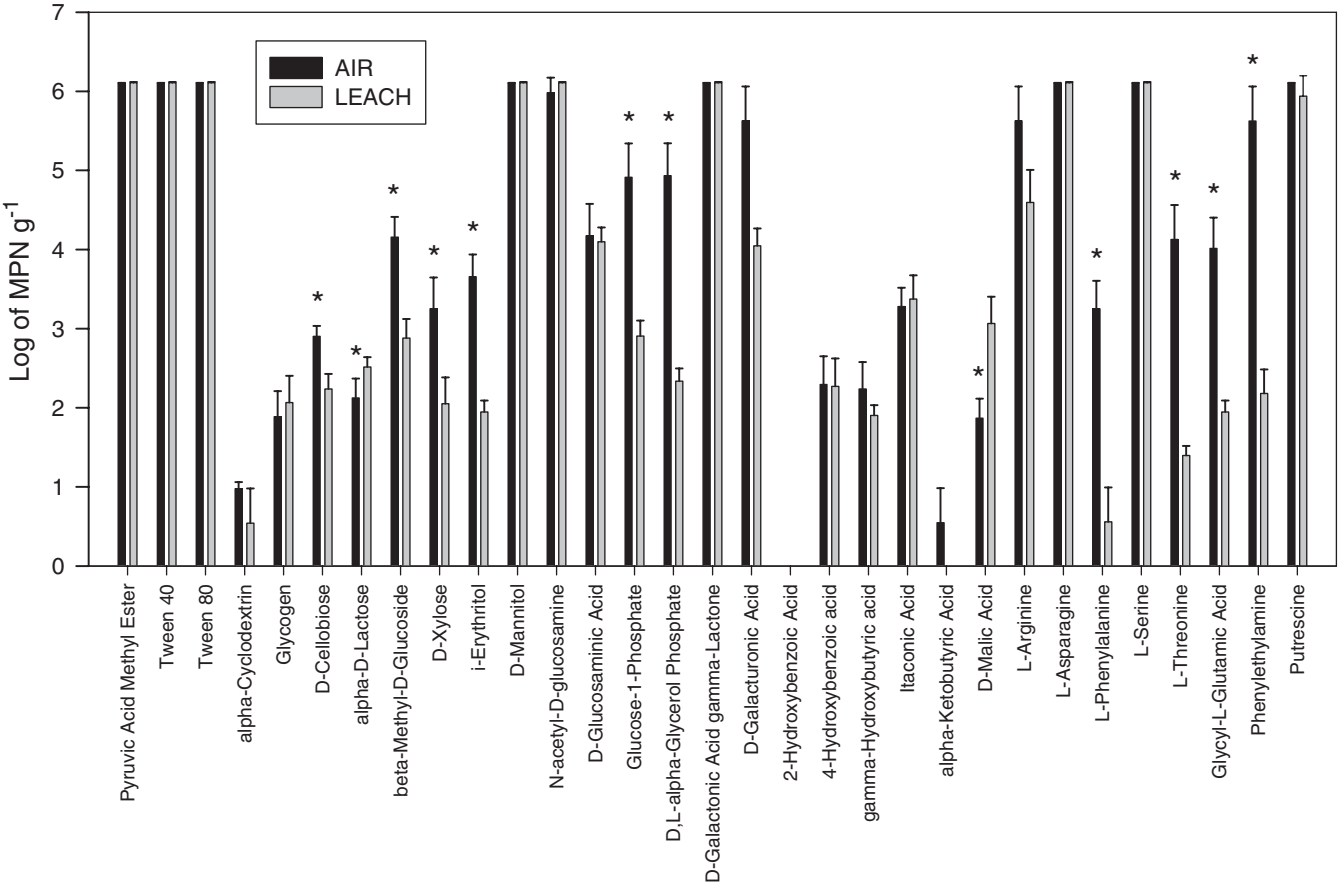

Fig. 2. Most-probable number (MPN) of microorganisms in aerated (AIR) and unaerated (LEACH) leachfield soil from a depth of 0 to 4 cm that grew on Biolog EcoPlate substrates. Values are means (n = 3). Bars represent one standard deviation. *Significant difference between treatments at $P < 0.05$.

ences in the concentration of DNA extracted from soil and by the average number of microorganisms per substrate detected in the CLPP assay. Lower microbial biomass in LEACH soil may be responsible for incomplete use of BOD_5 (Potts et al., 2004), and probably reflects electron acceptor limitations. Microbial biomass decreased with depth in both treatments, and was lower in LEACH than in AIR treatment. Lower microbial biomass with depth is typical of surface soils, where inputs of matter and energy are unidirectional, and has been observed for numbers of aerobic bacteria in soil absorption systems (Pell and Nyberg, 1989; Pell et al., 1990). Differences in biomass between AIR and LEACH treatments would be expected as a result of the differential availability of electron acceptors (Fuhrmann, 2004).

The AIR soil had greater microbial diversity, as indicated by a higher species richness based on PLFA markers and 16S rRNA gene fragments in AIR than in LEACH soil at both depths. In addition to fewer total DNA fragments from LEACH soil that separated as distinct bands using DGGE, the phylotypes amplified most strongly were different between the LEACH and AIR treatments (data not shown). Furthermore, the separation of LEACH and AIR soils by cluster analyses suggests that, despite common phylotypes, there are distinct differences in the dominant community members between these treatments.

The bulk of the microbial community in both treatments consisted of proteobacteria, a group that uses a wide variety of C sources, adapts quickly to changes in environmental conditions, and dominates the microbial community in activated sludge (Snaidr et al., 1997; Wagner et al., 1993). It includes enteric bacteria, *Pseudomonas* species, and chemolithotrophs. The AIR soil had the largest proportion of PLFA markers within the actinomycete/SRB group. Actinomycetes require oxic conditions, prefer a near-neutral pH, and are capable of using a number of the polysaccharides, proteins, and fats found in domestic wastewater. As such, they would be expected to thrive in AIR leachfield soil. Some midchain branched saturated PLFAs found in actinomycetes are also associated with SRB, such as *Desulfobacter* (Dowling et al., 1986), which should be found preferentially in LEACH soil. This was not the case, suggesting that the difference between AIR and LEACH treatments is attributable to actinomycetes. Kristiansen (1981b) found that streptomycetes were present in sand-filter trenches, although their numbers did not change in response to loading with septic tank effluent.

The contribution of anaerobic Gram-negative bacteria/firmicutes to the community in LEACH soil was roughly twice that in AIR soil. These organisms can be of fecal origin, with members ranging from strictly aerobic to anaerobic. Their contribution to the LEACH soil microbial community probably reflects the lower O_2 concentration in this treatment.

A number of factors probably contribute to lower diversity in LEACH soil. Ecosystems in which physicochemical factors dominate tend to have lower microbial diversity (Atlas and Bartha, 1998). In our study, the persistent low levels of O_2 limit the microbial community to those that prefer or can tolerate these conditions, including microaerophilic, obligate anaerobic, and facultative anaerobic organisms. In addition, the presence of elevated concentrations of H_2S (Potts et al., 2004)—which is toxic to many aerobic microorganisms (Postgate, 1984)—further restricts the community to those microorganisms capable of either detoxifying or otherwise tolerating high concentrations of this gas. A higher membrane stress index in LEACH than AIR soil suggests that toxicity may be involved in determining the structure of the LEACH microbial community. Differences in the size of the population of the microbivorous fauna may also have a differential effect on microbial community composition. The microbial community structure of soil changes in response to the presence of microbivorous nematodes (Griffiths et al., 1999) and protozoa (Rønn et al., 2002). Selective grazing by protozoa also affects the composition of the microbial community in activated sludge (Güde, 1979). Soil pH, which can exert selective pressure on the microbial community, ranged from 5.9 in AIR soil to 6.4 in LEACH soil, suggesting that it was not an important determinant of microbial community structure.

In contrast to the results of the culture-independent assays, CLPP analysis of the microbial community found little difference in functional diversity between AIR and LEACH soil. The reason for this discrepancy probably lies in the subset of the microbial community that was evaluated by these different methods. As employed in this study, the EcoPlate–MPN method would detect only those microorganisms capable of growing under the aerobic conditions of the assay—that is, aerobes and facultative anaerobes. The wide range of electron acceptors used by these microorganisms makes them more likely to be found in both AIR and LEACH soil under the conditions of the assay.

The difference in starvation index between AIR and LEACH soil is puzzling, given the considerable amount of organic C present in septic tank effluent. A number of factors can lead to an elevated starvation index. For example, the cyclopropyl content of phospholipids fatty acids increases during the stationary growth phase (Kieft et al., 1994). Other conditions known to result in elevated cyclopropyl levels include low C concentrations, high acidity, low O_2 levels, and high temperature, as observed in pure cultures of *E. coli* by Knivett and Cullen (1965). Given the high concentration of C in wastewater applied equally to both treatments, high levels of dissolved O_2 found in AIR drainage water (Potts et al., 2004), the relatively mild temperatures under which the experiment was conducted, and small differences in soil pH, other explanations must be considered.

The paradox of "starvation in the midst of plenty" may be explained by differences in microbial growth phase. Assuming the lysimeters are at steady state with respect to the size of the microbial population (a reasonable assumption after 2 mo under the same conditions [Pell and Nyberg, 1989]), the microbial population in AIR soil may be kept in the exponential growth phase by the frequent input of substrates. Under these circumstances,

the size of the population would be controlled by the amount of C input into the system. The complete absence of BOD_5 in the effluent of AIR lysimeters (Potts et al., 2004) indicates that C availability limits microbial growth, supporting the contention that the microbial population is in the exponential growth phase. The grazing pressure exerted by larger numbers of microbivorous fauna in AIR soil may further contribute to the starvation phenomenon by keeping a fraction of the microbial population in a constant state of growth. In contrast, the relatively high BOD_5 in the drainage water from LEACH lysimeters (Potts et al., 2004) suggests that factors other than C availability, such as availability of electron acceptors or toxicity of H_2S, limit the size of the population. Lower grazing pressure by microbivorous fauna may further contribute to the differences between AIR and LEACH treatments.

The controlled conditions of our experiment allowed us to evaluate the role of aeration in shaping the faunal and microbial communities of leachfield soils. The various spatial and temporal heterogeneities in wastewater and in soil physical, chemical, and biological properties and processes encountered under field conditions undoubtedly also affect the structure and function of these communities. This is particularly likely in shallow trench leachfields, where the abundance of autochthonous microflora and fauna is high and temperature variations are not as dampened and uniform as in conventional fields with greater depth. Our results indicate that the response of the faunal and microbial communities to aeration warrants further examination in the field. Establishment of clear links between the leachfield soil community and the biochemical processes involved in wastewater treatment will advance our understanding of how these systems function and enhance our ability to optimize their water quality functions.

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