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Distribution of methanotrophs in managed and highly disturbed watersheds

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Abstract

Potential impacts of mechanized infantry training activities on the distribution of methanotrophs were investigated in two watersheds on Fort Benning, a US Army installation in southwest Georgia, USA. The Bonham Creek watershed shows significant impacts from intensive training exercises, including severe erosion and deposition of sediments in bottomlands. The Sally Branch watershed is a managed watershed with only limited exposure to mechanized training activity and much less erosion. A clone library of the gene encoding particulate methane monooxygenase, *pmoA*, was constructed from DNA extracted from Sally Branch bottomland and upland samples. Libraries were constructed from only one watershed in the interest of decreasing the amount of sequencing, and Sally Branch was chosen for construction of these libraries because relatively undisturbed environments generally exhibit higher levels of diversity. The Sally Branch *pmoA* libraries were dominated by a deeply rooted lineage related to Type I methanotrophs (the “Benning soil cluster γ ,” BSC γ). Sequences clustering with known Type II methanotrophs were restricted to samples taken from the Sally Branch bottomland sample. Terminal restriction fragment length polymorphism (T-RFLP) analysis of *pmoA* was applied to samples taken from transects located in upland and bottomland sites within the two watersheds. Observed T-RFs matched well with T-RFs predicted from sequences obtained from the clone library, with few exceptions. Principal components analysis revealed that in both watersheds most T-RFLPs from upland samples clustered separately from bottomland samples. Upland and bottomland T-RFLPs clustered separately in the Sally Branch transect; however, some Bonham Creek bottomland T-RFLPs clustered within the upland cluster, suggesting mixing of upland with bottomland soils in this watershed. Assemblages were, in general, similar between the eroded and managed watersheds, although erosional soil movement likely resulted in mixing of upland assemblages with bottomland assemblages in the Bonham Creek watershed.

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1. Introduction

The impact of land use practices on the structure and function of microbial communities is currently of great interest, particularly with regard to potential

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effects on nutrient cycling and ecosystem functioning. Due to their responsiveness to environmental conditions, various aspects of microbial communities have been used as indicators of environmental quality (Rice et al., 1996; Paerl et al., 2003; Ellis et al., 2002) and ecosystem integrity (Peacock et al., 2001a). Much of this research has focused on use of general microbial indicators such as biomass or on gross structure of microbial communities through analysis of biomarkers such as phospholipid fatty acids (PLFA; Peacock et al., 2001a,b). Analysis of the response of individual groups of microorganisms to environmental perturbations may provide greater sensitivity to environmental changes than gross microbial measurements (Castro et al., 2002, 2004, 2005; Stephen et al., 2001), and provide greater insight into the effects of these perturbations on microbially mediated processes.

Among those microbial groups that have been suggested as indicators of ecosystem function and quality are methanotrophs, methane-oxidizing bacteria that are widespread in most soils. Methanotrophs are important sinks for approximately 700 Tg of methane emitted annually from various sources (Reeburgh et al., 1993), and are an important part of the carbon cycle in many soils.

Most methanotrophs can be divided into two broad physiological and phylogenetic groups, the Types I and II methanotrophs. Type I methanotrophs belong to the γ -proteobacteria and Type II are members of the α -proteobacteria (Hanson and Hanson, 1996). Both types coexist in many environments, although Type I methanotrophs typically dominate in high oxygen, low methane environments (Henckel et al., 2000b) and Type II methanotrophs tend to dominate under high methane conditions. Both types have been found in forests, although Type I methanotrophs have been reported to be more active than Type II strains in some forests (Knief et al., 2003). With few exceptions (Dunfield et al., 2003), both types harbor characteristic forms of the particulate methane monooxygenase gene (*pmoA*), and most Type II species typically also encode a soluble methane monooxygenase. These genes are sufficiently conserved between representative strains to be of use in constructing phylogenies that compare well with 16S rRNA phylogenies (Auman et al., 2000; Costello and Lidstrom, 1999).

The availability of conserved genes such as those encoding the particulate and soluble methane mono-

oxygenases make them attractive targets for non-culture based studies of the distribution and activities of methanotrophs in soils. Distribution of these genotypes has been studied in a variety of environments, including forests (Bourne et al., 2001; Henckel et al., 2000a; Knief et al., 2003), peat bogs (Kravchenko, 2002; Morris et al., 2002), aquifers (Newby et al., 2004) and rice paddies (Henckel et al., 1999). The relatively high diversity of methanotrophs, the availability of conserved genetic sequences and their importance in ecosystem functioning make methanotrophs strong candidates for indicator organisms in studies on the effects of land use on ecosystem properties.

Ecosystem stability is of great concern at military training facilities, particularly those involved in intensive training where the natural landforms are highly susceptible to soil loss. Movement of tracked vehicles across hillsides removes natural vegetation cover, and accelerates processes such as erosion. Decreased vegetation and increased erosion would significantly change rhizosphere nutrient dynamics and soil porosity, may impact rates of methanogenesis, and subsequently affect activities and compositions of assemblages of methanotrophs.

Ft. Benning, a 72,000 ha US Army installation in southwestern Georgia, USA, is the Army's primary infantry training facility. In addition, it supports a brigade of mechanized infantry of the regular army, which trains with tanks and other tracked armored vehicles. The entire area has been subjected to farming and disturbance since the early 19th century, but within the base are many less-impacted areas that are recovering, and therefore relatively undisturbed. These relatively undisturbed areas of the base are often adjacent to areas that have been highly impacted by training exercises (Bhat et al., submitted for publication; Perkins et al., submitted for publication). Highly disturbed areas are characterized by high levels of erosion (Perkins et al., submitted for publication) and relatively low numbers of plant species and surface coverage by plants (Dale et al., 2002). A previous study on the distribution of PLFA profiles utilized artificial neural networks to correlate microbial community composition with degree of traffic impact at Ft. Benning, indicating the response of the microbial community in general to training practices (Peacock et al., 2001a). Analysis of

PLFA provides a very good overview of microbial groups present in a sample; however, this approach lacks the resolution of approaches intended to characterize the variability within specific genetic elements, such as *pmoA*.

In this study, we investigated relationships between the degree of recent impact and the distribution of methanotrophs in a low impact and high impact watershed pair. The Sally Branch watershed has not been subjected to extensive training, and many upland areas are managed as pine plantations. Bonham Creek watershed is highly impacted, with a high degree of erosion observed in upland sites with concomitant high sedimentation in bottomland sites. A non-culture based approach, terminal restriction fragment length polymorphism (T-RFLP) analysis of the *pmoA* gene, was used to screen samples taken from two transects (upland and bottomland) in each watershed for comparison of potential effects of military training on methanotrophic assemblages. This study was intended to investigate the potential application of *pmoA* genotypes as an indicator of disturbance at this training facility.

2. Materials and methods

2.1. Site description and sampling

The study area is within the Ft. Benning military reservation in west-central Georgia, in the Carolina and Georgia sand hills major land resource area (USDA, 2000). Upland soils in the area are primarily well to excessively drained Ultisols and Entisols, supporting forests of slash (*Pinus elliotii*), long leaf (*P. palustris*) and loblolly (*P. taeda*) pines. Excessively drained Lakeland soils (Entisol) of sandhill communities are associated with long leaf pine, turkey oaks (*Quercus laevis*), blackjack oaks (*Q. marilandica*) and post oaks (*Q. stellata*) near ridgetops in the central and northern portion of the reservation. Wetlands and hydric soils are generally restricted to bottomlands along streams and creeks. Military related impacts result from the direct removal of or damage to vegetation, earthmoving activities and ground disturbance from vehicles. The mechanized forces in particular use tracked and wheeled vehicles that cause soil disturbance and

movement that may result in soil erosion and stream sedimentation. This study focused on two watersheds characterized by different land uses.

High and low impact regions were identified by land use, degree of disturbance and characteristic vegetation. The Bonham Creek watershed had supported extensive tank warfare training and was considered to be highly impacted. The Sally Branch watershed had not experienced intensive mechanized training and was considered to be minimally impacted. One upland transect and one bottomland transect were selected within each watershed (Table 1). Upland transects were 400 m long and soil samples were collected every 20 m. Bottomland transects were 25 m long and samples were collected every 5 m perpendicular to the creek. High and low impact bottomland transects were located downslope from the high and low impact upland transects, respectively. Five 20-cm deep cores were taken from each upland sampling site and manually homogenized to create one composite sample per site. Similarly, five replicate 10-cm deep cores were taken at each bottomland site. A total of 70 samples were collected and individual samples manually homogenized by mixing on bench top, sub-sampled from composite samples and stored at -80°C . Soil characteristics were provided by the Wetland Biogeochemistry Lab at University of Florida.

2.2. DNA extraction from soil

DNA was directly extracted and purified from 250 mg soil samples using an UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach, CA) according to manufacturer's instructions. The mass of DNA within 5 μl of extracted DNA was estimated by electrophoresis through a 0.7% agarose gel stained in ethidium bromide. DNA was aliquoted and stored at -20°C .

2.3. PCR amplification of *pmoA*

PCRs were conducted using the primer sets A189 (Holmes et al., 1995) combined with A650 (Bourne et al., 2001) or mb661 (Costello and Lidstrom, 1999). PCR reactions of 20 μl were set up containing approximately 1 μl of soil DNA, 0.4 μM of each primer, 7.4 μl of distilled water and 10 μl \times HotStarTaq Master Mix (Qiagen, Valencia, CA). PCR

Table 1
Site characteristics

Sample ^a	Site description	Disturbance	pH	Total carbon (g/kg)	Total nitrogen (g/kg)
BU0	Sandhill	Low	7.3	11.2	0.4
BU01-BU05	Sandhill	Low	5.5	10.3	0.3
BU07	Crater	Moderate	5.1	17.1	0.5
BU08-BU09	Patchy grass	Moderate	5.7	4.3	0.6
BU10	Sparse grass	Moderate	5.8	8.5	0.2
BU12	Sparse grass	Severe	5.6	2.5	0.1
BU13	Tree island	Severe	5.4	2.3	0.1
BU14	Sparse grass	Severe	5.3	2.0	0.1
SU01-SU05	Sandhill	Low	5.3–6.1	7.0–14.8	0.2–0.6
SU08-SU20	Planted pines	Low	5.1–5.6	7.1–13.0	0.1–0.4
BB10	Edge, vegetated, seep	Severe	5.0	27.4	1.0
BB12	Hummock, sparse litter	Severe	5.3	4.1	0.2
BB14	Hummock, few litter	Severe	5.4	3.9	0.2
BB15	Hummock, heavy litter	Severe	4.9	4.9	0.2
BB20	Ecotone, litter, seep	Severe	5.1	91.0	4.2
BB21	Hummock, grass, moss	Severe	5.6	5.9	0.3
BB22	Hummock, heavy litter	Severe	5.0	3.6	0.2
BB23	Streamside, sand, little litter	Severe	5.3	5.5	0.2
BB24	Hummock, sand/loam	Severe	5.4	6.3	0.2
BB25	Edge, litter, seep	Severe	5.1	19.8	0.6
BB30	Ecotone	Severe	5.3	9.3	0.3
BB31	Elevated, bamboo	Severe	5.4	4.4	0.2
BB32	Elevated, shrubs, heavy litter	Severe	5.6	2.4	0.1
BB33	Sand bar, sparse vegetation	Severe	5.5	1.6	0
BB34	Hummock	Severe	5.8	2.0	0.1
BB35	Ecotone, some bamboo	Severe	5.7	4.2	0.1
SB12, SB20, SB22-SB24	Depression	Low	4.4–4.8	50.7–290.1	2.5–12.7
SB33	Flat	Low	5.5	74.9	3.5
SB34	Hummock	Low	4.7	266.0	12.0
RH11 ^b	Bare ground	Severe	5.3	2.9	ND ^c

^a Sample designations refer to watershed and transect: B is Bonham Creek, S is Sally Branch; U is upland transect; B is bottomland transect.

^b Reference sample: bare ground, highly eroded.

^c Not determined.

was carried out in a GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems, Norwalk, CT) with the following cycling parameters 15 min at 95 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, with a final extension step for 7 min at 72 °C. PCR products were electrophoresed through 1% agarose gels to confirm the size.

2.4. Cloning, sequencing and phylogenetic analysis

Clone libraries for *pmoA* from representative low impact bottomland and low impact upland soils were constructed using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The clone libraries were screened by

restriction fragment length polymorphism (RFLP) analysis using *HhaI* digestion of PCR amplification products of clones screened with the corresponding primer set. Clones representative of unique RFLP patterns were selected and their plasmid DNAs were purified and sequenced using cloning vector primers (TOPO TA cloning kit, Invitrogen) at the DNA Sequencing Core Laboratory at the University of Florida.

Sequences were aligned against available genes in the National Center for Biotechnology Information Nucleotide Database (NCBI; <http://www.ncbi.nlm.nih.gov/>) using the Pileup function of the Genetic Computer Group sequence analysis package (GCG, Madison, WI). Phylogenetic analysis was constructed using maximum parsimony with PAUP* Version 4.0b8

(D. L. Swofford, Sinauer Associates, Sunderland, MA) and neighbor joining using the Jukes and Cantor algorithms (Van de Peer and de Wachter, 1994). Bootstrap analyses were conducted with 100 resamplings. Only branches with bootstrap values higher than 50% are shown. *pmoA* sequences for Sally Branch upland clones are listed under the GenBank accession numbers AY662347–AY662367, and sequences for Sally Branch bottomland clones are listed under accession numbers AY662368–AY662389. The sequences were later used in selection of restriction enzymes for T-RFLP.

2.5. T-RFLP analysis

PCRs were performed on 20 μ l reactions in triplicate using A189 and fluorescently labeled A650 with 6-FAM (6 carboxyfluorescein) in the 5' position (Invitrogen). PCRs were carried out using the following cycling parameters: 15 min at 95 °C, 30 cycles of 30 s at 94 °C, 30 s at 49 °C, 30 s at 72 °C, with a final extension step for 7 min at 72 °C. The triplicate PCR reactions were pooled and the pooled reactions were cleaned and concentrated using QIAquick PCR purification kit (Qiagen) to a final volume of 30 μ l following the manufacturer's instructions. Restriction enzymes were selected in silico based on manually aligned sequences using CloneMap Version 2.11 (CGC Scientific Inc., Ballwin, MO) on each sequence and the enzyme producing the greatest discrimination between sequences was selected for use in T-RFLP (the restriction enzyme with greater degree of discrimination for *pmoA* was *HhaI*). Approximately 50 ng of PCR product was added to the mixture with approximately 3 U (0.3 μ l) of restriction enzyme (Promega, Madison WI), 1 μ l restriction buffer, 1 μ g bovine serum albumin and deionized water to make a final volume of 10 μ l. Enzymatic digestions were carried out at 37 °C overnight.

One microliter of digestion product was used in terminal restriction fragment (t-RF) detection by the DNA Sequencing Core Laboratory (University of Florida). Briefly, 1 μ l of digested DNA was mixed with 2.5 μ l deionized formamide, 0.5 μ l ROX-labeled GenScan 500-bp internal size standard (Applied Biosystems, Perkin-Elmer Corporation, Norwalk, CT) and 0.5 μ l of loading buffer (50 mM EDTA,

50 mg/ml blue dextran). The samples were denatured by heating at 95 °C for 3 min and immediately transferred to ice. One microliter of denatured digests were electrophoresed in a 36 cm, 5% polyacrylamide gel containing 7 M urea for 3 h at 3000 V on an ABI 377 Genetic Analyzer (Applied Biosystems) with filter set A and a well-to-read length of 36 cm. T-RFLP profiles were analyzed using GeneScan Version 2.1 software (Applied Biosystems). The sizes in base pairs (bp) of terminal restriction fragments were estimated by reference to internal standard by using local southern method included in GeneScan Version 2.1 (Applied Biosystems). T-RFLP results, including peak size, peak fluorescence height and area and scan size were exported to Excel (Microsoft Corporation, Redmond, WA) for data analysis.

2.6. T-RFLP data analysis

Principal components analysis (PCA) was performed using the relative abundance of individual peaks normalized by the total area of all T-RFs with the Multivariate Statistical Package (MSVP Version 3.12d, Kovach Computing Services, Wales, UK). All T-RFs observed were included in this analysis.

Relationships between the frequency of individual T-RFs and various site characteristics were statistically analyzed and plotted by PC SAS (SAS Inst., 2003). GLM analysis for the significant differences of means was performed. Significant differences were observed at the 0.05 level. Population normality was tested for each variable before using parametric statistics for comparisons and testing.

3. Results and discussion

3.1. *pmoA* clone libraries

pmoA clone libraries were constructed from samples representative of the Sally Branch upland and bottomland transects (Fig. 1). Sally Branch soils were chosen for library construction due to their low level of disturbance. Low disturbance sites are generally characterized by higher biological diversity than are high disturbance sites (Atlas and Bartha, 1993), and was predicted that most genotypes observed in Sally Branch samples are present in the

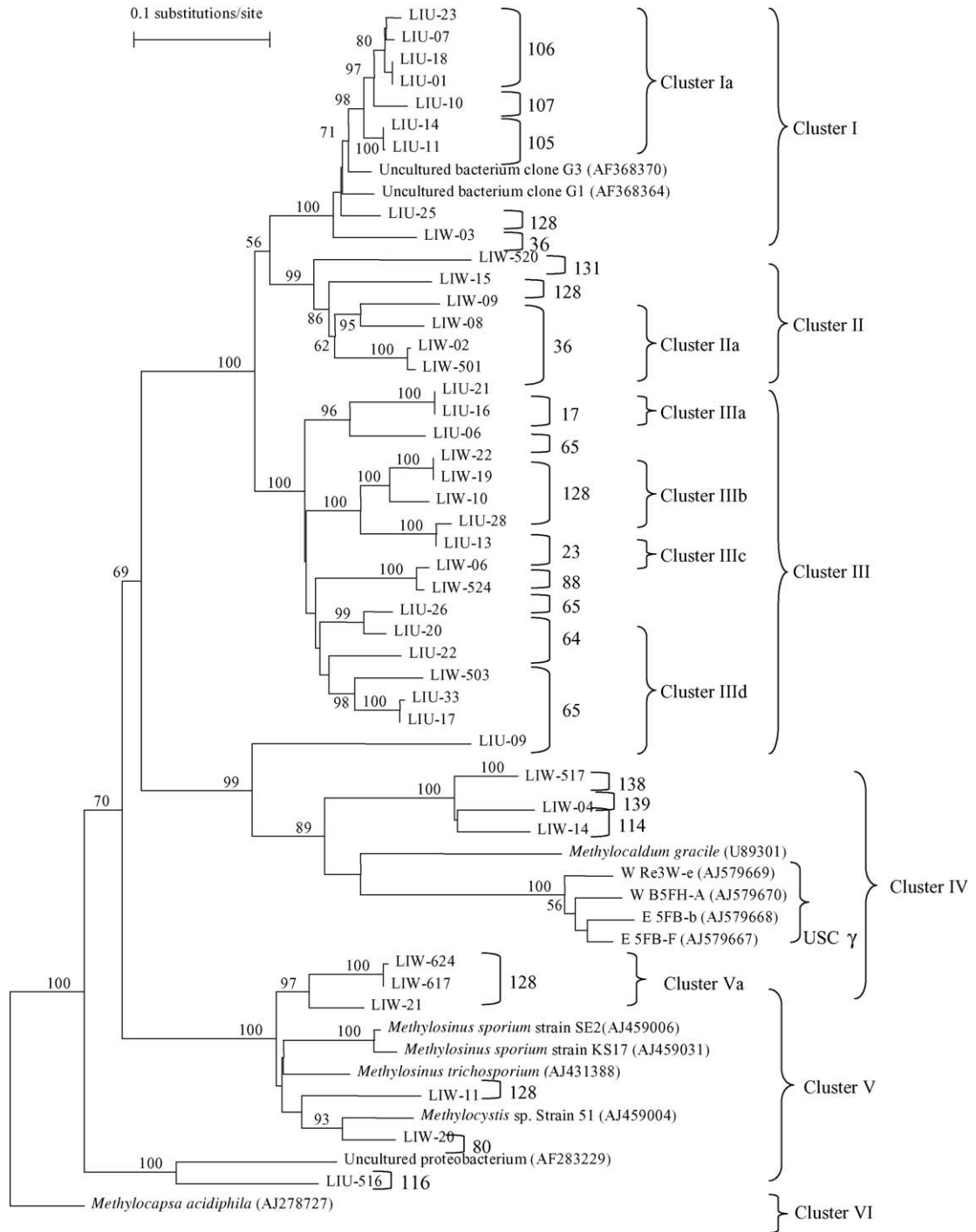


Fig. 1. Neighbor-joining phylogenetic tree of Sally Branch *pmoA* sequences. LIU clone numbers refer to upland samples; LIW refers to bottomland samples. Numbers at branch points represent bootstrap values after 100 resamplings. Numbers outside of left most brackets indicate predicted T-RF sizes. Scale bar represents 10 nt change per 100 sequence positions.

Bonham Creek samples. The assumption that Bonham Creek shared most sequences with Sally Branch was confirmed via T-RFLP analysis, presented below in Section 3.2.

The great majority (31 out of 41) of sequenced clones were grouped in Clusters I–III and were not associated with cultured representatives (Fig. 1). The closest cultured relative for these sequences is *Methylocaldum gracile*, a Type I methanotroph in Cluster IV that branches outside of the major branch for Clusters I–III. The deep branch isolating Clusters I–III from Cluster IV is supported by strong bootstrap values and indicates a major lineage within the Type I methanotrophs. These clusters, collectively termed the “Benning soil cluster γ ” (BSC γ), branch outside the recently reported “upland soil cluster γ ” (USC γ) (Knief et al., 2003), which falls in Cluster IV of our tree (Fig. 1). Representatives of this cluster have been reported previously (Auman et al., 2000; Bourne et al., 2001), but this is the first report of a library dominated by these sequences.

Representatives of upland and bottomland libraries were segregated in most major branches, e.g., Cluster Ia is comprised exclusively of upland clones and Cluster IIa is dominated by bottomland sequences. Type I methanotrophs have been reported to out-compete Type II methanotrophs under conditions of high oxygen and low methane concentrations (Henckel et al., 2000b), as might be expected from upland soils.

Five out of 41 clones sequenced grouped within Cluster V, defined by the Type II methanotrophic genera *Methylosinus* and *Methylocystis*. This group is not closely related to the previously described “forest sequence cluster,” a significant group of uncultured Type II methanotrophs identified in European soils (Bourne et al., 2001; Henckel et al., 2000a). Cluster V is exclusively comprised of bottomland clones; Type II methanotrophs typically dominate Type I methanotrophs in areas with higher methane concentrations, as might be expected for bottomlands (Hanson and Hanson, 1996). One upland clone (LIU-516; Cluster VI) branched outside of Types I and II clusters and was not associated with previously cultivated strains.

Most previous forest soil *pmoA* libraries have been constructed from European soils (Holmes et al., 1999; Henckel et al., 2000b) that contained much greater organic carbon contents than did the soils of this study

(Table 1). Organic carbon content of soil is roughly correlated with methanogenesis rates, and it is likely that methane produced in this soil is low relative to most previous studies. Sandy, well drained, low organic carbon soils such as those of the Ft. Benning watersheds are likely to be characterized by low CH₄:O₂ concentration ratios relative to finer textured soils reported in other studies (Holmes et al., 1999; Henckel et al., 2000b), which may select for methanotrophs with high affinities for methane. Bender and Conrad (1992) hypothesized that, although low affinity methanotrophs (micromolar K_m) dominate culture collections, high affinity methanotrophs (nanomolar K_m) may be largely responsible for much of the methane oxidation that occurs in soils. A number of studies have demonstrated that Type I methanotrophs are active under low CH₄:O₂ concentration ratios (Henckel et al., 2000b; Knief et al., 2003). It is possible that BSC γ methanotrophs are particularly well adapted to the low methane concentrations likely to be present in the very well drained, low organic carbon forest soils of the southeastern United States. Without culturable relatives, it is not possible to make inferences regarding possible ecological roles for these groups beyond general inferences for Type I methanotrophs.

3.2. T-RFLP analysis

T-RFLP is a useful approach to screening distributions of genotypes in large numbers of samples (Horz et al., 2000; Liu et al., 1997; Marsh, 1999). While subject to limitations (Dunbar et al., 2001; Osborn et al., 2000), T-RFLP analysis can provide information on complex assemblages of functional genotypes. Sequences of *pmoA* clones presented in Fig. 1 were used to design and evaluate a T-RFLP system for screening samples from the Sally Branch and Bonham Creek watersheds. Predicted T-RF lengths for individual clones are presented in Fig. 1 immediately to the right of the clones, and groupings within major clusters (e.g., Clusters Ia, IIa, IIIa, etc.) represent clones predicted to yield similar T-RFs (± 2 bp). Predicted T-RFs were consistent with major phylogenetic groupings, although some overlap was observed between individual clones and broader groups: Clone LIW-03 (Cluster I) shares a predicted T-RF of 36 bp with Cluster IIa; Clone LIW-11 (Cluster

V) shares T-RF 128 bp with Cluster Va; Clone LIU-09 (Cluster III) shares T-RF 138 and 139 bp with Clone LIW-517 (Cluster IV) and Clone LIW-11 (Cluster V) shares T-RF 128 bp with Cluster Va. In general, however, this T-RFLP system provides good resolution between major subgroups observed in the library.

T-RFLP analyses were conducted on a total of 70 soil samples plus one reference sample (Table 1), from the Bonham Creek watershed, 13 samples were analyzed from the upland transect, and 16 from the bottomland transect. For Sally Branch watershed, 18 samples were analyzed from the upland transect and 23 from the bottomland transect and one highly eroded reference site from Bonham Creek (RH11). A total of 21 T-RFs from all samples were observed, with numbers of T-RFs ranging from 5 to 13 for individual samples. Most T-RFs predicted from the library were observed, although 5 primarily minor predicted groups were not observed: T-RFs 78, 114, 127, 128 and 131. The absence of these predicted T-RFs from observed T-RFLPs was likely due to the greater sensitivity of clone libraries than of T-RFLP analysis. These strains were undoubtedly present in the samples because they were present in the clone libraries, but T-RFLP is not sufficiently sensitive to detect low abundance groups. Conversely, five T-RFs not predicted from the clone libraries were observed in T-RFLPs (Table 2).

Lowered species diversity is frequently cited as an indication of stress (Atlas, 1993), such that it was predicted that samples taken from Sally Branch would harbor greater richness of *pmoA* genotypes than similar samples taken from Bonham Creek. This prediction was not supported from the T-RFLP data, in which an average of between 8 and 10 T-RFs with no significant differences observed between the samples from the four transects (data not shown).

Principal components analysis was applied to T-RFLP data from the two watersheds and significant differences between PCAs of methanotrophic assemblages within a given watershed were observed (Fig. 2). Groupings of points were identified as individual clusters in these figures. The clusters ascribed are somewhat subjective and it is certainly possible to imagine other schemes for grouping these points. Upland samples were completely separated from bottomland samples in the Sally Branch watershed (Fig. 2A), although the bottomland samples formed two distinct clusters (1 and 2) that were largely

independent of vegetation type, but no mixing of bottomland and upland samples was observed. This is contrasted with the Bonham Creek watershed, where significant overlap between bottomland and upland T-RFLPs was observed (Fig. 2B). Two separate clusters comprised exclusively of several bottomland samples were observed (Clusters 1 and 2), but several bottomland samples also grouped among upland samples (Cluster 3).

These general trends are supported when all data are combined (Fig. 2C), all bottomland samples cluster in two separate groups (Clusters 1 and 2), with some mixing of Bonham Creek bottomland samples within the diffuse upland cluster (Cluster 3). The separation of T-RFLPs into bottomland and upland clusters, independent of watershed, indicates that land management effects on the distribution of methanotrophs were not as strong as landscape position at these sites. These data suggest that methanotrophs in these watersheds are relatively stable, and that the eroded conditions do not differ from the less disturbed areas significantly enough to select for different groups of methanotrophs. Species presence does not imply activities (Knief et al., 2003); however, it is quite possible that different groups are responsible for oxidation of methane in the different watersheds.

It is not known at this time why the bottomland assemblages of both watersheds split into two divergent clusters. Cluster 1 of the Bonham Creek watershed (Fig. 2B) was comprised of adjacent samples taken between ecotones (BB30–BB35; Table 1) and Cluster 2 (BB14, BB20 and BB21) were also adjacent samples, although not from similar vegetation. The Sally Branch bottomland Cluster 1 was composed of samples taken from adjacent sites (SB12, SB20, SB22 and SB24) within a depression, and Cluster 2 (SB23, SB34 and SB33) included samples from contiguous ecotones, although SB23 appears to be an outlier from adjacent samples that grouped in Cluster 1.

The robustness of these groupings is not clear at this time. Even though groupings assigned to these PCAs were subjective, it is clear that considerable overlap exists between the Bonham Creek bottomland and upland points at the center of Fig. 2B and C, and that mixing is not observed in Sally Branch. Additional data from Sally Branch bottomlands would be of great value in testing these groupings.

Table 2
Correlations between frequencies of individual T-RFs and sample type

Treatments	TRFs											
	23 (IIIc) ^a	26	30	36 (I, IIa)	48	66 (IIIId)	68 (IIIId)	80 (V)	84	107 (Ia)	139 (IV)	161
Upland	0.028 A	0.018 A	0.086 B	0.0194 A	0.0202 A	0.0251 A	0.0008 B	0.0488 A	0.009358 A	0.07379 A	0.1501 A	0.017 A
Bottomland	0.031 A	0.030 A	0.243 A	0.0078 A	0.0338 A	0.0024 A	0.0225 A	0.0006 A	0.004248 A	0.0000 B	0.0136 B	0.0008 A
L.S.D. (0.05)	0.044	0.033	0.158	0.0951	0.0432	0.0307	0.0184	0.0936	0.0137	0.0577	0.1287	0.0424
<i>P</i> -value (significance)												
Landscape	0.883	0.4547	0.046	0.7993	0.5162	0.1319	0.0177	0.3307	0.4454	0.011	0.0411	0.4323
Low	0.0229 A	0.0183 A	0.1025 A	0.0203 A	0.0246 A	0.0226 A	0.0045 A	0.0459 A	0.0094 A	0.060776 A	0.1414 A	0.0169 A
Severe	0.0275 A	0.0227 A	0.1226 A	0.0036 A	0.0165 A	0.0161 A	0.0000 A	0.0285 A	0.0000 A	0.07971 A	0.0164 A	0.0000 A
Moderate	0.0852 A	0.0301 A	0.1588 A	0.0018 A	0.0025 A	0.0172 A	0.0018 A	0.0285 A	0.00606 A	0.07284 A	0.0952 A	0.0019 A
L.S.D. (0.05)	0.0684	0.0518	0.2473	0.1494	0.0678	0.0482	0.0289	0.1599	0.0215	0.0906	0.2022	0.0665
<i>P</i> -value (significance)												
Disturbance	0.0770	0.8355	0.8531	0.9278	0.6904	0.9294	0.9355	0.8104	0.6133	0.8838	0.4378	0.7479
Sandhill	0.0160 AB	0.0119 A	0.0417 A	0.0019 B	0.0359 A	0.0488 A	0.0020 A	0.1500 A	0.0025 B	0.0483 A	0.0952 AB	0.0368 A
Sandhill, slight disturbance	0.0346 AB	0.0000 A	0.0101 A	0.2687 A	0.0086 A	0.0281 A	0.0000 A	0.0011 A	0.0298 A	0.0754 A	0.2105 AB	0.0019 A
Depression	0.0315 AB	0.0298 A	0.2427 A	0.0018 B	0.0338 A	0.0024 A	0.0226 A	0.0006 A	0.0043 B	0.0000 A	0.0136 B	0.0008 A
Sparse Grass	0.0275 AB	0.0227 A	0.1226 A	0.0036 B	0.0165 A	0.0161 A	0.0000 A	0.0000 A	0.0000 B	0.0797 A	0.0164 AB	0.0000 A
Patchy Grass	0.0852 A	0.0301 A	0.1588 A	0.0018 B	0.0025 A	0.0172 A	0.0018 A	0.0285 A	0.0061 B	0.0728 A	0.0952 AB	0.0019 A
Sandhill, moderate disturbance	0.0627 AB	0.0506 A	0.2024 A	0.0000 B	0.0105 A	0.0182 A	0.0000 A	0.0649 A	0.0085 B	0.0548 A	0.2236 A	0.0043 A
Planted pines	0.0117 B	0.0122 A	0.0677 A	0.0000 B	0.0216 A	0.0185 A	0.0004 A	0.0178 A	0.0118 AE	0.0890 A	0.1823 AB	0.0192 A
L.S.D. (0.05)	0.0695	0.0523	0.2558	0.1171	0.0721	0.0484	0.0312	0.1501	0.0207	0.0940	0.2082	0.0699
<i>P</i> -value (significance)												
Vegetation type	0.1953	0.5333	0.3159	0.0045	0.9259	0.3368	0.5167	0.1395	0.0220	0.2173	0.0148	0.8043
Bonham Creek Upland	0.0454 A	0.0301 A	0.13680 A	0.0392 A	0.0096 B	0.0259 A	0.0004 B	0.0230 A	0.0094 A	0.0803 A	0.1432 A	0.0016 A
Bonham Creek Bottomland	0.0061 B	0.0129 A	0.0999 A	0.0101A	0.0451 A	0.0094 A	0.0390 A	0.0310 A	0.0121 A	0.0038 B	0.0366 B	0.0066 A
Sally Branch Upland	0.0137 B	0.0128 A	0.0625 A	0.0006 A	0.0262 A	0.0263 A	0.0009 B	0.0629 A	0.0089 A	0.0711 A	0.1487 A	0.0262 A
Sally Branch Bottomland	0.0226 AB	0.0213 A	0.1780 A	0.0134 A	0.0536 A	0.0036 A	0.0364 A	0.0460 A	0.0117 A	0.0000 B	0.0197 B	0.0084 A
L.S.D. (0.05)	0.0312	0.0256	0.1341	0.0598	0.0407	0.0241	0.0269	0.0723	0.0128	0.0383	0.0889	0.0285
<i>P</i> -value (significance)												
Transect	0.042	0.3856	0.3806	0.5161	0.1535	0.1554	0.0017	0.5943	0.9279	<0.0001	0.0039	0.2098

Within each column, means that share the same letter are not significantly different at $P = 0.05$.

^a Numbers within parentheses refer to cluster presented in Fig. 1.

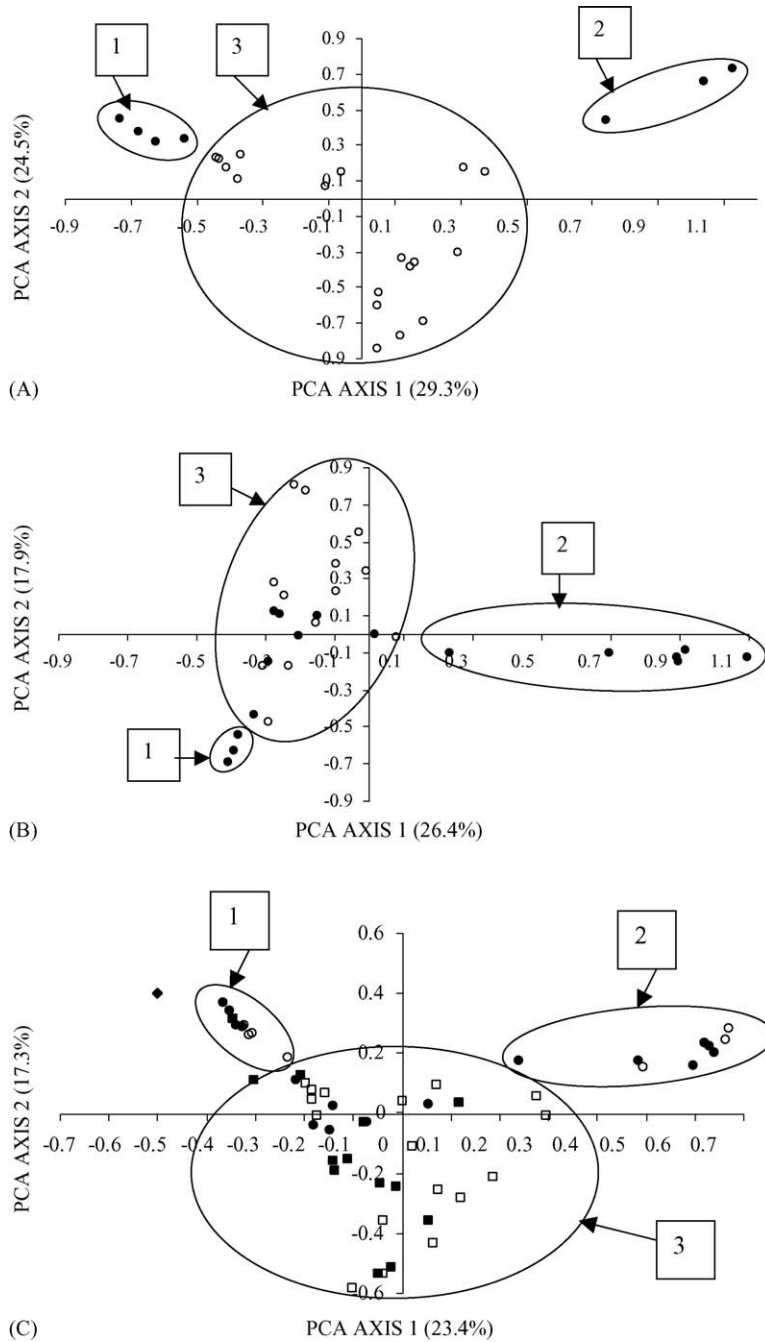


Fig. 2. PCA of *pmoA* T-RFLPs. (A) Sally Branch watershed; (B) Bonham Creek watershed; (C) combined Sally Branch and Bonham Creek watersheds. (A and B) (○) represent upland samples; (●) represent bottomland samples. (C) (□) represent Sally Branch upland samples, (○) represent Sally Branch bottomlands; (■) represent Bonham Creek upland samples, (●) represent Bonham Creek bottomland samples, (◆) represents reference sample RH-1 (Table 1). Numbers in boxes refer to clusters discussed in text.

Differences between the Sally Branch and Bonham Creek PCAs are likely related to difference in land use impacts between the two watersheds. Erosion of Bonham Creek uplands has been well documented (Bhat et al., 2004; Perkins et al., 2004), with transport of soil down slopes into bottomlands. Transport of soil bacteria, including methanotrophs, would be expected to occur with erosion, resulting in mixing of upland with bottomland communities. Uplands and bottomlands in forested watersheds not subjected to significant amounts of erosion would be more completely separated, as observed with the Sally Branch PCA.

Correlation of relative frequencies of individual T-RFs with environmental parameters, including transect, subjective level of disturbance, dominant plant community and watershed is presented in Table 2. Individual T-RFs within the expected ± 2 bp margin of error are presented as a single T-RF if both T-RFs exhibit similar trends. If trends between T-RFs with ± 2 bp exhibited different trends (e.g., T-RFs 66 and 68; Table 2), both T-RFs are presented.

The strongest correlations among predicted T-RFs were observed for: T-RF 23 (Cluster IIIc), which showed a clear enrichment in grassy patches of the upland Bonham Creek transect relative to Bonham Creek bottomlands or the managed pines of the Sally Branch upland transect; T-RF 68 (Cluster IIIc), positively correlated with the bottomlands of both watersheds and T-RFs 107 (Cluster Ia) and 139 (Cluster IV), positively correlated with the upland transects of both watersheds. Even though the transects crossed a number of plant community types (Table 1), the relative frequencies of most individual T-RFs did not correlate strongly with individual plant community types (Table 2). A few significant correlations between T-RF frequency and level of disturbance or general sample type was noted, e.g., the negative correlation between T-RF 23 (IIIc) and planted pines (Table 2); however, this was not typical.

These data indicate that individual genotypes were present in both transects, and that the distinctions between the different PCA clusters outlined in Fig. 2 were likely due to the presence or absence of a few genotypes in each assemblage. No significant differences in the frequencies of individual T-RFs were observed between the Bonham Creek and Sally Branch transects (Table 2), although a few T-RFs

correlated with combined upland or bottomland transects. This indicates that our preliminary assumption that these Bonham Creek and Sally Branch could serve as paired watersheds for the purpose of comparison was appropriate.

4. Conclusions

T-RFLP analysis of *pmoA* sequences in DNA from soil samples taken from transects in two watersheds at Ft. Benning suggests impact of land use on the distribution of methanotrophs was largely a result of transport of upland methanotrophs to bottomlands as a result of erosion in the highly impacted Bonham Creek watershed. Upland and bottomland assemblages in the relatively undisturbed Sally Branch watershed were distinct, with no evident mixing. T-RFLPs from Sally Branch and Bonham Creek upland transects clustered together; however, indicating that land use differences between the two watersheds did not significantly affect the distribution of methanotrophs. Implications for impact of erosion on functioning of methanotroph assemblages suggest the likelihood of diminished methane oxidation rates as strains adapted to upland conditions are transported to bottomlands. Transects in both watersheds crossed a number of different plant community types (Tanner et al., submitted for publication), although the composition of methanotroph assemblages did not appear to be related to the dominant vegetation or watershed. More work is required to investigate relationships between land use, methanogenesis, methane oxidation and environmental factors that control distribution of individual species in highly disturbed forest soils. Erosion and associated effects, including redistribution of methanotrophs, are likely indicators of declining ecosystem function and health at Ft. Benning.

Application of *pmoA* diversity as a general indicator of ecosystem health in other forests will require more development, and any conclusions regarding distribution of specific genotypes or T-RFs are likely to be site-specific. The Sally Branch clone libraries in this study were dominated by clusters representing BSC γ , sequences that have not been described in libraries from other forest soils. Conversely, *pmoA* libraries from other forest soils have been shown to harbor sequences that do not form

significant fractions of the libraries constructed in this study. No *pmoA* genotype or T-RF in this study was specifically associated with impact, but rather with translocation of upland sequences into bottomland environments. Studies in a variety of environments, including those without significant slopes and clearly distinct communities as reported here, are required to establish *pmoA* genotype distribution as a general indicator of ecological impact.

It is likely that the most sensitive molecular microbial indicator of a particular stress or ecosystem health will be dependent on the stressor and the specific environment. For example, Castro et al. (2005) recently demonstrated that distribution of specific genes associated with sulfate reducing bacteria and methanogens provided sensitive indicators of nutrient impact in a wetland soil. A fundamental knowledge of the general ecology of the site and the microbial process most likely affected by the impact would aid in identification of potential molecular microbial indicators.

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