Abstract

Acidification of forest ecosystems due to acid deposition has been shown to decrease acid neutralizing capacity (ANC) and adversely affect microbial community structure, function, and diversity. In this study, I describe the effects of acidification on soil methane oxidizing communities in the Northeastern United States and provides evidence of changing chemical and biological indicators of acid stress in forested headwater catchments. Biological methane oxidation in soils is an important global sink of methane, a potent greenhouse gas, and can contribute up to 15% of total methane consumption annually. Methane oxidation rates are sensitive to changes in pH, but the effects of acid deposition on natural methane oxidizing communities are unknown.

Reduced strong acid inputs have not been accompanied by increases in ANC as expected, and many remote forest soils and streams remain chronically acidic. Stream water chemistry was investigated to determine what is suppressing the ANC and contributing to acidification of streams. Naturally occurring organic acids, measured with dissolved organic carbon (DOC), showed significant relationships when regressed against ANC.

In situ hybridization (FISH) with rRNA-targeted, fluorescently labeled oligonucleotide probes was used with confocal scanning laser microscopy to enumerate and compare methanotrophic bacteria in forest soils affected by varying amounts of atmospheric acid deposition. Surface soil horizons showed relatively low numbers of methanotrophs in all seasons, but organic and mineral horizons varied widely. Type II methanotrophs showed a moderate correlation with soil pH.

Terminal restriction fragment (TRF) analysis was conducted on environmental 16S rRNA gene samples to determine fingerprints of microbial methane oxidizing communities. Inherent differences exist between the tested methanotroph communities and known strains of methanotrophs. Samples exposed to higher levels of acid deposition did not show lower numbers of methane oxidizers, but suggested a shift in community structure to favor unknown acid tolerant species.

Indicators of Acid Stress in Northeastern Forest Soil Methane Oxidizing Communities

A thesis by

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Committee Approval Page

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Chapter 1: Introduction

1.1 Atmospheric methane

Methane is a greenhouse gas that has been increasing in the atmosphere by about 1% each year for the past few decades, mainly due to anthropogenic activities (Cicerone and Oremland 1988). Because methane is 26 times more efficient than carbon dioxide at absorbing infrared radiation, this exponential increase has raised concerns about the global warming potential of methane (Schlesinger 1997, Reay 2001). A significant amount of methane is degraded photochemically in the atmosphere (~450 Tg yr^{-1}). In addition, biological methane oxidation in soils is also an important sink of methane gas, consuming approximately 30 Tg yr^{-1} (Table 1.1). Aerable soils can contribute up to 15% of total methane consumption annually (Born et al. 1990).

The most significant natural source of atmospheric methane is methanogenesis from anoxic soils and sediments. Most of the methane generated in anoxic lower soil layers is oxidized by bacteria in the aerobic layers above before it enters the atmosphere (~700 Tg y^{-1}) (Nedwell 1995, Holmes et al. 1999). Some aerobic microorganisms in soils, believed to be methanotrophs, can also oxidize methane directly from the atmosphere, but the organisms responsible for this oxidation have not yet been identified (Reay 2001). Currently, however, the methanotrophs' only known function in soil is to oxidize methane escaping from anoxic soils. This effective natural sink of methane could potentially make communities of methanotrophs very important in mediating methane release to the atmosphere and reducing the contribution of methane to global warming.

Table 1.1. Global methane budget including estimated sources and sinks of methane in the atmosphere in units of 10^{12}g CH_4 yr^{-1} (From Schlesinger 1997).

Sources	**Range**	**Likely**
Natural		
Wetlands		
Tropics	30-80	65
Northern	20-60	40
Others	5-15	10
Termites	10-50	20
Ocean	5-50	10
Freshwater	1-25	5
Geological	5-15	10
Total		160
Anthropogenic		
Fossil fuel related		
Coal mines	15-45	30
Natural gas	25-50	40
Petroleum industry	5-30	15
Coal combustion	5-30	15
Waste management system		
Landfills	20-70	40
Animal waste	20-30	25
Domestic sewage treatment	15-80	25
Enteric fermentation	65-100	85
Biomass burning	20-80	40
Rice paddies	20-100	60
Total		375
Total sources		535
Sinks		
Reaction witih OH	330-560	445
Removal in stratosphere	25-55	40
Removal by soils	15-45	30
Total sinks		515
Atmospheric increase	30-35	30

1.2 Methane oxidation

The methane oxidizing bacteria (MOB) found in soils fall into two distinct groups, methanotrophs and nitrifiers. Methanotrophs are able to assimilate methane into biomass and use the oxidation of methane exclusively as their sole source of energy. In one study, 31 to 43% of methane oxidized by MOB was assimilated into all major cell macromolecules while the remainder was collected as respirated CO_2 (Roslev et al. 1997). Almost all bacteria that utilize methane for carbon and energy are obligate methanotrophs (Hanson and Hanson 1996). Alternatively, nitrification is a key process in the global nitrogen cycle that can affect methane oxidation (Schlesinger 1997; Vitousek et al. 1997). However, nitrifiers are only able to cooxidize minimal amounts of methane for short periods of time with no significant benefit to the cell (Holmes et al. 1999). Thus, these organisms have only a minor influence on total methane oxidation in soils.

The first step in methane oxidation by MOB is the conversion of methane to methanol catalyzed by the enzyme methane monooxygenase (MMO). There are two types of MMO: a membrane-bound or particulate type (pMMO) found in nearly all known methanotrophs and a soluble or cytoplasmic enzyme (sMMO) characteristic of only certain methanotrophs. The sMMO has been reported to maintain significantly higher rates of methane oxidation than the pMMO (Hanson and Hanson 1996). Methanotrophs in pure culture show sustained methane uptake at normal atmospheric concentrations when methanol is added (Benstead et al. 1998) and some cultured methanotrophs are able to maintain the ability to oxidize methane at normal rates after being deprived of methane for up to ten generations (Hanson and Hanson 1996). It has

been proposed that methanol in soil may be important for methane oxidation by methanotrophs in the environment (McDonald and Murrell 1997).

The broad enzymatic specificity of monooxygenases also enables methanotrophs to oxidize ammonia and nitrifiers to oxidize methane, although at minimal rates (Jiang and Bakken 1999). The oxidation of ammonia by nitrifiers is carried out by ammonia monooxygenase (AMO), which can substitute methane for ammonia as its substrate (Bodelier and Frenzel 1999). The genes encoding pMMO and AMO contain highly conserved regions in the active site subunits, suggesting the enzymes may be evolutionarily related (Holmes et al. 1995). Group-specific oligonucleotide probes complementary to 16S rRNA and pMMO sequences have been developed to detect methanotrophs and nitrifiers in uncultured environmental samples (Bourne et al. 2001; Costello and Lidstrom 1999; Dedysh et al. 2001; Gulledge et al. 2001; Hanson and Hanson 1996; Horz et al. 2001; Murell et al. 1998).

Methanotrophic bacteria have been observed to produce and consume nitrogen oxides (Ren et al. 2000) and fix atmospheric N (Auman et al. 2001). Methanotrophs are also able to degrade a variety of common halogenated aliphatic compounds, such as trichloroethylene (TCE) and chloroform (Hanson and Hanson 1996). The non-specificity of the MMO enables substrate substitution and cometabolism of these compounds (Mahendra 2001). This cometabolic activity can be utilized in the bioremediation of contaminated sites.

1.3 Classification

Methanotrophs are classified into two main subgroups based on their methane

assimilation pathways. Hanson and Hanson (1996) have described the utilization of methane by methanotrophs in detail. All methanotrophs oxidize methane to methanol utilizing either pMMO or sMMO and then convert methanol to formaldehyde using a periplasmic methanol dehydrogenase. Type I methanotrophs come from the γ-subdivision of the *Proteobacteria* and utilize the ribulose monophosphate (RuMP) pathway for formaldehyde fixation. Type II methanotrophs are found in the α-subdivision of the *Proteobacteria* and utilize formaldehyde through the serine pathway. A third subgroup, called type X and characterized by *Methylococcus capsulatus*, contains enzymes from both the RuMP and serine pathways. Type I methanotrophs include the genera *Methylomonas, Methylocaldum, Methylomicrobium, Methylobacter, Methylosarcina, Methylosphaera, Methylothermus, and Methylococcus* (Bowman et al. 1993, Costello et al. 2002). Type II methanotrophs traditionally include the genera *Methylocystis, Methylosinus,* and *Methylocella* (Dedysh et al. 2000, Eller et al. 2001). In addition, *Methylocapsa acidophila* B2, a proposed type III methanotroph because of its novel intercytoplasmic membrane structure, has been recently isolated from *Sphagnum* peat (Dedysh et al. 2002).

1.4 Temperature and moisture

Temperature and moisture are important factors for microorganisms in general and are suggested to be key factors for the growth of MOB in the environment. Significant seasonal differences in methane oxidation rates were observed at experimental field sites with high rates during summer, low rates during winter, and moderate rates during spring and autumn (Steinkamp et al. 2001). Rates of methane

oxidation in the field were positively correlated with soil temperature and negatively correlated with soil moisture, with temperature being a stronger modulator below soil temperatures of 10^{o}C (Prieme and Christensen 1997, Steinkamp et al. 2001). Since methanotrophic activity in soils can be reduced by extremes in temperature and moisture, the mineral soil may provide a more stable environment for methane oxidizers than the organic horizons (Schnell and King 1996).

Most methanotrophs in pure culture are mesophiles with optimal growth at 25^{o}C. In one study, the percentage of methane assimilated by MOB increased between temperatures of 5 to 20^{o}C, increased slightly between 20 and 30^{o}C, and decreased above 30^{o}C (Roslev et al. 1997). Type X methanotrophs can grow at up to 50 ^{o}C and some psychrophilic type I strains have also been identified (Hanson and Hanson 1996). Several new genera of thermotolerant and thermophilic methanotrophs have also been identified, including *Methylocaldum* and *Methylothermus,* respectively (Bodrossy et al. 1997, Bodrossy et al. 1999, Trotsenko and Kmelenina 2002).

1.5 Acid deposition

Acid deposition has become a critical environmental problem affecting forested ecosystems throughout the world. Air pollution travels across large geographic areas and contributes to biological stress and ecosystem degradation (Schlesinger 1997). Sulfate deposition, the major contributor to acid rain, has declined in recent years due to the implementation of 1990 Amendments to the Clean Air Act (Fig.1.1) (Driscoll et al. 2001). Other anthropogenic contributors to acid rain, principally NO_3^-, remain high in

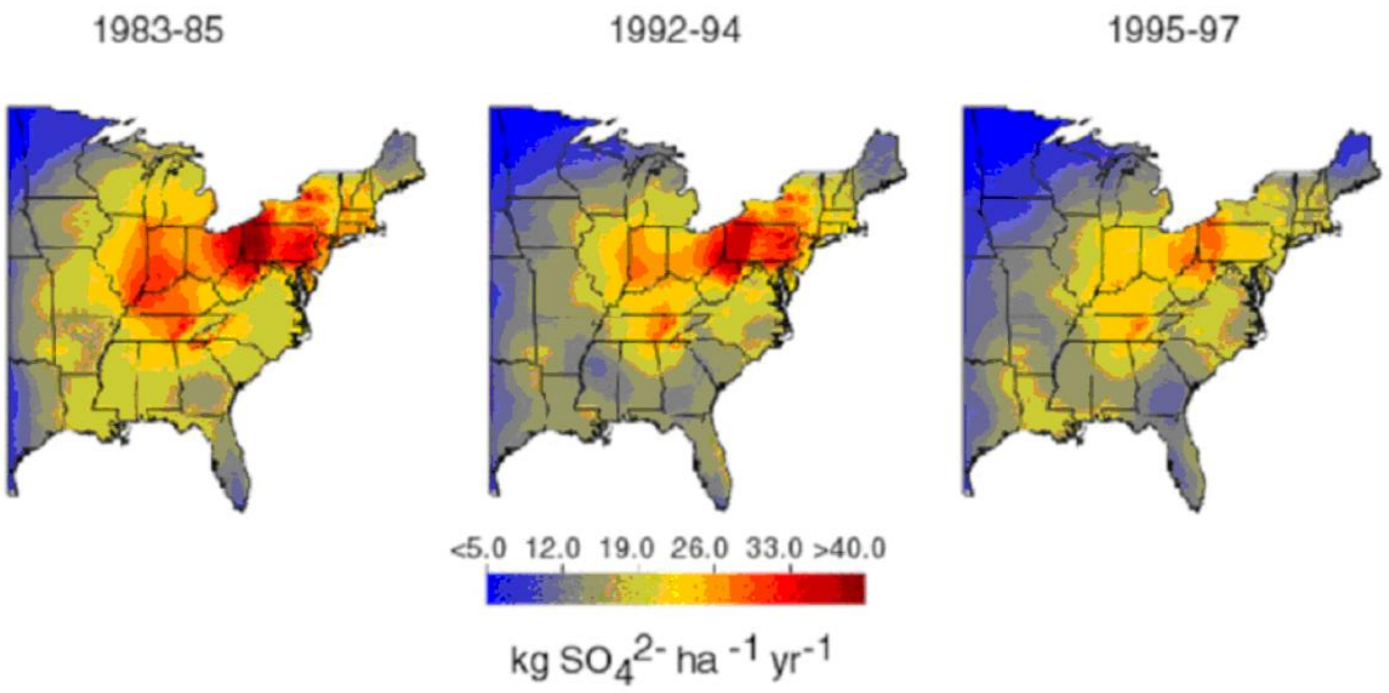


Figure 1.1. Annual wet deposition of SO_4^{2-} (in kg SO_4^{2-} ha^{-1} yr^{-1}) in the Eastern United States for 1983-1985, 1992-1994, and 1995-1997 (From Driscoll et al. 2001).

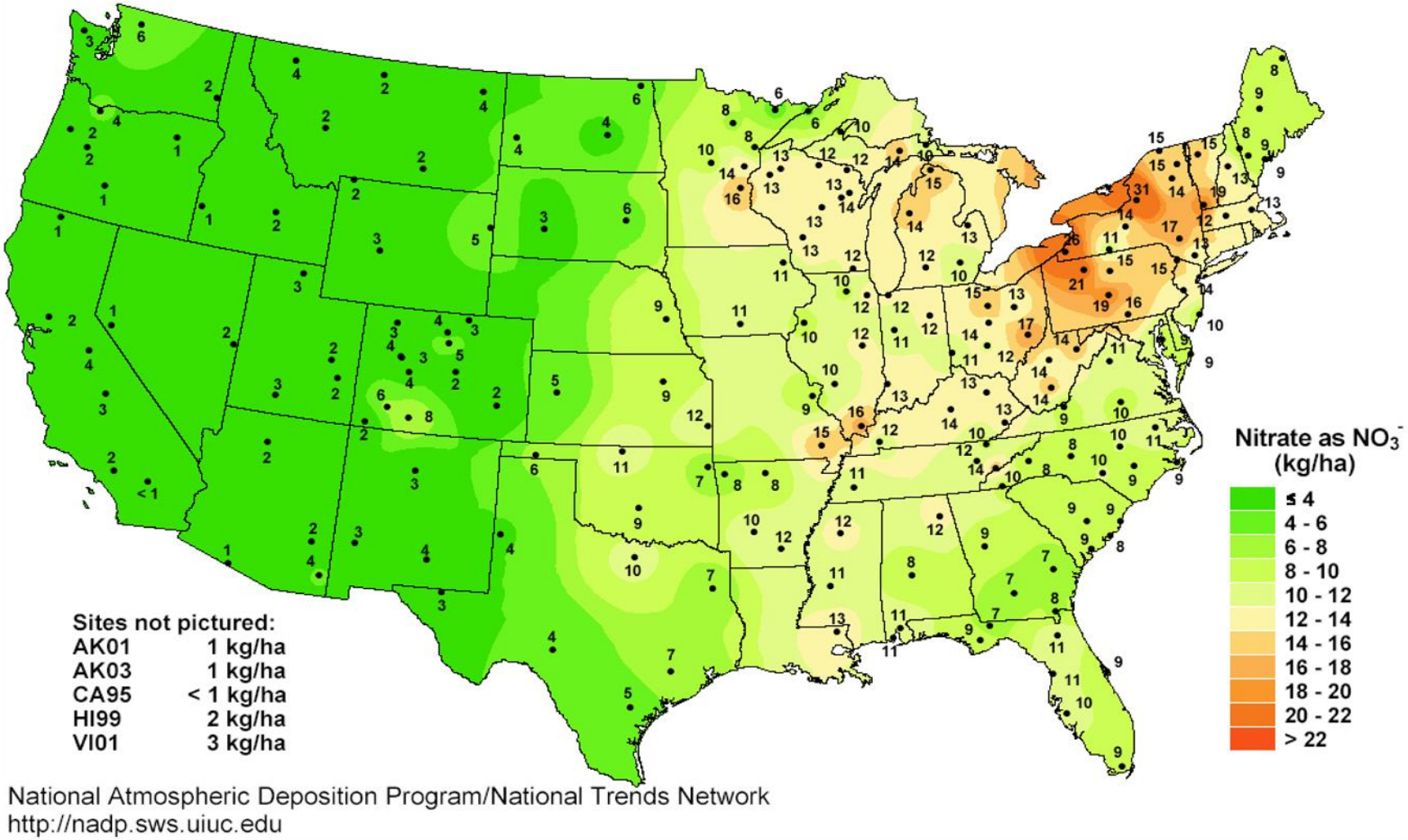


Figure 1.2. Annual wet deposition of NO3- (in kg NO3- ha-1 yr-1) in the United States for 2002. From the National Atmospheric Deposition Program/National Trends Network. http://nadp.sws.uiuc.edu, February 2004.

the Northeastern U.S. (Fig. 1.2) (Driscoll et al. 2003). Sulfate, ammonium, and nitrate deposition are important inputs to forested ecosystems and are major contributors to soil acidification (Likens and Bormann 1995). Acid deposition is known to decrease soil acid neutralizing capacity, leach base cations, and mobilize pH-dependent toxic metals, such as Al^{3+}, all of which can have serious deleterious effects on forested ecosystems (Schlesinger 1997).

Global land-use change and atmospheric pollution has led to widespread soil disturbance. Physical disturbance of the soil can decrease methane oxidation and assimilation by 50 to 58% (Roslev et al. 1997). The fact that the magnitude of the observed atmospheric increase is equivalent to the estimated soil methane sink of about 20 to 60 Tg yr^{-1} heightens the importance of soil methane oxidation in the global methane cycle (Holmes et al. 1999).

Many soil microorganisms are sensitive to extremes in pH (Pennanen et al. 1998). Previous studies have investigated the effect of pH on methane oxidation. Methanotroph communities appear to be able to oxidize methane in a wide range of pH (Hanson and Hanson 1996). Similar rates of methane oxidation have been observed in soils pH 3.5 to 8.0. Dedysh et al. (2000) described methane oxidation in peat soils, which was present at pH 4.0 to 6.0 and decreased sharply at values above and below this range. The pH optima and minima for methane oxidation were slightly lower in acidic soils and oxidation rates were highest in acidic soils at pH 5.0-5.5. These studies suggest that some methanotrophs are at least partially adapted to acidic environments. How methanotroph communities respond to various levels of chronic acid deposition is not yet known.

Acidophilic MOB have been isolated from acidic *Sphagnum* peat bogs by Dedysh et al. (1998), and have pH values ranging from 3.5 to 5.0. The methane monoxygenase subunit, *mmo*X, and 16S rRNA gene sequence data indicated that these acidophilic strains represent a lineage of MOB only moderately related to *Methylosinus-Methylocystis* spp. The acidophilic strains isolated from peat were most closely related to *Beijerinckia indica*, a common heterotrophic bacterium in acid soils. After further study, a new genus, *Methylocella*, and a new species, *Methylocella palustris*, was identified for three strains of methane oxidizing bacteria without pMMO isolated from acidic *Sphagnum* peat bogs (Dedysh et al. 2000). With 96.5% 16S rDNA sequence similarity to *B. indica*, these methanotrophs represent a new type of MOB that may be present in acid forest soils.

1.6 Nitrogen

Differences in N cycling may play an important role in regulating the strength of soil methane sinks (Tlustos et al. 1998). Maximum methane oxidation rates tend to coincide with low average ammonium and nitrate concentrations (Prieme and Christensen 1997). Nitrogen additions can significantly decrease the methane uptake rate in temperate forest soils (Steudler et al. 1989) and other ecosystems (Mosier et al. 1991). Complete short-term inhibitions of methane oxidation were noticed immediately after ammonium additions (Bronson and Mosier 1994). Inhibitory effects were observed in approximately inverse proportion to the amount of ammonium added (Roslev et al. 1997, Tlustos et al. 1998). Repeated application of NH_4^+ was found to result in strong, long-term inhibition of methane oxidation (Hutsch et al. 1994).

In pure cultures of the methanotrophs *Methylomicrobium album* BG8 and *Methlyosinus trichosporium* OB3b, methane oxidation was non-competitively inhibited by NO_2^- produced from NH_4^+ (King and Schnell 1994a). However, in soils with strong nitrification, accumulation of NO_2^- is small and of little importance for inhibition of methane oxidation (Hutsch 1998). Nitrate-based fertilizers appear to have little effect on oxidation of methane (Hanson and Hanson 1996, Hutsch 1998). Reay et al. (2001) have hypothesized that ammonium may be a competitive inhibitor for methane in methanotrophs or that high ammonium concentrations inhibit methane oxidation by favoring nitrifiers over methanotrophs. It remains unclear whether the low methane oxidation potentials are due to direct inhibition by ammonium or indirect competition by nitrifiers (King and Schnell 1994b).

In some studies, however, N additions had no inhibitory effect on methane oxidation (Castro et al. 1995, Goldman et al. 1995, Gulledge et al. 1997). The reasons for conflicting results are not yet clear, but it has been hypothesized that potential effects of N additions on methane oxidation may depend on the N status of the forest ecosystem (Steinkamp et al. 2001). As an effect of excessive N deposition from the atmosphere, the N status of forests can shift from N limited to N saturated (Aber et al. 1998). A higher N immobilization capacity of N saturated soils may protect methane oxidizers from exposure to the inhibitory effects of ammonium (Gulledge et al. 1997). On the other hand, chronic additions of N could cause alterations in the microbial communities in soil, possibly resulting in the suppression of methanotrophic bacteria by boosting populations of nitrifiers (Adamsen and King 1993). Differences like these might be reconciled by an

in-depth study of the methane oxidizing and nitrifying communities across an N deposition gradient.

1.7 Objectives of this study

Culture-dependent detection methods for community analyses of environmental samples are inadequate because most organisms are difficult to culture in the laboratory and these methods often underestimate the numbers and diversity of bacterial communities (Amann et al. 1996; Holmes et al. 1999). Among culture-independent techniques, terminal restriction fragment length polymorphism (TRFLP) analysis and fluorescent *in situ* hybridization (FISH) in combination with confocal scanning laser microscopy are powerful tools that allow the detection, enumeration, and characterization of bacterial cells and communities directly in environmental samples (Amann et al. 1995; Christensen et al. 1999; Zarda et al. 1997). In this study, TRFLP with group specific 16S rRNA and functional gene primers combined with FISH using rRNA-targeted oligonucleotide probes were used to characterize type I and type II methanotroph communities in acidic forest soils in the northeastern U.S. Since atmospheric deposition causes acidification and nutrient fertilization (Driscoll et al. 2001) and methane oxidation in soil is known to be sensitive to pH and nitrogen addition (Hutsch et al. 1994), it is important to determine how the soil bacterial communities are changing due to chronic inputs of acid and nutrients and what are the associated effects on the function of methane oxidation. If relationships exist between methanotroph community ecology and the acid-base status of soils, it may be possible to relate differences in belowground microbial diversity to ecosystem acid stress.

Chapter 2: Stream Water and Soil Chemistry Analysis

2.1 Introduction

Atmospheric deposition of sulfate, nitrate, and ammonia has caused the acidification of forests and surface waters in the northeastern United States. Because acid deposition is highly variable across space and time, study sites need to reflect the differences in acid input across the entire study area. In order to isolate the effects of acid deposition in this study, it was necessary to minimize potential natural differences between sites. Four study sites were chosen along a southwest to northeast acid deposition transect. The physical characteristics of the selected study sites, such as climate, vegetation, elevation, slope, and aspect, were secured allowing the variation in acid deposition inputs to become the major variable. Soil and water samples were collected during the height of the summer growing season (May-July 2002 and 2003) and in the dormant winter season (January-February 2003).

The effects of dissolved organic acids on the acid-base chemistry of stream water were studied in further detail at the Hubbard Brook Experimental Forest (HBEF). Acid deposition and stream water chemistry have been studied in detail at the HBEF for four decades. Driscoll et al. (1988) noted that it was difficult to estimate what conditions were like before monitoring began, and during the entire first two decades of this record, the HBEF system was subject to inputs of strong acids from the atmosphere. Considerable debate exists as to the exact effects of deposition on the acidification of surface waters and the relative contributions of naturally occurring organic acids and bedrock mineral weathering. Attempts were made to understand potential baseline conditions by comparing the HBEF with a less-affected areas (Driscoll et al. 1988; Peters 1985), modeling deposition and stream water trends with a biogeochemical models (Driscoll et

al. 1994; Gbondo-Tugbawa and Driscoll 2003), and restoring the calcium pool loss of an entire small watershed (WS1) with addition of wollastonite (Driscoll 2000). The implication of atmospheric inputs as a major source of acidification in the 1980s led to legislation aimed at reducing source emissions of acidifying sulfur compounds. Recent decreases in strong acid anion deposition have resulted in significantly lower concentrations of SO_4^{2-} and NO_3^- in stream water (Stoddard et al. 1999), however, reduced strong acid inputs have not been accompanied by large increases in ANC as expected, and most streams remain chronically acidic (Driscoll et al. 1998; Driscoll et al. 2001). Naturally occurring organic acids may be suppressing the ANC and contributing to acidification in streams at Hubbard Brook. The objective of this study was to investigate the influence of DOC on the acidification of headwater streams by analyzing long-term data stream water chemistry from WS6 and WS1 at the HBEF.

2.2 Description of Study Sites

Major features of the climate of the study sites include broad ranges in daily and annual air temperature, and nearly uniform monthly precipitation. The major air flow patterns over the northeastern U.S. are continental polar air from subarctic North America, with occasional maritime tropical air from the Caribbean and Gulf of Mexico from the south or maritime air from the North Atlantic out of the east. Despite the proximity of the study sites to the ocean, their climates are predominantly continental. Summers are short and cool and winters are long and cold. The forest soil usually remains unfrozen during winter because of a thick organic layer and deep snow pack. Higher elevations generally experience colder temperatures and more precipitation than

lower elevations. More precipitation with the same concentration of acid anion leads to effectively higher inputs of acid deposition at high elevation. This observation provides the opportunity to study potential intra-site elevation differences in soil bacterial communities. To encompass the full extent of acid deposition in the northeastern U.S., four sites were chosen: Bear Brook Watershed, Maine; HBEF, New Hampshire; Lye Brook Wilderness, Vermont; and Biscuit Brook, New York. (Fig. 2.1).

The Bear Brook Watershed in Maine (BBWM) is the site of a long-term forested paired stream catchment study (Norton et al. 1999). The BBWM, located in eastern Maine approximately 50 km from the Atlantic Ocean, lies on the southeast slope of Lead Mountain ($44^{o}52'$ N, $68^{o}06'$W) between 265 and 475 meters. East Bear Brook drains a 10.2 hectare contiguous biogeochemical reference watershed and contains low dissolved organic carbon (DOC) and low ANC (Norton et al. 1999). The bedrock is quartzite and gneiss with granitic intrusions. Soils are coarse-loamy, frigid Typic Haplorthods 0 to 1 meters thick formed from dense basal till. Soils are acidic with low base saturation, low cation exchange capacity, and low sulfate adsorption capacity. Vegetation is predominantly a northern hardwood forest characterized by American beech (*Fagus grandifolia*), yellow birch (*Betula alleghaniensis*), sugar maple (*Acer saccharum*), and red maple (*Acer rubrum*). Red spruce (*Picea rubens*) dominates and balsam fir (*Abies balsamea*) is a minor component at high elevations and ridgetops. Average annual air temperature is 4.9^{o}C and annual precipitation is about 1.3 meters. An atmospheric monitoring site near the BBWM in Acadia National Park received a moderately low amount of atmospheric acid deposition (Table 2.1). For this study, three sites were

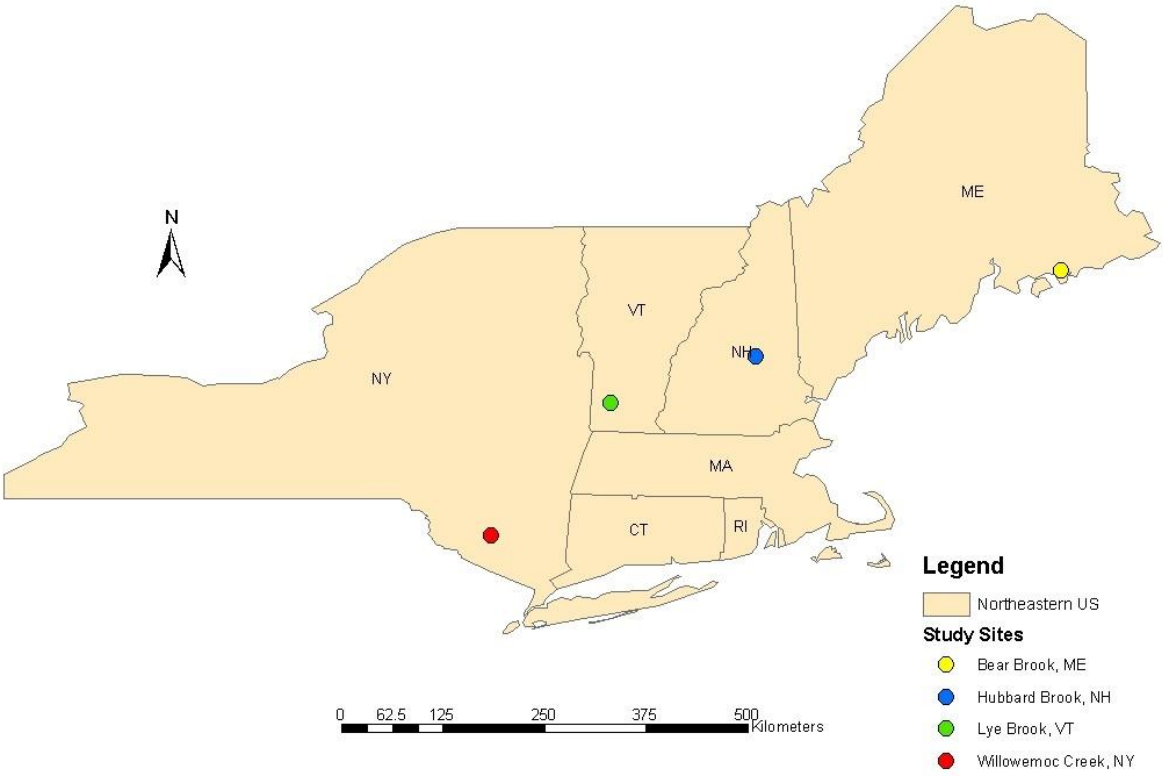


Figure 2.1. Study sites in the northeastern U.S. Bear Brook, ME (N44.51.67, W68.06.30); Hubbard Brook, NH (N43.57.03, W71.44.35); Lye Brook, VT (N43.05.16, W73.05.02); Willowemoc Creek, NY (N48.51.52, W74.37.60).

Table 2.1. Average annual atmospheric deposition in kg ha^{-1} yr^{-1} in 2002. (http://nadp.sws.uiuc.edu/sites/ntnmap.asp). Total S and N wet deposition is the sum of wet SO_4^{2-}, NO_3^-, NH_4^+ in kg ha^{-1} yr^{-1}.

National Atmospheric Deposition Program Site	**SO_4^{2-} wet deposition (kg ha^{-1} yr^{-1})**	**NO_3^- wet deposition (kg ha^{-1} yr^{-1})**	**NH_4^+ wet deposition (kg ha^{-1} yr^{-1})**	**Total S & N wet deposition (kg ha^{-1} yr^{-1})**
Acadia National Park, ME	14.32	10.29	1.87	26.48
Hubbard Brook, NH	13.35	12.85	2.21	28.41
Bennington, VT	23.08	18.95	3.74	45.77
Biscuit Brook, NY	20.92	16.74	3.30	40.96

designated on a longitudinal gradient east of East Bear Brook watershed at approximately 265, 365, and 465 meters.

The HBEF encompasses 3160 hectares in the White Mountains of New Hampshire (43°56'N, 71°45'W) approximately 116 km

from the Atlantic Ocean. The HBEF includes a continuous on-site research program dedicated to the long-term study of forest and aquatic ecosystems. The forest was established by the USDA Forest Service Northeastern Research Station in 1955 as a major center for hydrologic research in New England. Sampling of bulk deposition and stream chemistry in the south-facing watershed six (WS6) began in 1963 and remains the longest continuous record at the HBEF. Likens and Bormann (1995) have described the physical characteristics of the HBEF as well as long-term chemical trends. Data from 13.2 ha WS6 is considered a biogeochemical reference for the experimental watersheds. The forest at the HBEF is underlain by a complex assemblage of metasedimentary and igneous rocks. Soils at the study sites are predominantly well-drained acidic Spodosols, more specifically, Typic Haplorthods, derived from glacial till, with sandy loam textures. Northern hardwood forest characterized by American beech (*Fagus grandifolia*), sugar maple (*Acer saccharum*), and yellow birch (*Betula alleghaniensis*) dominates at low and mid elevations. White birch (*Betula papyrifera*), red spruce (*Picea rubens*), and balsam fir (*Abies balsamea*) are dominant at high elevations and ridgetops. Annual precipitation averages approximately 1.4 meters, of which about one third is snow. A snowpack usually persists from mid-December until mid-April, with a peak depth in March of about 1.2 meters. The mean air temperature in July is 19^{o}C and in January is –9^{o}C. In 2002, the HBEF received a moderately low amount of atmospheric acid deposition (Table 2.1). For this study, four sites were designated along a longitudinal transect west of WS6 at approximately 550, 640, 730, and 790 meters elevation.

The Lye Brook Wilderness (LBW), at 6274 hectares, is the second largest wilderness area in the Green Mountain National Forest, Vermont (43^{o}15'N, 73^{o}05'W).

The LBW is located in the USDA Forest Service Manchester Ranger District and is designated a Class I Air Quality Area under the Clean Air Act. Under this designation, air quality values related to scenic beauty, vegetation, wildlife, water, and odor must be protected from deterioration due to air pollution. Bedrock at the LBW is igneous and metamorphic rock. Soils are sandy loam textured, well-drained acidic Spodosols derived from glacial till. Vegetation consists of American beech (*Fagus grandifolia*), sugar maple (*Acer saccharum*), and yellow birch (*Betula alleghaniensis*) at low elevations. At middle and high elevations, red spruce (*Picea rubens*) and balsam fir (*Abies balsamea*) are dominant. Precipitation at the LBW is about 1.4 meters annually, one third of which falls as snow. A monitoring site near the LBW in Bennington, Vermont, received a high amount of atmospheric acid deposition (Table 2.1). Three locations at the LBW site were chosen on a south-facing slope at approximately 580, 670, and 760 meters.

The Willowemoc Creek (WC) study site in New York covers about 20 hectares near Mongaup Mountain. The bedrock under the Catskills region of New York is sedimentary and metamorphic rock rich in basic cations. Although the WC receives a higher amount of atmospheric acid deposition among the study sites (Table 2.1), soil and surface waters in the region are well buffered with higher stream acid neutralizing capacity (ANC). Vegetation is dominated by American beech (*Fagus grandifolia*), sugar maple (*Acer saccharum*), and yellow birch (*Betula alleghaniensis*). Three sites were measured at WC on an elevational gradient at approximately 520, 610, and 700 meters.

2.3 Measurements

Eighty water samples were collected for chemical analyses. Duplicate 500 mL samples were collected from five elevations at each study site or about every 200 meters along an adjacent stream. All samples were transported to the laboratory on ice, stored at $4^{o}C$, and analyzed according to standard methods. Solution pH was measured in the field the same day the sample was collected using a Fisher Scientific Accumet AP61 portable pH meter calibrated with pH 7.0 and 4.0 buffer solutions (Fisher Scientific). Acid neutralizing capacity (ANC) was measured by titrating 50.0 mL of sample on a Metrohm 716 DMS Titrino and pH Meter-Ross General Purpose Sure-Flow, Standard Size Combination Electrode calibrated with pH 4.0, 7.0, 10.0 buffer solutions (Fisher Scientific) and analyzed with Brinkmann Titrino Workcell 4.3. Dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) were measured on a Tekmar-Dohrmann Phoenix 8000 UV-Persulfate TOC Analyzer calibrated with 1.0, 3.0, 5.0, and 10.0 ppm KHP for DOC and 1.0, 3.0, 5.0, and 10.0 ppm $NaHCO_3$ for DIC. Total nitrogen (Tot-N) was measured on the Bran Luebbe Auto Analyzer 3 with Compact Sampler and AACE 5.24 calibrated with 5.0, 25.0, 50.0 125.0, and 250.0 umol/L KNO_3. Ammonium (NH_4^+) was measured on a Technicon Auto Analyzer with a Proportioning Pump and Labtronics Guelph NewAnalyzer Program 2.50.001 calibrated with 1.0, 2.5, 5.0, 12.5, and 25 umol/L NH_4Cl. Nitrate (NO_3^-), chloride (Cl^-), sulfate (SO_4^{2-}), total fluoride (Tot-F), and total phosphorus (Tot-P) were measured on a Dionex Ion Chromatograph with an AS40 Autosampler, CD20 Conductivity Detector, GP40 Gradient Pump, and Peaknet 4.11 calibrated with 0.375, 0.750, 1.5, 3.75, 7.5, and 15.0 umol/L NaF; 5.0, 10.0, 20.0, 50.0, 100.0, and 200.0 umol/L NaCl; 5.0, 10.0, 20.0, 50.0,

100.0, and 200.0 umol/L KNO_3; 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 umol/L KH_2PO_4; and 5.0, 10.0, 20.0, 50.0, 100.0, and 200.0 umol/L K_2SO_4.

Seventy-five soil samples were collected for chemical and microbiological analyses. Approximately 50-100 g of three soil horizons were collected by soil cores or pits at three elevations at each study site. A fourth, higher spruce-fir dominated sample was collected at the HBEF. To reduce the number of samples, soil horizons from the most microbially active area of each pit or core were pooled to produce three sample horizons (Oi/Oe, Oa/A, E/B) at each sample location. The location of each soil core or pit was determined using a global positioning system (Leica System 500). All samples were transported to the laboratory on ice and stored at -20^{o}C or 4^{o}C. Soil pH was measured in the laboratory by mixing 4.0 grams of organic soil or 20.0 grams of mineral soil in 19.80 mL deionized water, stirring every fifteen minutes for one hour, and measuring pH with a Fisher Scientific Accumet 10 pH meter. Exchangeable hydrogen was measured by adding 4.0 mL 0.1 N calcium chloride to the solutions, stirring every fifteen minutes for one hour, and measuring pH. Calcium chloride dissociates completely and the calcium ions replace the hydrogen ions on the soil exchange matrix, releasing H^+ into solution where it can be measured directly.

In addition, stream water data from 1996-2003 were obtained for the reference watershed 6 (WS6) and experimental watershed 1 (WS1) at HBEF, West Thornton, NH (Driscoll, personal communication). South-facing, 11.8 ha WS1 was amended with 45 tons of Wollastonite ($CaSiO_3$) by helicopter in October 1999. Regression analyses of stream water constituents were performed at a 95% confidence level using $p < 0.05$ as the criterion for determining significant relationships. Surface water was classified as

containing high or low concentrations of naturally occurring organic acids based on the concentration of dissolved organic carbon (DOC; > 500 or < 500 umol of C/L, respectively). The chemical equilibrium model program MINEQL+ (Version 4.0, Environmental Research Software, 1998) was used to calculate ANC based on annual averages of commonly measured stream water constituents (including Na, K^+, Ca^{2+}, Mg^{2+}, Al_T, NH_4^+, F_T, Cl^-, NO_3^-, SO_4^{2-}, DIC, DOC, and pH).

2.4 Results and Discussion

Soil pH for all samples listed in Table 2.2 show a pH range from 3.32 to 4.93. Soil exchangeable acidity in Table 2.3 show a wide variability among horizons, elevations, and sites. Soil chemistry probably plays a vital role in microbial diversity, along with other characteristics of the soil environment.

2.4.1 Soil Horizon Thickness

Each year, forests produce large amounts of organic material in the form of leaves, needles, stems, branches, trunks, and roots. Microorganisms and microfauna responsible for decomposing this abundance of organic material are not able to process it all and some accumulation occurs. Soil depths at the study sites, including unweathered till, were highly variable and averaged approximately 1.0 meter, surface to bedrock. Soils on the ridgetops sometimes consisted of a thin accumulation of organic matter, resting directly on bedrock. The separation between the soil forming zone and the unweathered till and bedrock below was typically distinct. At various places in the forest, the C horizon exists as an impermeable hardpan. These layers restrict root

Table 2.2. Soil pH by horizon at different elevations for each study site.

Soil pH				
Site	Elevation (m)	Oi/Oe	Oa/A	E/B
	265	4.42	4.04	4.81
	365	3.83	3.72	4.28
Bear Brook, ME	465	4.06	4.13	4.04
	550	4.22	4.16	4.51
	640	4.62	4.76	4.93
	730	4.17	4.62	4.65
Hubbard Brook, NH	790	4.32	3.83	3.76
	580	3.65	3.32	3.93
	670	3.71	3.31	4.04
Lye Brook, VT	760	3.67	3.43	4.47
	520	4.10	3.90	4.04
	610	3.67	4.37	4.47
Willowemoc Creek, NY	700	4.51	4.57	4.70

development and downward water movement. Rocks of all sizes can be scattered throughout the soil profile. A prominent feature of the surface topography throughout the northern hardwood forest is the rough pit-and-mound appearance caused by the uprooting of trees (Likens and Bormann 1995). These natural disturbances mix mineral soil from below with nutrient-rich organic surface layers or deposit the lower mineral layers directly on top of the forest floor humus layers, creating buried horizons. These activities

also affect weathering and biogeochemical cycles. Sampling soil in an obvious pit or mound was avoided. At the four study sites, which were representative of northern hardwood forests in general, the forest floor consisted of undecomposed and partially decomposed organic material and was referred to as Oi/Oe. A layer of highly decomposed rich organic soil, or Oa/A, was found below the forest floor. Between the

Table 2.3. Soil exchangeable hydrogen of soil samples by horizon and elevation for each of the study sites.

		Exchangeable Hydrogen (umol/L)		
Site	Elevation (m)	Oi/Oe	Oa/A	E/B
	265	120.5	171.8	31.3
	365	190.9	384.9	98.9
Bear Brook, ME	465	301.9	314.9	127.6
	550	94.6	159.9	56.2
	640	45.2	92.3	16.4
	730	131.9	71.5	39.3
Hubbard Brook, NH	790	138.3	278.7	243.1
	580	484.1	901.8	329.2
	670	262.1	890.6	289.0
Lye Brook, VT	760	253.9	724.9	49.3
	520	177.6	352.7	132.7
	610	462.3	112.2	55.2
Willowemoc Creek, NY	700	92.1	60.2	32.5

organic material and the bedrock was a layer of weathered mineral soil (E/B). Soil horizon depths are shown in Figure 2.2.

2.4.2 Soil Solution Chemistry

Acid neutralizing capacity (ANC), the sum of base cations minus acid anions in solution, is a potentially important chemical indicator of ecosystem acid stress. Recent studies have classified lakes with negative ANC values as chronically acidic and lakes with ANC values of 50 ueq/L or less as vulnerable to episodic acidification (Driscoll et al. 2001). In exploring the link between chemical factors and biological effects of acid inputs, it is important to understand the existing chemical controls on ANC. Soil water draining the HBEF reference WS6 was examined for patterns associated with ANC. Soil water lysimeter data from 2002 were obtained for the reference watershed (WS6) at HBEF (Driscoll, personal communication). Soil water ANC in 2002 ranged from –295.2 to 241.2 ueq/L in the pH range 3.44 to 5.82. As an example of an acid forest soil ecosystem, soil water constituent concentrations and standard deviations for 2001 to 2002 at HBEF are listed in Table 2.5. Notably, DOC concentrations in soil at HBEF ranged from 180.0 to 8504.0 umol C/L. Regression of ANC versus dissolved organic carbon (DOC) showed that 42% of the variation in ANC in soil water (R^2=0.4198; Fig. 2.3) could be explained by DOC concentration. DOC was high at LBW (>400 umol C/L), high at BBWM and HBEF (75-130 umol C/L), and low at WC (<50 umol C/L). DOC was negatively correlated with ANC (Fig. 2.4).

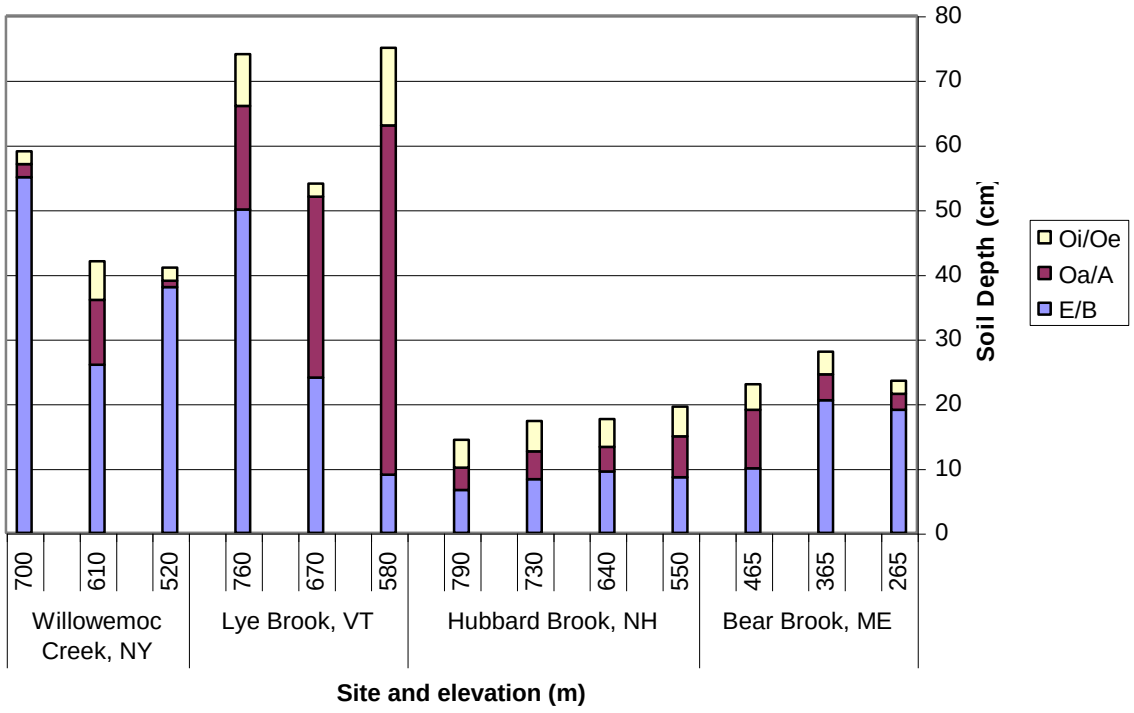


Figure 2.2. Soil horizon thickness from the study sites. Mineral soil measurements reflect the biologically active area of the mineral soil. It was assumed that the mineral soil extended downward to the glacial till, but this depth was not measured.

Table 2.4. Soil water constituent concentrations and standard deviations for six sites in Hubbard Brook Experimental Forest Watershed 6 during the study period 2001-2002.

Soil Water Constituent	Concentration Range	Average ± Standard Deviation n=309
pH	3.59 – 5.82	4.48 ± 0.31
ANC (ueq L^{-1})	-295.2 – 39.1	-36.7 ± 45.4
SO_4^{2-} (ueq L^{-1})	5.4 – 58.9	32.7 ± 12.2
NO_3^- (umol L^{-1})	0.0 – 164.2	14.7 ± 27.9
NH_4^+ (umol L^{-1})	0.2 – 62.2	3.9 ± 6.6
Total N (umol L^{-1})	5.6 – 222.4	43.9 ± 43.2
DIC (umol C L^{-1})	72.8 – 1233.1	313.3 ± 200.8
DOC (umol C L^{-1})	180.0 – 4395.8	862.6 ± 717.1

2.4.3 Stream Chemistry

Water flow through soil is a major mechanism of chemical transport to stream water. Water flow paths and differences in water source control the chemistry of drainage water in forested ecosystems (Pellerin et al. 2002). Baseflow involves chemical weathering reactions that consume hydrogen ions from the upper soil horizons, releasing ANC (Neal et al. 1989). Micropore flow allows longer water residence time, resulting in higher ANC (Peters and Murdoch 1985) and lower dissolved organic carbon (McGlynn et al. 1999). Stream water chemistry reflects flow type, soil solution chemistry, and soil biological activity and is therefore an important indicator of forest ecosystem processes. Measured stream water quality constituents are shown in Tables 2.6.a., 2.6.b., and 2.7.

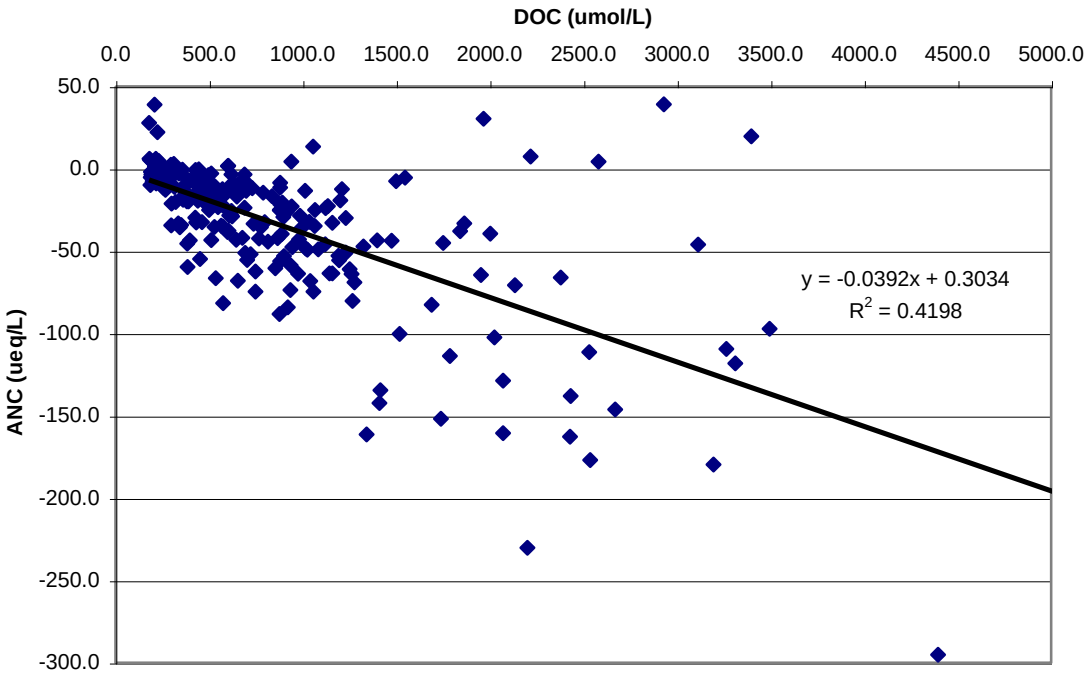


Figure 2.3. Soil water acid neutralizing capacity (ANC) as a function of dissolved organic carbon (DOC) at Hubbard Brook Experimental Forest Watershed 6 during 2001-2002.

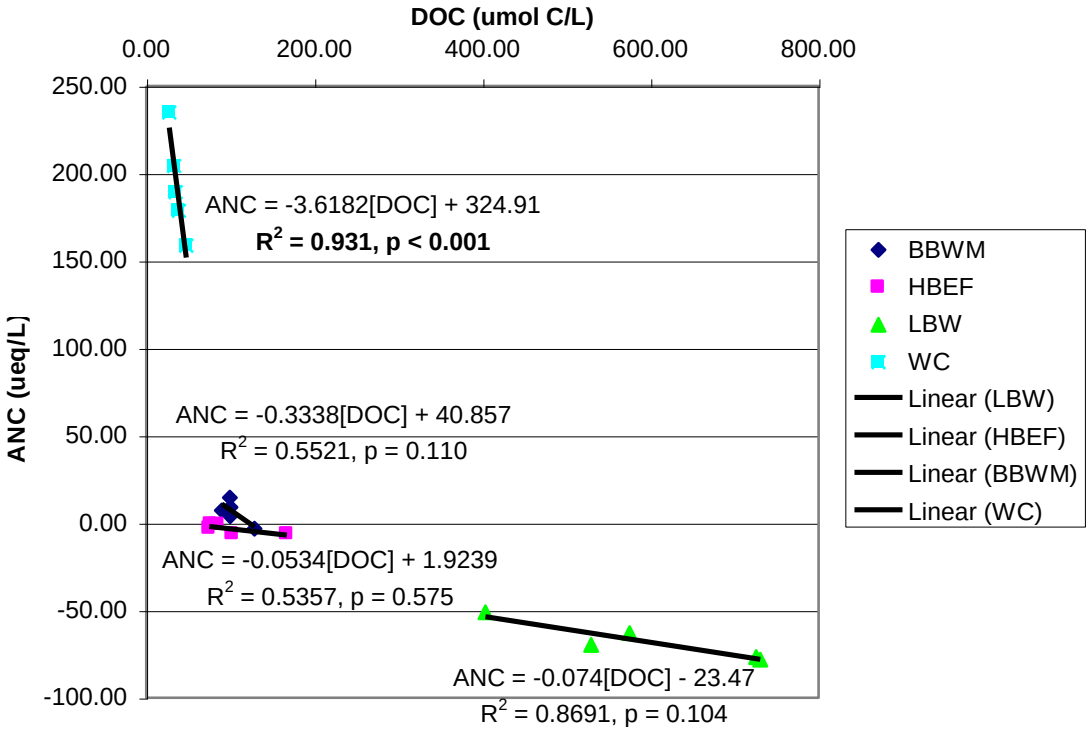


Figure 2.4. Stream water acid neutralizing capacity (ANC; ueq/L) as a function of dissolved organic carbon (DOC; umol C/L) at the Bear Brook Watershed (BBWM); Hubbard Brook Experimental Forest (HBEF); Lye Brook Wilderness (LBW); and Willowemoc Creek (WC), including regression coefficients (R^2) and significance (*p*).

Stream water pH was acidic (pH<5.0) at all study sites, least acidic at WC and most acidic at LBW. Stream pH at BBMW and HBEF was moderate and showed a clear pattern of decreases with increase in elevation at HBEF, but this pattern was not observed at the other sites. Stream water ANC was high at WC (>150 ueq/L), sensitive to episodic acidification at BBWM (0-15 ueq/L), and chronically acidic at HBEF and LBW (<0 ueq/L). Under these conditions, any input of acid from atmospheric deposition would directly lower surface water pH at the HBEF and LBW. Stream water F^- and Cl^- were near zero. Stream water SO_4^{2-} was higher than expected at BBWM, disrupting the pattern of high to low S deposition southwest to northeast. Since sulfate deposition is very low in Maine (Table 2.1) another source of SO_4^{2-}, such mineral weathering, may account for high stream water concentrations. Stream water PO_4^{3-} was below the detection limit of 10 ppb for all samples measured. Stream water NO_3^- was extremely low at all sites except WC, where NO_3^- was in the range of 21-35 umol/L. Although these sites receive nitrate from atmospheric deposition, little enters the stream because it is readily taken up by organisms and used for growth. Stream water NH_4^+ was very low at all the study sites. Most of the ammonium that is deposited on these watersheds from the atmosphere, which is small (Table 2.1), is nitrified or used by plants before it can reach the stream. Total N in stream water includes both inorganic and organic species of N and is very low at these sites. DIC was high at BBWM and WC (>120 umol C/L), and low at HBEF and LBW (<85 umol C/L). DIC is an indicator of microbial activity or weathering in the soil with high DIC indicative of high chemical or biological activity.

Microbial activity and root respiration in soil produce relatively large amounts of CO_2. The increased concentrations of CO_2 cause the pCO_2 to rise above atmospheric

Table 2.5.a. Stream water pH, acid neutralizing capacity, fluoride, chloride, and sulfate from samples collected along elevation gradients at the Bear Brook Watershed (BBWM); Hubbard Brook Experimental Forest (HBEF); Lye Brook Wilderness (LBW); and Willowemoc Creek (WC) in 2002.

site	elevation	field pH	ANC	F^-	Cl^-	SO_4^{2-}
	m		μeq/L	μmol/L	μmol/L	μeq/L
BBWM	265	5.32	9	1	83	103
BBWM	315	5.48	14	2	81	103
BBWM	365	5.25	4	1	81	106
BBWM	415	5.36	7	1	88	107
BBWM	465	4.94	-3	0	68	104
HBEF	550	5.00	-1	2	11	85
HBEF	595	5.00	-1	2	11	87
HBEF	640	4.91	-3	1	10	88
HBEF	685	4.68	-6	1	9	84
HBEF	730	4.54	-6	0	8	79
LBW	580	4.13	-63	1	13	110
LBW	625	4.05	-76	1	13	109
LBW	670	4.04	-78	1	13	110
LBW	715	4.17	-51	1	14	126
LBW	760	4.09	-70	1	15	128
WC	520	5.90	156	1	17	123
WC	565	5.85	179	1	17	124
WC	610	5.94	189	1	12	85
WC	655	5.93	204	1	18	126
WC	700	5.92	234	5	17	73

levels. Based on Henry's Law, an increased partial pressure of CO_2 causes it to dissolve more in solution. The effect was higher concentrations of DIC in soil water, which ranged from 73 to 1233 umol/L at HBEF in 2002, with an annual average of 312 umol/L (Table 2.5). The DIC showed a sharp increase at the spruce-fir elevation during the autumn due to the rapid metabolism of new leaf litter by forest soil microorganisms. As the water moved out of the soil into the stream, the pCO_2 dropped back to atmospheric levels and the excess CO_2 degassed. Stream water DIC concentrations in the stream ranged from 21 to 230 umol/L with an annual average of 49.9 umol/L (Table 2.6). Carbon dioxide gas contributes directly to aquatic acidity by dissolving in streams in the

Table 2.5.b. Stream water nitrate, ammonium, total N, dissolved inorganic carbon, and dissolved carbon from samples collected along elevation gradients at the Bear Brook Watershed (BBWM); Hubbard Brook Experimental Forest (HBEF); Lye Brook Wilderness (LBW); and Willowemoc Creek (WC) in 2002.

site	elevation	NO_3^-	NH_4^+	Tot N	DIC	DOC
	m	μmol/L	μmol/L	μmol/L	μmolC/L	μmolC/L
BBWM	265	0	1	6	121	100
BBWM	315	0	1	5	140	100
BBWM	365	0	1	4	147	100
BBWM	415	0	1	3	138	90
BBWM	465	0	1	5	190	129
HBEF	550	0	1	2	35	85
HBEF	595	0	1	2	32	77
HBEF	640	0	1	3	31	75
HBEF	685	1	1	3	40	102
HBEF	730	1	1	8	82	167
LBW	580	0	0	8	30	575
LBW	625	0	1	9	25	726
LBW	670	0	1	10	29	731
LBW	715	0	0	6	53	404
LBW	760	0	0	7	53	529
WC	520	27	1	25	226	48
WC	565	29	1	25	246	39
WC	610	21	1	27	264	35
WC	655	35	1	29	290	33
WC	700	24	1	31	609	27

form of carbonic acid. However, since CO_2 is not a charged species, it has no effect on the ANC of aquatic systems.

In addition to the stream water data collected for the four study sites, data wereobtained for the reference watershed (WS6) and experimental watershed 1 (WS1) at the HBEF from 1996-2003. Stream water in WS6 during 1996-2003 had DOC ranging from 22 to 952 μmol of C/L and ANC ranging from –111 to 45 μeq/L (Fig. 2.5). Stream water in Watershed 1 during this time had DOC values ranging from 26 to 984 μmol of

Table 2.6. Constituent concentrations and standard deviations for stream water data obtained from Hubbard Brook Experimental Forest Watershed 6 in 2002.

Soil Water Constituent	Concentration Range	Average and Standard Deviation
pH	4.18 – 5.31	4.84 ± 0.33
ANC (meq L^{-1})	-78 – 5	-16 ± 18
SO_4^{2-} (mmol L^{-1})	28 – 51	40 ± 7
NO_3^- (mmol L^{-1})	0 – 12	3 ± 4
NH_4^+ (mmol L^{-1})	0 – 5	2 ± 1
Total N (mmol L^{-1})	3 – 27	11 ± 6
DIC (mmol C L^{-1})	20 – 230	50 ± 36
DOC (mmol C L^{-1})	89 – 1389	290 ± 277

C/L and ANC values ranging from –83 to 27 meq/L (Fig. 2.6). DOC showed a significant relationship when regressed against ANC in WS6 (R^2=0.62, p<0.002; Fig. 2.7) and in WS1 (R^2=0.15, p<0.001; Fig. 2.8). In both WS6 and WS1, five out of eight years showed DOC having a significant influence on ANC (Table 2.8). WS6 seemed to show a decrease in relationship between DOC and ANC between 1996-1999 and an increase in significance during 2000-2003. During the former period in WS6, DOC explained 55% of the variation in ANC in stream water (R^2=0.56, Fig.2.9), but the

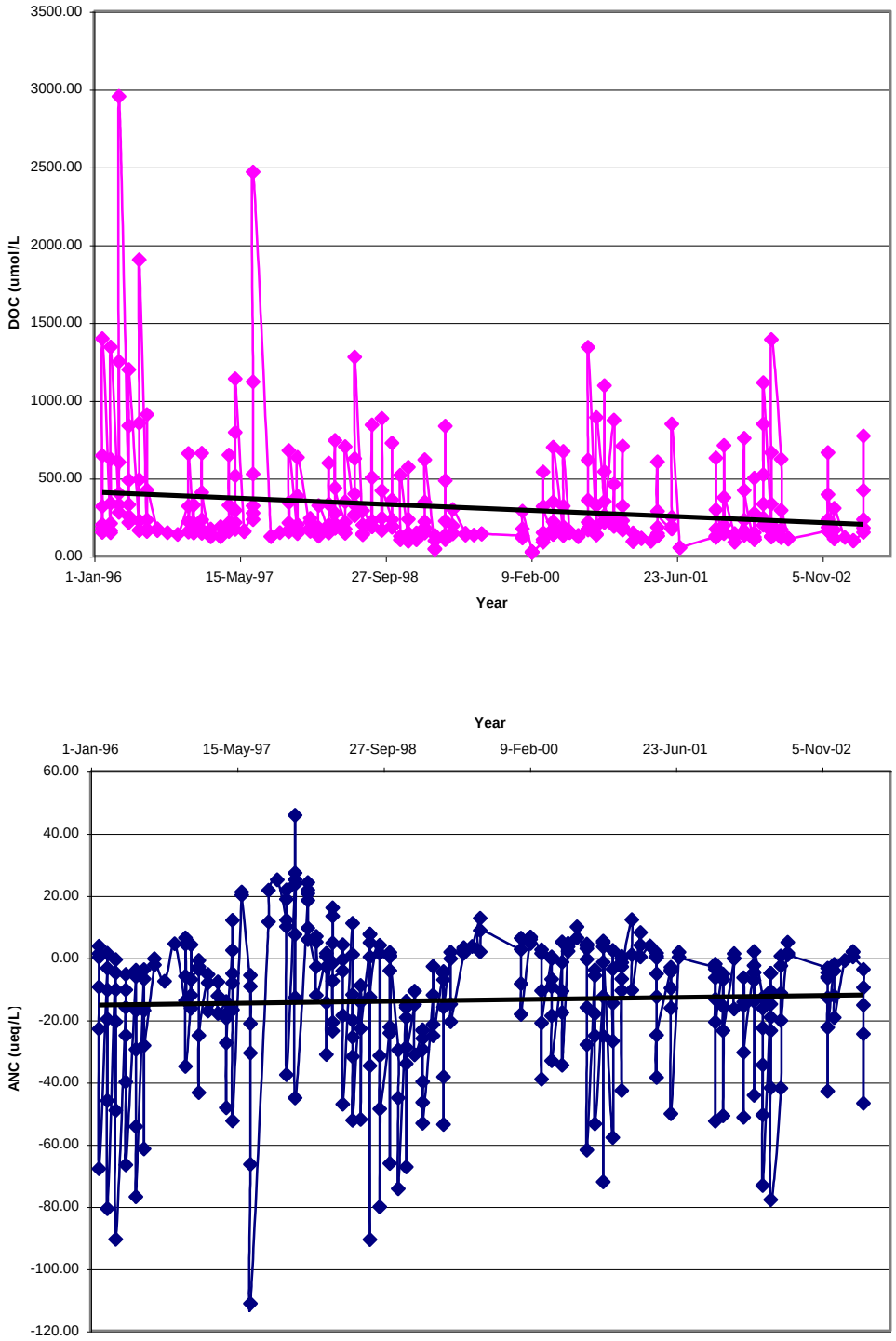


Figure 2.5. Concentrations of dissolved organic carbon (DOC) and acid neutralizing capacity (ANC) at the Hubbard Brook Experimental Forest (HBEF) Watershed 6 during the period 1996-2003.

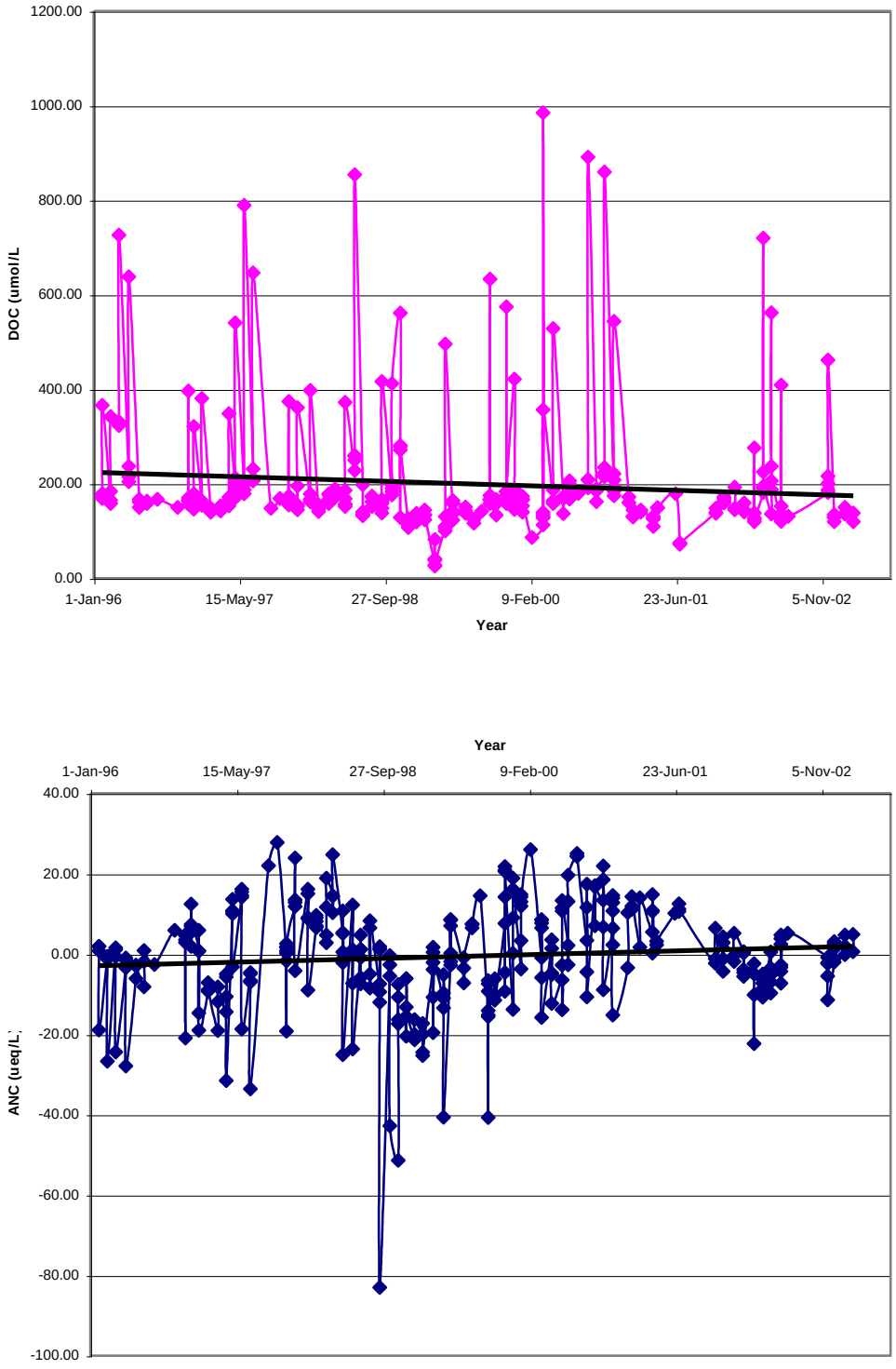


Figure 2.6. Concentrations of dissolved organic carbon (DOC) and acid neutralizing capacity (ANC) at the Hubbard Brook Experimental Forest (HBEF) Watershed 1 during the period 1996-2003.

relationship was not significant. However, during the more recent period, a stronger, significant relationship developed between the two variables (R^2=0.87, $p<0.001$; Fig. 9).

In WS6, periodic decreases in ANC corresponded to spikes in DOC (Fig. 2.5). WS1 showed several decreases in ANC, in particular a major decline in August 1998 (Fig. 2.6) that did not correspond to increases in DOC possibly due to a severe winter ice storm. After the addition of Wollastonite in October 1999, ANC remained around zero ueq/L while DOC continued to fluctuate, occasionally to high levels. The addition of wollastonite, a soluble form of calcium increased the base status of soil in WS1. The new pool of available calcium raised the ANC slightly and buffered the chemistry of soil and stream water from fluctuations in ANC due to natural variations in acid anions. The wollastonite treatment had a leveling-off effect on ANC and may have temporarily reduced the influence of naturally occurring organic acids on ANC in WS1. However, in the four years following the wollastonite treatment, the regression relationships became steadily stronger just as they did in the reference WS6. In the first two years following treatment, the relationship between ANC and DOC was statistically significant in both experimental and control watersheds (Table 2.8). The third year after treatment, the regression relationships in both watersheds became stronger, but were not statistically significant. These observations show that wollastonite dampened the natural fluctuations in ANC, but did not affect the natural cycle of relationships with DOC. WS6, with depleted base cation pool, was always more strongly influenced by naturally occurring dissolved organic acids than WS1 and the regression patterns were consistent in both watersheds. Since acid deposition was consistently decreasing during the study period,

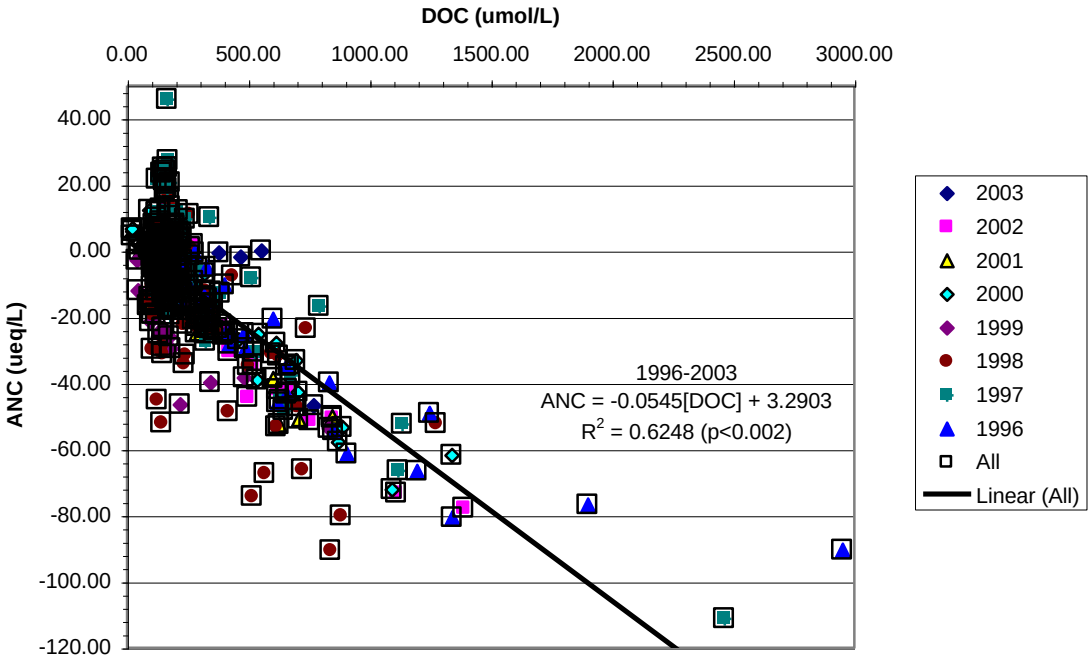


Figure 2.7. Stream water acid neutralizing capacity (ANC) as a function of dissolved organic carbon (DOC) at Hubbard Brook Experimental Forest, New Hampshire, Watershed 6 during 1996-2003.

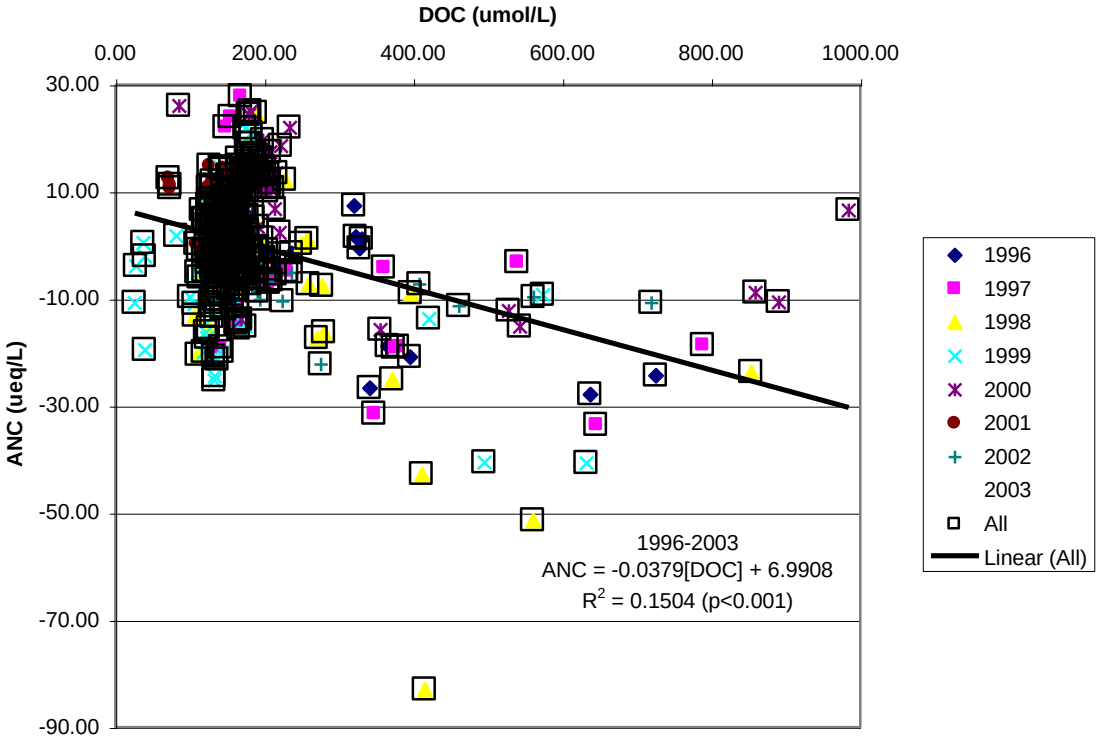


Figure 2.8. Stream water acid neutralizing capacity (ANC) as a function of dissolved organic carbon (DOC) at Hubbard Brook Experimental Forest, New Hampshire, Watershed 1 during 1996-2003.

Table 2.7. Regression relationships and statistical significance between acid neutralizing capacity and dissolved organic carbon in reference watershed 6 and experimental watershed 1 stream water at the Hubbard Brook Experimental Forest, New Hampshire.

	Watershed 6			Watershed 1		
Year	n	R^2	p	n	R^2	p
1996	48	0.83	0.929	34	**0.55**	**<0.001**
1997	45	**0.67**	**<0.001**	45	**0.22**	**0.008**
1998	67	**0.47**	**0.040**	62	**0.32**	**<0.001**
1999	33	0.50	0.932	55	0.10	0.684
2000	62	**0.88**	**<0.001**	53	**0.14**	**<0.001**
2001	34	**0.90**	**<0.001**	26	**0.18**	**0.002**
2002	49	0.90	0.091	45	0.29	0.356
2003	9	**0.97**	**0.003**	5	0.63	0.086

* Model R^2 relates stream acid neutralizing capacity to dissolved organic carbon.
** Significant correlations ($P < 0.05$) are shown in bold.

these results suggest that a more overarching factor, perhaps inter-annual climate variation, exerts control on ANC-DOC relationships at the HBEF.

2.5 Organic Acids

Naturally occurring organic acids include a complex mixture of fulvic, humic, carboxylic, and amino acids, carbohydrates, and hydrocarbons, which vary greatly in their molecular weight and acid-base characteristics (Thurman 1985). Strongly acidic functional groups on organic acids behave similarly to inorganic strong acids and have powerful effects on the chemistry of waters draining podzolic soils (Johnson et al. 1984). Dissociation of naturally occurring organic acids could significantly reduce the acid neutralizing capacity (ANC) of dilute surface waters, however, weakly acidic organic acids do not alter ANC (Driscoll et al. 1989). Measurement of dissolved organic carbon (DOC) is a way to determine the concentration of organic acids and is a crude measure of organic acidity.

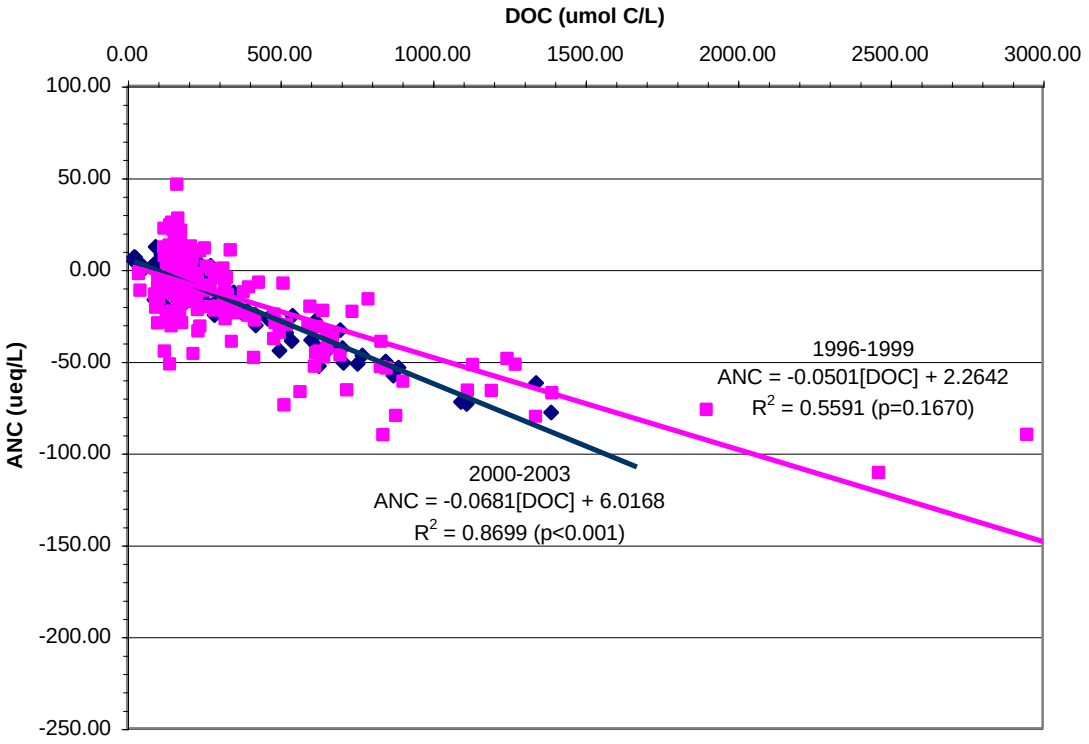


Figure 2.9. Stream water acid neutralizing capacity (ANC) as a function of dissolved organic carbon (DOC) at Hubbard Brook Experimental Forest, New Hampshire Watershed 6 during 1996-2003.

Dissolved organic carbon plays important roles in soil nutrient cycling (Qualls et al. 1991), metal complexation reactions (Pohlman et al. 1988), and belowground carbon cycling (Jandl and Sollins 1997). The large flux of DOC in water passing through the forest floor is thought to originate from microbial degradation of decaying organic matter (McDowell and Likens 1988). Production and consumption of DOC in the forest floor is complicated because macromolecules are not subject to direct root uptake and require microbial degradation (Qualls et al. 2002). Major sources of DOC include microbial activity, root exudates, and soil organic matter (SOM) leaching (Hagedorn et al 2002), although mechanisms controlling leaching of DOC have not been clearly defined (Qualls et al. 2000). Sources of new C make minor contributions to the DOC of the soil solution (Hagedorn et al. 2002). Only 27% of the C in fresh deciduous litter was soluble in water (Qualls et al. 1991), but turnover of DOC in the litter layer and release from labile C pools can be rapid (Park et al. 2002; McDowell 1985; Yano et al. 2000). Environmental controls on microbial activity, such as soil temperature and moisture, could exert major controls on the production of DOC in forest soils (Christ and David 1996). Decomposition rates can also be seasonally suppressed by fluctuations in soil moisture (Wagener et al. 1998).

Soil properties have a great influence on DOC release (Krug and Frink 1983; Hagedorn et al. 2002). Dissolved materials pass readily through sandy soils, but clay soils resist such exports (Wagener et al. 1998). In the mineral soil, DOC is not easily biodegradable due to less favorable biological conditions. DOC is removed from the mineral horizons primarily by adsorption to Fe and Al hydroxides (Qualls et al. 2002) and complexation of organic ligands to inorganic species of aluminum (Pohlman and

McColl 1988; Driscoll et al. 2003). Amendment of NH_4NO_3 reduces the cumulative DOC release (Park et al. 2002) and inputs of strong acid from atmospheric deposition are thought to immobilize organic acids (Driscoll et al. 1989).

Elevation differences within the watersheds are sufficient to cause temperature and moisture variations that affect dissolved nutrient cycling (McDowell and Likens 1988). Wet areas at high elevations, especially in WS6, supply naturally occurring organic acids to soil solution. Concentrations of DOC were consistently highest in high elevation samples, providing a strong DOC source for streams. Each autumn, leaf litter accumulated on the forest floor and released large amounts of naturally occurring organic acids into solution (McDowell 1985). Warm seasonal temperatures caused intense microbial activity in the upper soil horizons. Biological activities consumed, produced, and converted dissolved organic matter and released them into waters that drained the watersheds. Aquatic microorganisms probably metabolized some of the lower molecular weight DOC on its way downstream, but much of the DOC was adsorbed to the stream sediment where it served as organic ligands for soluble Al (Driscoll et al. 2003). In WS6, organic monomeric Al was a significant positive function of DOC while concentrations of both monomeric inorganic Al and DOC decreased most at mid elevation, suggesting aluminum-DOC complexation reactions were taking place in the stream sediment (Johnson et al. 2000; Palmer et al. 2003). At lower elevations, slope was reduced and soil water was able to stay in contact with zones of oxidation for longer periods of time, increasing the opportunity for DOC metabolism by soil microorganisms. Soil waters entering streams at lower elevations had lower DOC concentrations and tended to dilute the total stream DOC.

Naturally occurring dissolved organic acids play important roles in the water chemistry of the HBEF ecosystem. Application of the equilibrium model MINEQL+ provided a theoretical measure of ANC to which water chemistry data could be compared. ANC measurements were compared with theoretical ANC predictions in the range of pH existing at the HBEF. Actual ANC measurements deviated from theoretical predictions by an average of 2%. However, some predicted values were up to 25% from the actual data points, especially at low pH, and suggested a significant anion deficit in solution. As stated earlier, naturally occurring organic acids are a complex mixture of dissolved organic carbon compounds whose acid-base characteristics vary widely. In this case, some portion of the dissolved organic acids with strong-acting acid groups not accounted for by MINEQL+ could make up for the estimated anion deficit. The strong relationships between ANC and DOC shown earlier provide support for this hypothesis.

The evidence presented here provides a case for DOC playing a large role in the acid-base chemistry of base-poor watersheds at the HBEF affected by acid deposition. An anion deficit in solution suggested by MINEQL+ equilibrium modeling could be easily accounted for by strong acid functional groups on a wide variety of naturally occurring organic acids. Significant regression relationships indicate a shifting role of organic acids and their recent increased influence on the acidity of base-depleted headwater streams. Natural variation in the regression patterns suggests climatic control on the strength of ANC-DOC relationships.

2.6 Potential controls on microbial diversity

Several environmental and chemical factors distinguish the sites from each other. Acid deposition was highest at the WC and LBW, showing a stronger chemical stress on the soils of those sites than of the HBEF and BBWM. Soil depth was greatest at the LBW followed closely by the WC watershed. Soil moisture, temperature, exchangeable acidity, and gas diffusion are all directly related to soil depth and may play a large role in the quality of microbial habitats. The dissolved organic carbon – acid neutralizing capacity relationships were strongest at the WC and LBW (Fig. 2.4). The DOC values at these two sites seem to show extremes in concentrations, low DOC at the WC and high DOC at the LBW. The sites had similarities in state variables like vegetation, elevation, aspect, and slope. Only a certain number of factors remain to influence microbial populations. The presence of acid deposition is probably an important stressor driving the adaptation of microbial communities.

Although the relationship between atmospheric deposition and microbial diversity is not yet clear, the chemical patterns observed in detail at HBEF and factors at the other study sites give insight into potential drivers of microbial community structure. Atmospheric deposition has been shown to alter the soil chemical environment and influence microbial populations (Pennanen et al. 1998). Since atmospheric deposition causes acidification and nutrient fertilization (Driscoll et al. 2001) and the process of methane oxidation in aerable soils is known to be sensitive to pH and nitrogen fertilization (Hutsch et al. 1994), the next step is to determine how the soil bacterial communities are changing due to chronic inputs of acid and nutrients.

Chapter 3: Fluorescent *In Situ* Hybridization

3.1 Introduction

The study of methanotrophic bacteria is of particular interest because of their role in the natural consumption of methane, a potent greenhouse gas (Roslev et al. 1997). Anoxic soils in wetlands and rice fields are some of the most important sources of atmospheric methane (Benstead et al. 1998; Nedwell 1995). Only a small portion of the methane produced in these soils is actually released into the atmosphere because aerobic methane oxidizing bacteria consume a significant part of methane produced in these habitats (Bodelier and Frenzel 1999). Little is known about methane flux in forest soils. It is possible that methanotroph activity may make forest soils an important natural sink of methane (Reay et al. 2001), consuming a potentially large methane flux before it reaches the atmosphere.

Methanotrophs are classified into two physiologically distinct groups, type I methanotrophs, which use the particulate methane monooxygenase and the ribulose monophosphate pathway for carbon assimilation, and type II methanotrophs, which use the particulate and soluble methane monooxygenases and the serine cycle for carbon assimilation (Hanson and Hanson 1996). These two groups form phylogenetically related clusters in the γ- and α- subclasses of *Proteobacteria*, respectively (Murrell et al. 1998). Type I methanotrophs include the genera *Methylomonas, Methylocaldum, Methylomicrobium, Methylobacter, Methylosarcina, Methylosphaera, Methylothermus, and Methylococcus* (Bowman et al. 1993, Costello et al. 2002). Type II methanotrophs include the genera *Methylocystis, Methylosinus,* and *Methylocella* (Dedysh et al. 2000, Eller et al. 2001). *Methylocapsa acidophila* B2, a proposed type III methanotroph

because of its novel intercytoplasmic membrane structure, has recently been isolated from *Sphagnum* peat (Dedysh et al. 2002).

Culture-dependent detection methods for community analyses of environmental samples are inadequate because they often underestimate the numbers of bacteria and diversity of bacterial communities (Amann et al. 1996; Holmes et al. 1999). Among culture-independent techniques, fluorescent *in situ* hybridization (FISH) is a powerful tool that allows the detection and enumeration of bacterial cells directly in environmental samples (Amann et al. 1995; Christensen et al. 1999; Zarda et al. 1997). To date, reports on FISH-based techniques for the analysis of methanotrophs have only been applied to laboratory cultures, rice plant rhizospheres, and acidic *Sphagnum* peat (Bourne et al. 2000; Dedysh et al. 2001). In this study, FISH with rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy was used to visualize type I and type II methanotrophs in acidic forest soils in the northeastern U.S.

Although sulfate deposition has declined in recent years due to the implementation of 1990 amendments to the Clean Air Act, anthropogenic acid deposition remains elevated in the northeastern U.S. (Driscoll et al. 2001). Soil acidification due to acid deposition has been shown to decrease soil acid neutralizing capacity (ANC) and leach natural cations (Driscoll et al. 1988). Acid inputs can also affect microbial community structure, function, and diversity (Brumme and Borken 1999, Penannen et al. 1998, Steudler et al. 1989). Processes such as methane oxidation are known to be sensitive to pH (Dunfield et al. 1993). If relationships exist between methanotroph community ecology and the acid-base status of soils, it may be possible to relate differences in belowground microbial diversity to an ecosystem acid stress response. In

this study, soil microorganisms were counted by FISH and revealed the relative abundances of methanotrophs under a range of environmental conditions. Temperature, moisture, nutrient availability and oxygen saturation appear to remain the major controls over microbial abundance. Acid deposition does not seem to have an attenuating effect on methanotroph populations, but rather it may work to alter the community structure by favoring acid-tolerant species.

3.2 Study sites, soil sampling, and treatments

Seventy-five soil samples were collected between May 2002 and July 2003 from four sites in the northeastern U.S. affected by varying amounts of acid deposition. These sites were located on elevation gradients of south-facing slopes ranging from 250 to 850 m above sea level at Willowemoc Creek, NY (WC; N48.51.52, W74.37.60); the Lye Brook Wilderness, VT (LBW; N43.05.16, W73.05.02); the Hubbard Brook Experimental Forest, NH (HBEF; N43.57.03, W71.44.35); and East Bear Brook Watershed, ME (BBWM; N44.51.67, W68.06.30). Soils collected at these sites were Spodosols with evidence of leaching of iron and aluminum sesquioxides form the E horizon, if present. Approximately one hundred grams of bulk soil were collected separately from the organic (Oi/Oe), (Oa/A), and mineral (E/B) horizons in soil pits or cores excavated at each of three elevations (low, middle, high) in control watersheds. At the HBEF, samples were also collected at a fourth, higher elevation (790 m). Organic horizons ranged from 3 to 6.5 cm in depth and mineral horizon samples were collected at less than 28 cm below the surface (Table 3.1). Vegetation at each site was dependent on elevation. Hardwood species (*Fagus grandifolia*, *Acer saccharum*, *Betula alleghaniensis*) dominated the lower

Table 3.1. Soil horizon thickness in centimeters. Mineral soil measurements reflect the biologically active area of the mineral soil. It was assumed that the mineral soil extended downward to the bedrock, but this depth was not measured.

Site	Elevation (m)	Oi/Oe (cm)	Oa/A (cm)	E/B (cm)
Bear Brook, ME	265	2.0	2.5	19.0+
	365	3.5	4.0	20.5+
	465	4.0	9.0	10.0+
Hubbard Brook, NH	550	4.6	6.3	8.6+
	640	4.3	3.8	9.5+
	730	4.7	4.3	8.3+
	790	4.3	3.5	6.6+
Lye Brook, VT	580	12.0	54.0	9.0+
	670	2.0	28.0	24.0+
	760	8.0	16.0	50.0+
Willowemoc Creek, NY	520	2.0	1.0	38.0+
	610	6.0	10.0	26.0+
	700	2.0	2.0	55.0+

and middle elevations and mostly coniferous species (*Picea rubens*, *Abies balsamea*) were found at higher elevations.

All soil samples were collected in plastic bags, transported to the laboratory on ice, and sieved with a 2 mm diameter sieve. Samples were collected from HBEF and BBWM in late May 2002, and from the LBW and WC in July 2002. The 2002 samples were stored at –20ºC for up to 4 months before FISH analysis. Since freezing of the samples before analysis could lyse cells and reduce the cell counts, the decision was made to compare the results with fresh winter and summer season soils. Samples were taken from the HBEF in January and the LBW in February 2003, from unfrozen soil under the snow pack. Fresh summer samples were taken from the HBEF and BBWM in July 2003 and used for detailed FISH analysis. Samples from 2003 were stored at 4ºC, and portions fixed within 4 days of collection. Cell fixation and hybridization procedures were modified from Dedysh et al (2001). Soil (0.5 g) was suspended in 0.375 mL phosphate-buffered saline (PBS) containing 8.0 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.2 g/L NaH_2PO_4 (pH 7.0) and mixed with 1.125 mL of 4% paraformaldehyde at room temperature for 3 hours. The cells were collected by centrifugation (6,600 x g for 2 minutes) and washed twice with PBS to ensure complete removal of paraformaldehyde. A 10 uL aliquot of the fixed cell suspension was spotted onto Silane prep slides (Sigma) and allowed to air-dry overnight. Samples were dehydrated by successive passes through 50%, 70%, and 100% ethanol for 3 minutes each.

3.3 Hybridization with oligonucleotide probes

The 16S rRNA-targeted oligonucleotide probes chosen had reported group specificity for type I or type II methanotrophs (Dedysh et al. 2001). Probes M-84 and M-450 were applied in FISH with indocarbocyanine Cy3 and Cy5 labels, respectively (MWG Biotech, Table 3.2). A slip of BioRad filter paper soaked in hybridization buffer was placed in the hybridization chamber and the chamber was allowed to equilibrate at 45^{o}C for 30 minutes. A 7 uL aliquot of hybridization solution, composed of 0.9 M NaCl, 20 mM Tris-HCl (pH 7.0), 0.01% SDS, and 20% (or 30%) formamide, was added over the dried cell spot with 1 uL of each fluorescent probe solution (50 ng probe in nuclease-free water) and the universal DNA stain Acridine Orange (AO, 10 uM) or 4'-6-diamidine-2-phenyl indole (DAPI, 10 uM). Samples were incubated in the hybridization chamber for 2 h. Following hybridization, slides were washed for 10 min with washing buffer containing 20 uM Tris-HCl, 0.01% SDS, and 225 mM NaCl. Slides were then rinsed with deionized water, air dried, and viewed immediately.

Table 3.2. Characteristics of stain acridine orange (AO) and probes M-84 and M-450 with indocarbocyanine Cy3 and Cy5 labels used in fluorescent *in situ* hybridization.

Stain/Probe	**Target**	**Fluor**	**Excitation 1 (nm)**
Acridine Orange	All Bacteria	AO	458
M-84	Type I Methanotrophs	Cy3	543
M-450	Type II Methanotrophs	Cy5	633

3.4 Optimization of hybridization conditions

Hybridization stringency was tested by increasing the concentration of formamide in the hybridization buffer in 10% (vol/vol) steps. Each hybridization test was applied to methanotrophs in pure culture as well as frozen and fresh organic and mineral horizon environmental samples. An optimal hybridization temperature for M-84 and M-450 of 45ºC was found to be in agreement with Dedysh et al. (2001).

Positive controls of the type I methanotrophs, *Methylomicrobium album* BG8 and *Methylomonas rubra*, and the type II methanotrophs, *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP grown in culture were prepared to confirm functioning and specificity of the fluorescent probes. All samples were tested with probe M-84 and M-450 individually and in combination.

3.5 Microbial enumeration

Numbers of fixed cells in soil suspensions and pure culture were determined by simultaneously staining cell cultures and cell suspensions with Acridine Orange, M-84, and M-450. Obtaining a normally distributed statistically valid sample set would involve counting several hundred fields of view (Dedysh et al. 2001). Due to the number of samples being analyzed, the number of fields of view to be counted was limited to 5 random fields. Acridine orange stains every cell in the sample so total numbers could be counted (Fig 3.1). The number of target cells per gram of wet soil was determined by the procedure described by Dedysh et al. (2001). In this study and others, background

fluorescence of semi-decomposed soil organic material made counting every bacterium difficult. In contrast, the cells hybridized with M-450 and M-84 stood out sharply against the background and were easily counted (Fig. 3.1).

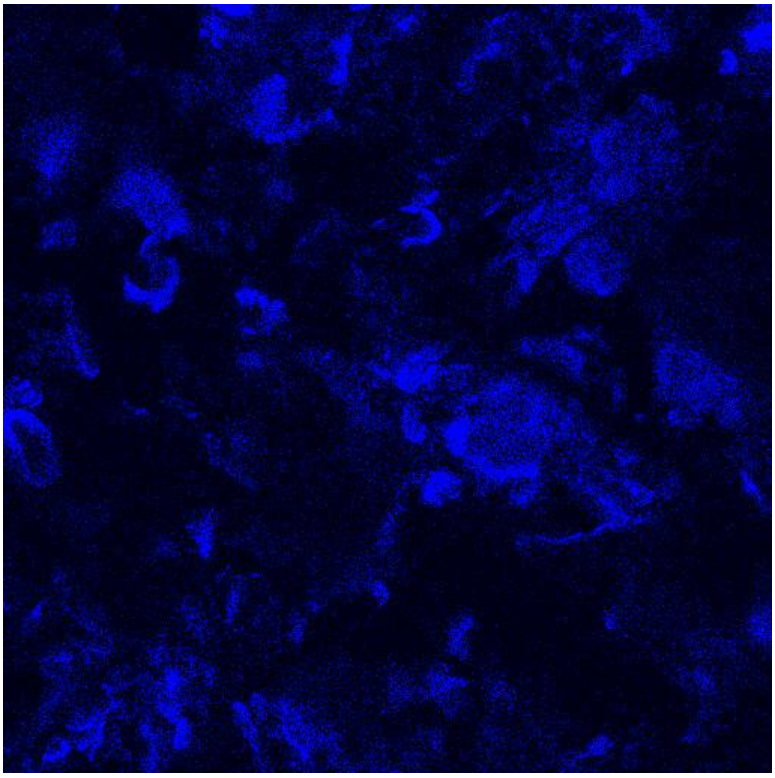
3.6 Confocal scanning laser microscopy

Hybridized cells were detected with a Zeiss Laser Scanning Microscope (LSM) 5 Pascal confocal laser microscope equipped with Helium/Neon lasers (excitation 418, 543, 633 nm). Fluorescent probes were observed under Plan-Neofluar 10x/0.3, Plan-Apochromat 20x/0.75, and C-Apochromat 63x/1.2 water corrective objectives using the DIC II filter set for AO stain (excitation 418, emission 458); Cy3 labeled probes (excitation 510-560 nm, emission 572.5 to 642.5 nm); and Cy5 labeled probes (excitation 649 nm, emission 670 nm). Zeiss Filter NU was used with a high-pressure mercury bulb (50 Watt) under 1000 x magnification for detection of DAPI stained cells (excitation 365 nm, emission 420 nm). Color jpeg files were manipulated with Adobe Photoshop.

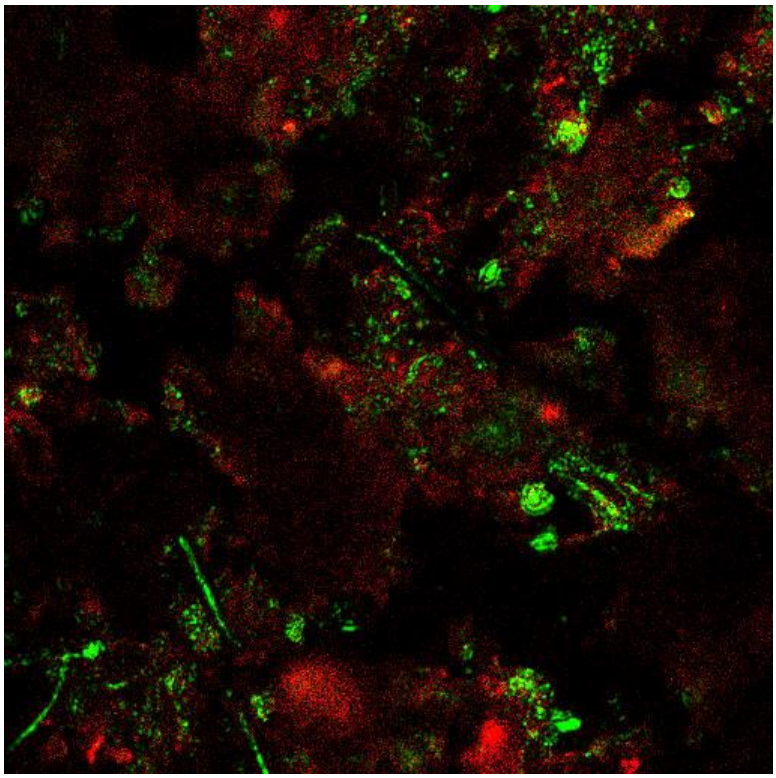
3.7 Results and discussion

3.7.1 Detection of methanotrophs.

Probe M-84 showed group-specific binding to target cells and high detection intensity in pure cultures and environmental samples. Probe M-84 was hybridized optimally with type I methanotrophs at a formamide concentration of 20%, at which two pure culture strains (*Methylomicrobium album* BG8 and *Methylomonas rubra*) were detected. Probe M-84 demonstrated type I methanotroph specificity by failing to bind to pure culture type II methanotrophs under any hybridization conditions. Probe M-450



a)



b)

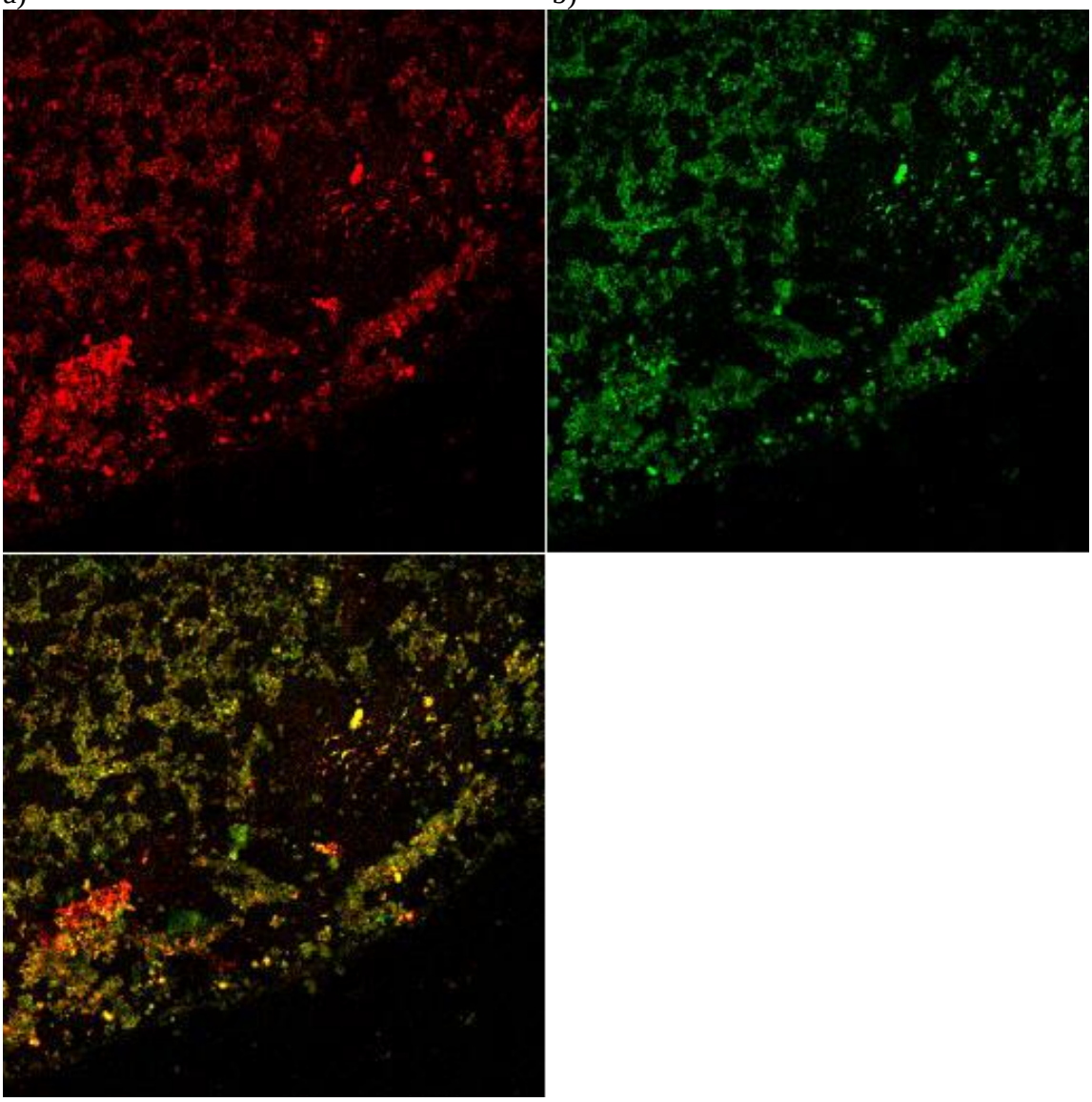
Figure 3.1. a) AO stained cells; and b) Probes M-84 (green) and M-450 (red) hybridized to methanotrophs in summer mineral soil from Bear Brook Watershed, ME.

showed group-specific binding to target cells and detection equal to M-84 under confocal microscopy. Two pure culture strains (*Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP) were detected by probe M-450 at an optimal formamide concentration of 30%. A weak non-specific signal was detected in all environmental samples when probe M-450 was applied with less than 30% formamide. Formamide is used in hybridization studies because every 1% increase in formamide concentration reduces the melting point of DNA by 0.7^{o}C (Dedysh et al. 2001). Hybridization can then take place at lower temperatures without loss of stringency. For Dedysh et al. (2001), probe M-450 also showed optimal hybridization with type II methanotrophs with a formamide concentration of 30%. After testing hybridization efficiencies, these observations proved consistent with our results. Type II probe M-450 did not give a hybridization signal when applied to either type I pure culture strain under any hybridization conditions.

3.7.2 Analysis of environmental samples

Probes M-84 and M-450 were applied to environmental samples from forest soils affected by varying amounts of acid deposition. Simultaneous detection of type I and type II methanotrophs was achieved by applying Cy3-labeled probe M-84 and Cy5-labeled probe M-450 in combination (Fig. 3.2). The hybridization signal of M-84 was distinct and well distinguished from the lower intensity non-specific autofluorescence of soil mineral and organic matter. The M-450 signal was also strong and at high detection gains the probes could often be confused with background emission, especially under

a) b)



c)

Figure 3.2. Probes M-450 (a), M-84 (b), and both detected simultaneously (b) hybridized to methanotrophs in summer organic soil from Willowemoc Creek, NY.

lower stringency conditions. The probes have been shown to possess a wide scope of binding capabilities that perfectly matches nearly all 16S rRNA sequences of methanotrophic strains in public databases (Dedysh et al. 2001). Since the probes are highly specific and hybridization conditions were optimized, it is likely that probes provided accurate counts of methanotrophs in FISH.

Under the snow pack at the LBW, the soil remained unfrozen and hosted methanotroph numbers similar to summer populations. However, soil horizons did show different numbers of cells in different seasons. In the LBW middle elevation Oi/Oe horizon fresh winter samples (Table 3.3) Type I methanotrophs numbered 49.0 ± 10.6 x 10^6 cells g^{-1} wet soil and Type II methanotrophs numbered 46.6 ± 14.3 x 10^6 cells g^{-1} wet soil, while at the HBEF middle elevation Oi/Oe horizon fresh summer samples (Table 3.4), type I methanotrophs numbered 32.5 ± 12.4 x 10^6 cells g^{-1} wet soil and Type II methanotrophs numbered 22.6 ± 12.8 x 10^6 cells g^{-1} wet soil. In summer, warm temperatures increase evapotranspiration rates and dry out the forest floor soil horizon. Such dry conditions may be less favorable to methanotroph populations. In the LBW middle elevation E/B horizon fresh winter samples (Table 3.3) Type I methanotrophs numbered 32.3 ± 3.6 x 10^6 cells g^{-1} wet soil and Type II methanotrophs numbered 32.7 ± 4.2 x 10^6 cells g^{-1} wet soil, while at the HBEF middle elevation Oi/Oe horizon fresh summer samples (Table 3.4) Type I methanotrophs numbered 45.5 ± 11.3 x 10^6 cells g^{-1} wet soil and Type II methanotrophs numbered 54.7 ± 18.3 x 10^6 cells g^{-1} wet soil. Cold winter temperatures may inhibit growth of methanotrophs in the coldest mineral soil horizon. The Oa/A horizon has consistent counts of methanotrophs in summer and winter seasons.

Table 3.3. Average cell counts for fresh 2003 winter soil samples at Lye Brook, VT ($x10^6$ cells g^{-1} wet soil).

Sample Elevation and Horizon	**Type I Methanotrophs**	**Type II Methanotrophs**
LBW Mid Oi/Oe	49.0 ± 10.6	46.6 ± 14.3
LBW Mid Oa/A	44.6 ± 4.2	46.8 ± 4.6
LBW Mid. E/B	32.3 ± 3.6	32.7 ± 4.2

Freezing the soil samples in the laboratory reduced the number of methanotrophs detected by FISH and gave overall lower numbers. When the soil was frozen, Type I methanotrophs in the middle elevation Oa/A horizon at the LBW decreased from 44.6 ± 4.2 x 10^6 cells g^{-1} wet soil (Table 3.3) to 10.4 ± 6.1 x 10^6 cells g^{-1} wet soil (Table 3.5) and type II methanotrophs decreased from 46.8 ± 4.6 x 10^6 cells g^{-1} wet soil (Table 3.3) to 6.2 ± 1.8 x 10^6 cells g^{-1} wet soil (Table 3.5). Similarly, at the HBEF, Type I methanotrophs in the middle elevation Oa/A horizon at the LBW decreased from 45.4 ± 11.3 x 10^6 cells g^{-1} wet soil (Table 3.4) to 10.3 ± 1.8 x 10^6 cells g^{-1} wet soil (Table 3.5) and type II methanotrophs decreased from 54.7 ± 18.3 x 10^6 cells g^{-1} wet soil (Table 3.4) to 16.6 ± 1.5 x 10^6 cells g^{-1} wet soil (Table 3.5). Methanotrophs in unfrozen soil contributed 0.07 to 0.27 % to total cell counts compared to only 0.010 to 0.045 % total cell counts in frozen soil (Table 3.4; Table 3.5). Fitzhugh et al. (2003) showed that experimental soil freezing events at the HBEF resulted in pronounced acidification of soil solutions. Such

Table 3.4. Average cell numbers from fresh 2003 summer soil samples at Hubbard Brook, NH (x 10^6 cells g^{-1} wet soil).

Sample Horizon	Elevation (m)	Type I Methanotrophs	Type II Methanotrophs	% Total AO Cell Count	
				Type I	Type II
Oi/Oe	550	14.4 ± 6.3	33.3 ± 16.0	0.10	0.23
Oa/A	550	39.8 ± 14.6	44.1 ± 7.7	0.21	0.23
E/B	550	40.7 ± 13.2	62.4 ± 24.9	0.17	0.25
Oi/Oe	640	32.5 ± 12.4	22.6 ± 12.8	0.16	0.12
Oa/A	640	40.0 ± 13.7	38.2 ± 16.5	0.13	0.13
E/B	640	45.4 ± 11.3	54.7 ± 18.3	0.19	0.23
Oi/Oe	730	39.1 ± 20.6	44.1 ± 22.0	0.26	0.30
Oa/A	730	45.5 ± 20.6	46.7 ± 23.9	0.26	0.27
E/B	730	31.8 ± 9.6	31.5 ± 10.6	0.10	0.10
Oi/Oe	790	25.0 ± 14.4	27.0 ± 14.8	0.17	0.18
Oa/A	790	17.5 ± 2.2	18.3 ± 6.8	0.07	0.08
E/B	790	16.8 ± 12.1	23.8 ± 8.1	0.07	0.09

differences in bacterial numbers in frozen compared to unfrozen soil suggest either methanotroph populations were particularly sensitive to cold cell rupture or were decreasing as a result of unfavorable soil chemical conditions caused by sudden acidification.

Type I and type II methanotrophs occurred in relatively similar numbers in the acid forest soils tested, especially at the HBEF (Table 3.4). Type I cells often occurred without type II cells, however, type II cells always occurred in close proximity to type I cells (Fig3.3). Type I and type II methanotrophs were often found in groups clustered around soil organic matter (Fig. 3.4). The association of type II to type I cells and organic matter suggest some unknown factors in the natural soil environment that encourage methanotrophs to live on these surfaces.

Table 3.5. Average cell numbers from frozen summer soil samples at Bear Brook, ME; Hubbard Brook, NH; Lye Brook, VT; and Willowemoc Creek, NY (x 10^6 cells g^{-1} wet soil).

Sample Site	**Sample Horizon**	**Type I**	**Type II**	**% Total DAPI Cell Count**	
				Type I	Type II
Bear Brook, ME	Oa/A	18.3 ± 1.3	13.1 ± 3.3	0.029	0.021
Bear Brook, ME	E/B	10.8 ± 7.2	7.0 ± 7.9	0.034	0.022
Hubbard Brook, NH	Oa/A	12.3 ± 3.8	13.1 ± 6.3	0.027	0.029
Hubbard Brook, NH	E/B	10.3 ± 1.8	16.6 ± 1.5	0.025	0.040
Lye Brook, VT	Oa/A	10.4 ± 6.1	6.2 ± 1.8	0.023	0.013
Lye Brook, VT	E/B	13.1 ± 6.1	9.9 ± 6.6	0.045	0.034
Willowemoc Creek, NY	Oa/A	11.9 ± 5.2	6.3 ± 4.9	0.019	0.010
Willowemoc Creek, NY	E/B	2.6 ± 2.1	3.2 ± 2.8	0.015	0.018

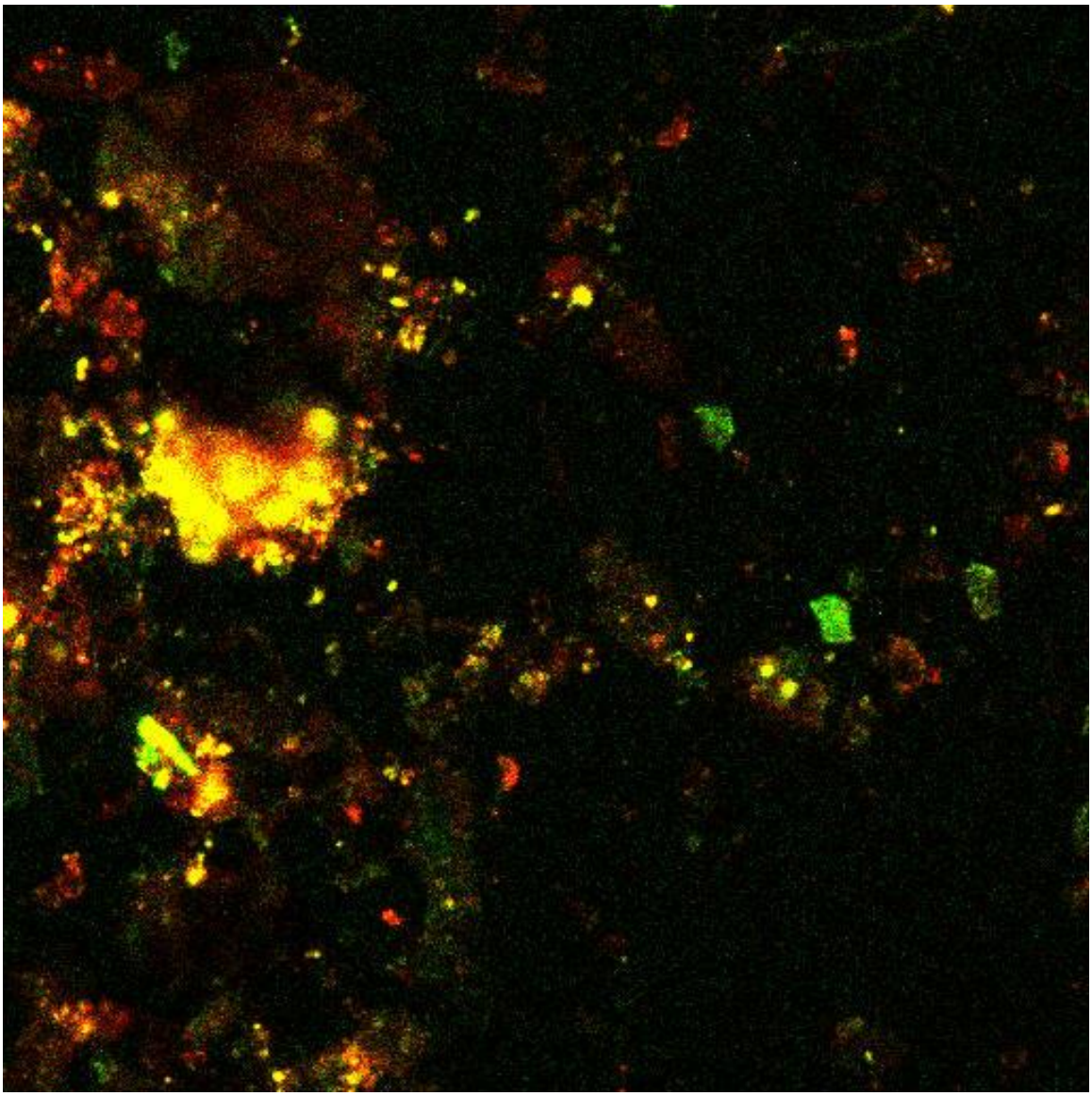
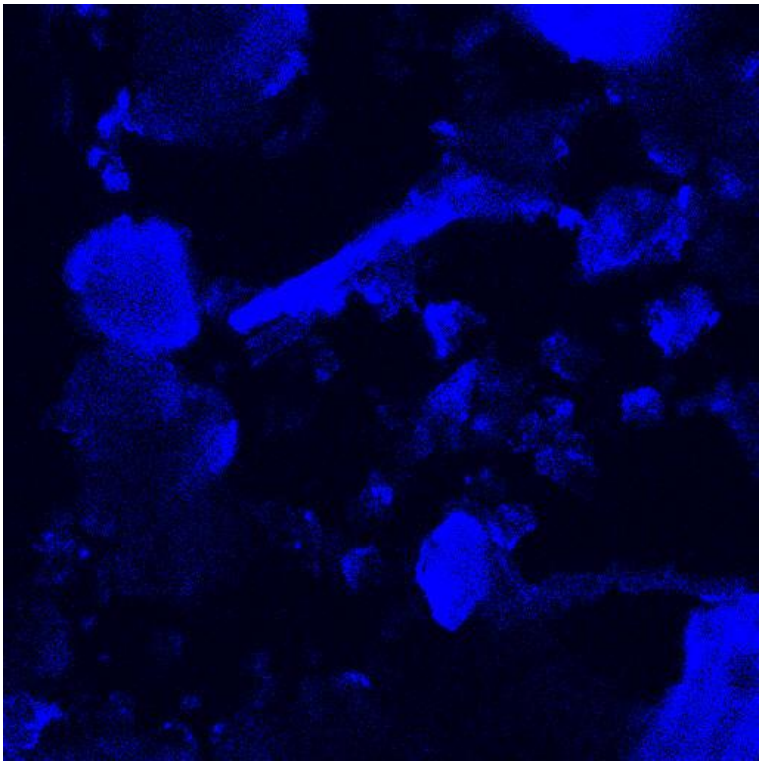
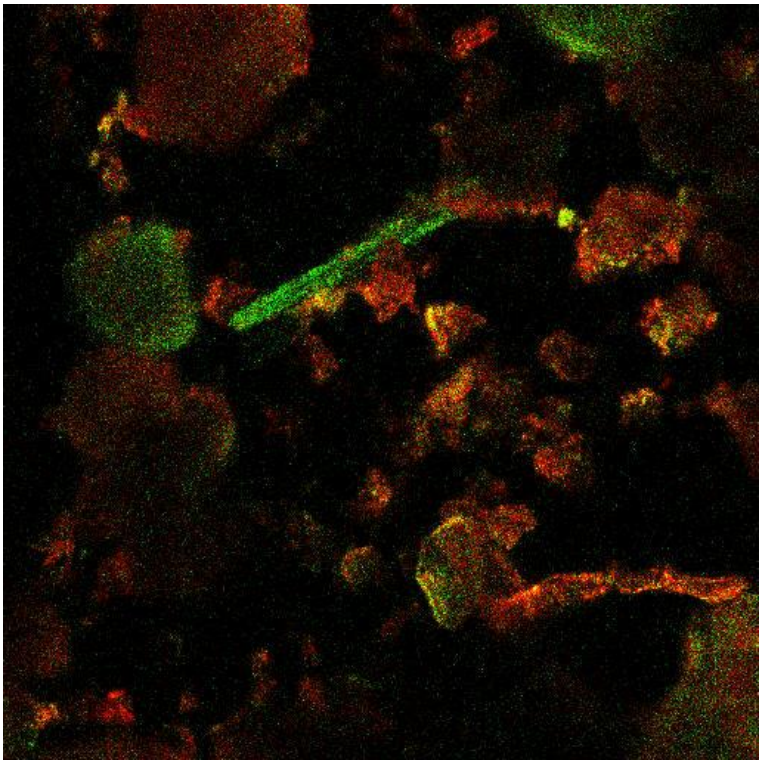


Figure 3.3. Probes M-84 (green) and M-450 (red) hybridized to methanotrophs in unfrozen winter organic soil from Lye Brook Wilderness, VT.



a)



b)

Figure 3.4. a) Acridine orange stained cells; b) Fluorescent probes M-84 and M-450 attached to type I (green) and type II (red) methane oxidizing cells, respectively, in an forest Oi/Oe soil sample from Hubbard Brook, NH.

Previous research has shown methanotroph abundance in acidic peat (pH 4.2) was on the order of 3.0×10^6 cells per gram of wet peat, which represented 0.8% of the total bacterial cell number (Dedysh et al. 2001). Based the enumeration data, methanotroph abundance in acid forest soils (6.2×10^7 cells/g wet soil) was up to 20 times higher than acidic *Sphagnum* peat. Although the relative abundance of methanotrophs in acidic forest soils was only 0.1 – 0.3%, their importance may still be large, due to the global extent of temperate forest ecosystems. These data suggest that the relative abundance of methanotrophs in the acid forest soil community is lower due to competition for resources in the highly diverse and productive northern hardwood forest soils. At the Hubbard Brook Experimental Forest, NH, methanotroph populations were lower in the surface Oi/Oe horizon and at high elevation (Fig. 3.5). Acid deposition enters the system in throughfall to the forest floor, increasing the acidity in the surface layer (Table 3.6).

Although acid deposition increases with elevation, it is also likely that environmental factors, like the adiabatic cooling effect and soil moisture content, are influencing the observed pattern. Type II methanotrophs showed a moderate correlation with soil pH (R^2=0.42) (Fig. 3.6). Other chemical factors, such as base saturation, soil pH, and total N have been shown to influence methane oxidation (Brumme and Borken 1999). Chemical changes associated with atmospheric deposition, including NH_4^+, NO_3^-, SO_4^{2-}, and H^+ additions, inhibit CH_4 oxidation (Bradford et al. 2001; Steudler et al. 1989). Soil pH at the sites was relatively low (Table 3.6) and varied due to intensity of acid deposition and soil base saturation. Soil pH can account for 42% of the variation in type I methanotroph abundance at HBEF, but seems to have little influence on type I

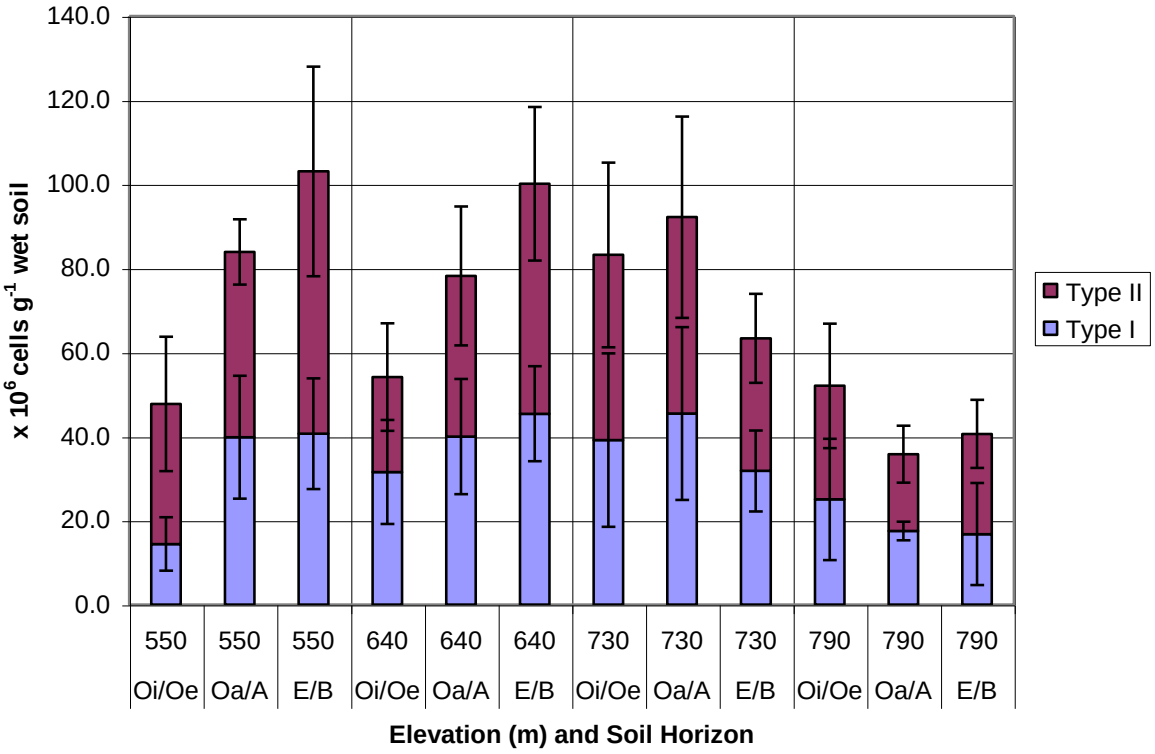


Figure 3.5. Methanotroph distribution over an elevation gradient in control watershed 6 2003 fresh summer soil at Hubbard Brook, NH.

Table 3.6. Average soil pH per horizon at the study sites.

Sample	pH Oi/Oe	pH Oa/A	pH E/B
Willowemoc Creek, NY	4.09	4.28	4.40
Lye Brook, VT	3.68	3.35	4.15
Hubbard Brook, NH	4.34	4.51	4.70
Bear Brook, ME	4.10	3.96	4.38

populations (Fig. 3.6). Methanotroph abundance compared at all four sites showed that the average type I and type II counts ranged between 0.1 to 0.45% of total cell counts (Table 3.5). Methanotrophs were found in relatively similar numbers throughout and overall abundance did not seem to vary with soil acidity alone (Fig. 3.7). It is likely that a complex assemblage of physical and chemical factors influences the abundance of methanotrophs in forest soils. Although the mechanisms are not clear, changes in the methane oxidation activity are likely caused by alteration in the structure of the methane oxidizing bacterial community (Reay et al. 2001). So, although total abundance of methanotrophs is not necessarily linked to acid deposition directly, soils environments impacted by acid deposition are likely influenced to favor acid tolerant methane oxidizing species.

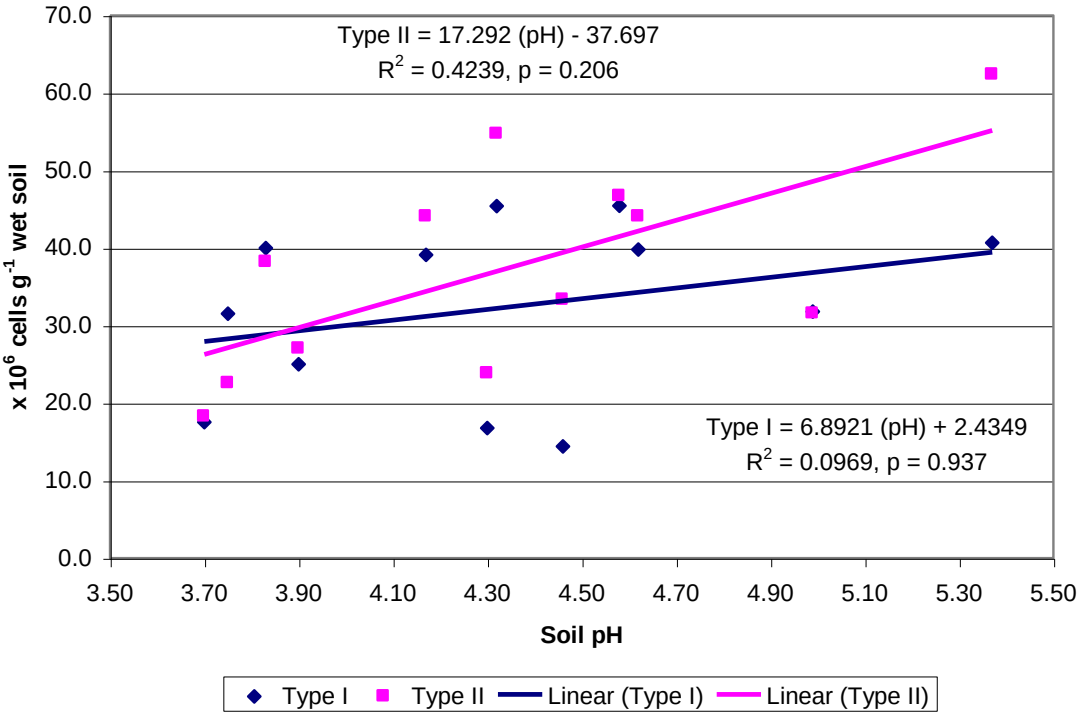


Figure 3.6. Methanotroph populations relative to pH in unfrozen 2003 summer samples from the Hubbard Brook Experimental Forest, NH. Type II methanotrophs showed a moderate correlation with soil pH.

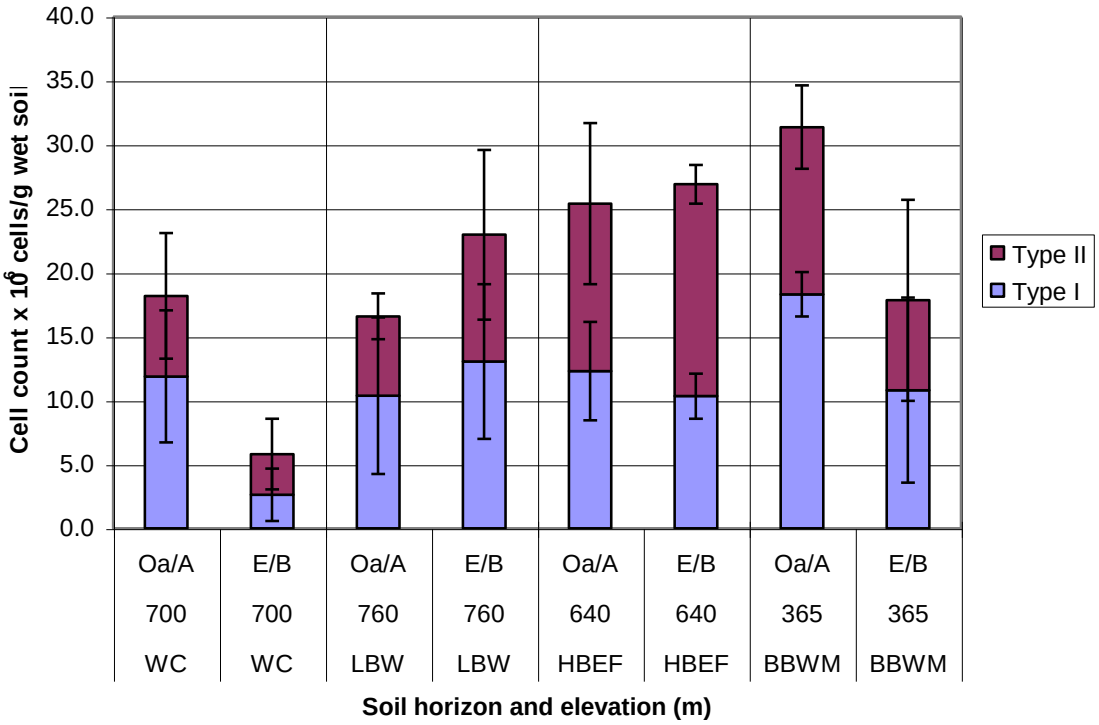


Figure 3.7. Methanotroph distribution in reference watershed frozen summer soils at Willowemoc Creek, NY; Lye Brook Wilderness, VT; Hubbard Brook Experimental Forest, NH; and East Bear Brook Watershed, ME.

3.8 Conclusions

FISH can be used to detect, distinguish, and count type I and type II methanotrophs in acidic forest soils. The advantage of FISH over other enumeration methods, such as phospholipid fatty-acid extraction (Costello et al. 2002), is the ability to count whole cells directly. Using FISH, I was able to examine the effects of temperature, moisture, elevation, and acid deposition on natural populations of methanotrophs. The tools described here for the detection and enumeration of methanotrophic bacteria in forest soil now allow the potential for assessment of the distribution and abundance of methanotrophs in other ecosystems.

Chapter 4: Terminal Restriction Fragment (TRF) Analysis

4.1 Introduction

Terminal restriction fragment length polymorphism (TRFLP or TRF) analysis is an important and relatively new tool in microbial ecology (Osborn et al. 2000; Ranjard et al. 2000). It can be widely used for profiling and screening microbial community structure in many habitats by targeting small subunit rRNA and functional genes (Bruce 1997; Liu et al. 1997; Horz et al. 2000; Lueders and Friedrich 2003; Rousseaux et al. 2003). TRFLP patterns are produced in a series of steps that combine the polymerase chain reaction (PCR), restriction enzyme digestion, and gel electrophoresis (Fig. 4.1) (Clement et al. 1998; Kitts 2001). DNA extracted from a soil sample is used in PCR with primers homologous to conserved regions in the 16S rRNA gene (Dunbar et al. 2000; Dunbar et al. 2001). One primer is labeled on the 5'-end with a fluorescent molecule, in this case 5-carboxyfluorescein (FAM). The amplicons are then digested with a restriction enzyme that has a tetranucleotide recognition sequence. Digested amplicon fragments are subjected to gel electrophoresis using a fluorescence detector so the fluorescently labeled terminal restriction fragments are measured. An automated fragment analysis program calculates fragment length in base pairs by comparing fragment peak retention time to DNA size standards. The program integrates the results and calculates fragment peak height and area. The patterns of peaks can be compared between sample horizons and study sites by statistical methods (Dollhopf et al. 2001). Individual peaks can be compared to predictions from an existing database of sequences.

TRF analysis on methanotroph communities has only been conducted on rice field soil and roots (Henckel et al. 1999). Ammonia-oxidizing communities have been examined by this method in many environments and because of the functional and

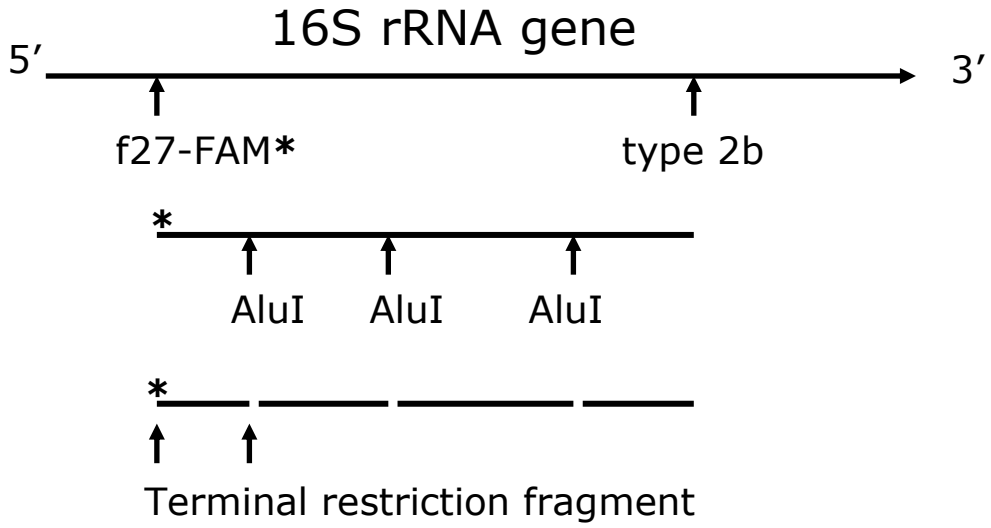


Figure 4.1 Conceptual diagram of restriction enzyme digestion of amplified PCR product.

genetic similarities of ammonia oxidizers to methane oxidizers, those results are particularly relevant to this study. Among forest applications, TRF analysis has never been conducted on northeastern hardwood forest soils, only southwestern pinyon-juniper woodland soils (Dunbar et al. 2001). However, TRF analysis has been proven in a wide variety of applications and should be a useful method for monitoring complex microbial communities in any soil environment (Ranjard et al. 2000). The objective of this study was to determine the diversity and community structure of methane oxidizing communities in northeastern hardwood forest ecosystems affected by an environmental stressor, acid deposition.

4.2 Polymerase Chain Reaction (PCR)

The first step in the TRF analysis process is amplification of environmental DNA using the polymerase chain reaction (PCR). The PCR utilizes the natural ability of the polymerase enzyme from *Thermus aquaticus*, an organism found in a Yellowstone National Park hot spring (Madigan et al. 2000). The *Taq* polymerase is able to synthesize strands of DNA from environmental template DNA and make millions of copies of a particular section of interest. The amplified sections can be used for many purposes, in this case digested with restriction enzymes and analyzed for the resulting terminal fragment lengths.

The PCR method has known biases when used to amplify genes from environmental samples (Bourne et al. 2001; Egert et al. 2003; Mummey et al. 2003). Problems with the PCR include inhibition by co-extracted contaminants, preferential amplification of some templates, formation of artifacts, and variations in copy number in

16S rRNA *rrn* operon (Wintzingerode et al. 1997). A major problem for this study was the co-extraction of humic acids with nucleic acids in the DNA extraction process. The humics strongly inhibit polymerase enzymes and need to be removed before amplification. Minimum inhibitory concentrations of commercially prepared humic acids on a polymerase enzyme was found to be 0.08 ug mL^{-1}, which is very low (Tebbe and Vahjen 1993). When attempts were made to purify samples from humic acid contamination, DNA was often lost in the process. Non-acetylated bovine serum albumin (BSA) can be added at 400 ng uL^{-1} in the PCR to increase the polymerase tolerance toward humic and fulvic acids by 10-1000 fold (Kreader 1996, McKeown 1994). Equal amplification efficiencies of DNA in environmental samples is not possible because all molecules are not equally accessible to primer hybridization, primer-template combinations do not form equally, extension efficiencies are not the same for all templates, and limitations by substrate exhaustion do not necessarily affect the elongation of each template equally (Suzuki and Giovannoni 1996). In short, it was difficult to know how much DNA was amplified by the PCR, so it needed to be compared to standard amounts of DNA by gel electrophoresis.

4.3 Study sites and samples

Seventy-five soil samples were collected between May 2002 and July 2003 from four sites in the northeastern U.S. affected by varying amounts of acid deposition. These sites were located on elevation gradients of south-facing slopes ranging from 250 to 850 m above sea level at Willowemoc Creek, NY (WC; N48.51.52, W74.37.60); the Lye Brook Wilderness, VT (LBW; N43.05.16, W73.05.02); the Hubbard Brook Experimental

Forest, NH (HBEF; N43.57.03, W71.44.35); and East Bear Brook Watershed, ME (BBWM; N44.51.67, W68.06.30). Soils collected at these sites were Spodosols with evidence of leaching of iron and aluminum sesquioxides form the E horizon, if present. Approximately one hundred grams of bulk soil were collected separately from the organic (Oi/Oe), (Oa/A), and mineral (E/B) horizons in soil pits or cores excavated at each of three elevations (low, middle, high) in control watersheds. At the HBEF, samples were also collected at a fourth, higher elevation (790 m).

4.4 DNA extractions

Total DNA was extracted from soil samples with standard procedures from commercial kits (MoBio) that utilize chemicals, heat, and bead beating in combination. These treatments are designed to lyse all the microbial cells in the sample and release their contents into the surrounding solution. Among the cell constituents is DNA, which can be isolated and used to determine the diversity and community structure of the soil bacterial community. Two sizes of kits were used to generate different amounts of extracted DNA. For the MoBio UltraClean Soil DNA Kit Mega Prep, approximately 10 grams of sieved soil was used. For the MoBio UltraClean Soil DNA Isolation Kit, 0.5 grams of sieved soil was used.

Several methods were used for purification of the extracted DNA. Sepharose 2B (Sigma) was placed in 4 mm diameter glass columns (2 mL). The extracted DNA was passed through the columns in 2 mL aliquots, collected separately and used in the PCR. Another purification involved passing the extracted DNA by electrophoresis through a composite gel. The top 2 cm of the gel consisted of 2% agarose and 1%

polyvinylpolypyrolidone (PVPP, Sigma) wells with normal 1% agarose base (adapted from Kowalchuk et al. 1997). Double-sized wells were loaded with 100 uL extracted DNA and electrophoresed at 120 mV for 60 minutes. Humic acids that can inhibit the PCR were bound by the PVPP in the top of the gel while the DNA migrated through the normal part of the gel toward the positive electrode. Bands of 1500 base pairs were cut from the gel and recovered with a gel extraction kit (MoBio). The other method of DNA purification used was dilution. Since the PCR needed only nanograms of DNA to function, the extracted DNA was simply diluted 1 uL in 199 uL deionized water to reduce the concentration of inhibitory humic acids.

4.5 Amplification

To amplify sections of 16S rRNA genes from methanotrophs genes, primer type 2b was employed with f27 (Costello and Lidstrom 1999). A total volume reaction of 25 uL was used and a megamix was created and distributed equally among the test samples (Table 4.1). Primers f27/type 2b amplify a section of the 16S rRNA gene from methanotrophs that is approximately 950 base pairs long. The PCR reaction was carried out in an MJ Research Peltier Thermo Cycler to provide the correct temperatures for the reactions (Fig. 4.2). With repeated cycling of these steps, the PCR creates millions of copies of the desired region of DNA (Fig 4.2). To verify a successful reaction, small aliquots of the product (5 uL) were run on a 1% agarose gel by electrophoresis (Fig. 4.3).

Table 4.1. Recipe for a 25 uL polymerase chain reaction.

Component	**Concentration**	**Amount per reaction (uL)**
Deionized H_2O	-	13.5
$MgCl_2$	25 mM	1.5
Bovine serum albumin (BSA)	5000 mg/L	0.5
f27 primer	10 mM	0.5
type 2b primer	10 mM	1.25
f27-FAM	10mM	1.00
ExTaq Buffer	10x	2.5
Nucleotides (DNTPs)	200 mM	2.5
ExTaq Polymerase	5 Units/uL	0.25
Sample DNA	1:200 dilution	1.5

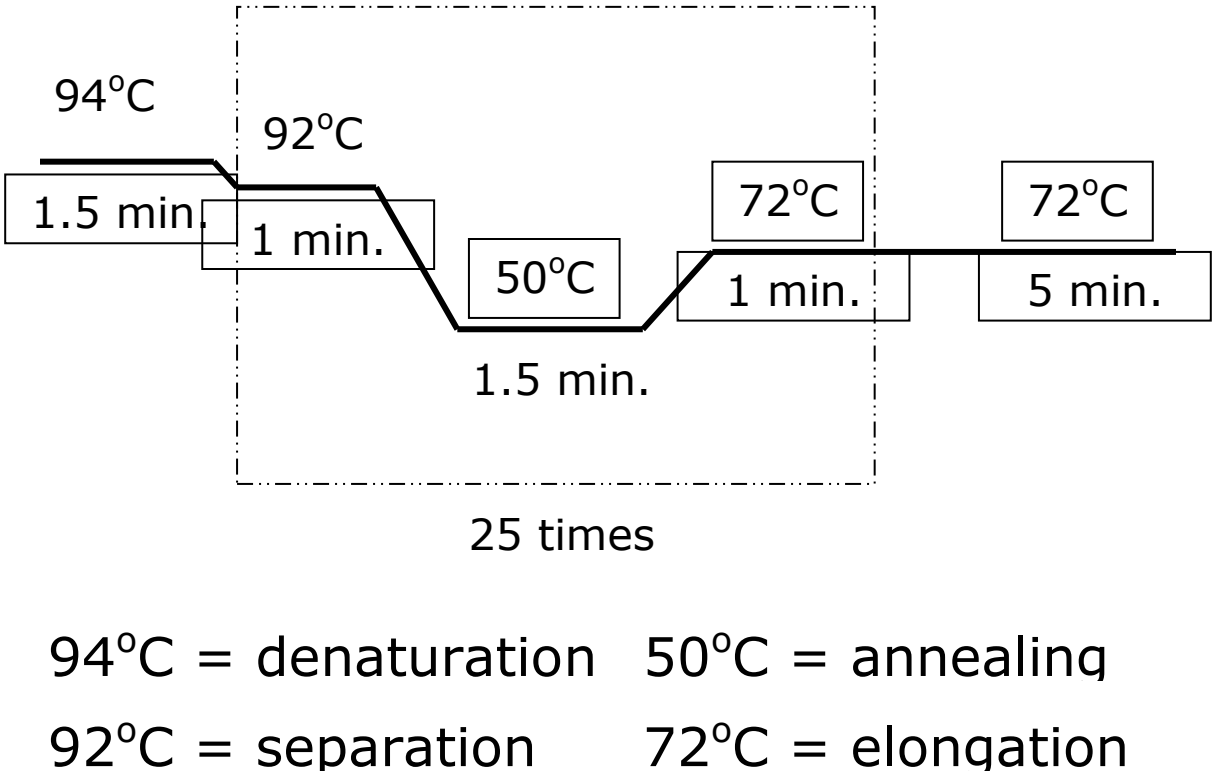


Figure 4.2. Conceptual diagram of the thermocycling of the polymerase chain reaction for 16S rDNA gene amplification from methanotrophs with primers f27 and type 2b.

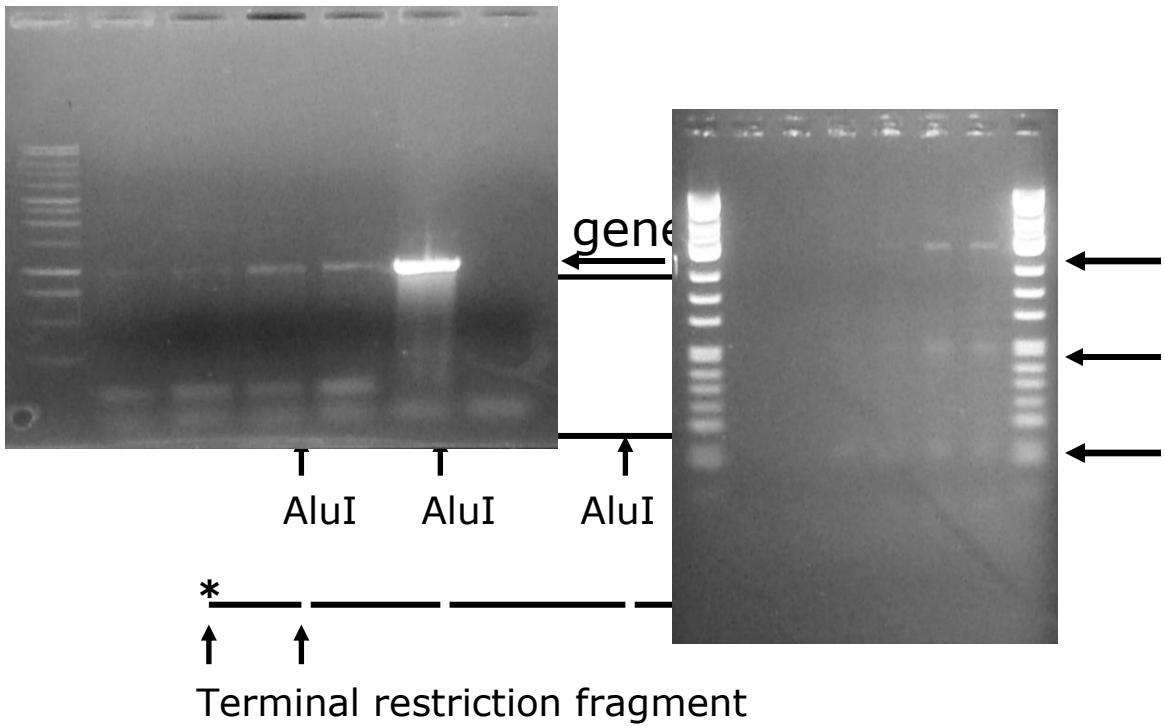


Figure 4.3. DNA fragments on electrophoresis gels indicating a) an approximately 950 base pair fragment amplified by labeled f27-type 2b primer set and b) fragments shown after restriction enzyme digestion with *Alu*I, labeled terminal fragment is shown with an arrow.

After amplification was verified, PCR products were purified with a QIAquick PCR Purification Kit (Qiagen Inc.). Five volumes of PB buffer, included with the kit, were added to 1 volume of PCR sample and centrifuged at 10,000 x g in a microcentrifuge spin column for 1 minute. The sample was washed with 750 uL ethanol buffer and centrifuged for 2 minutes. Purified DNA was recovered by centrifugation with 50 uL elution buffer.

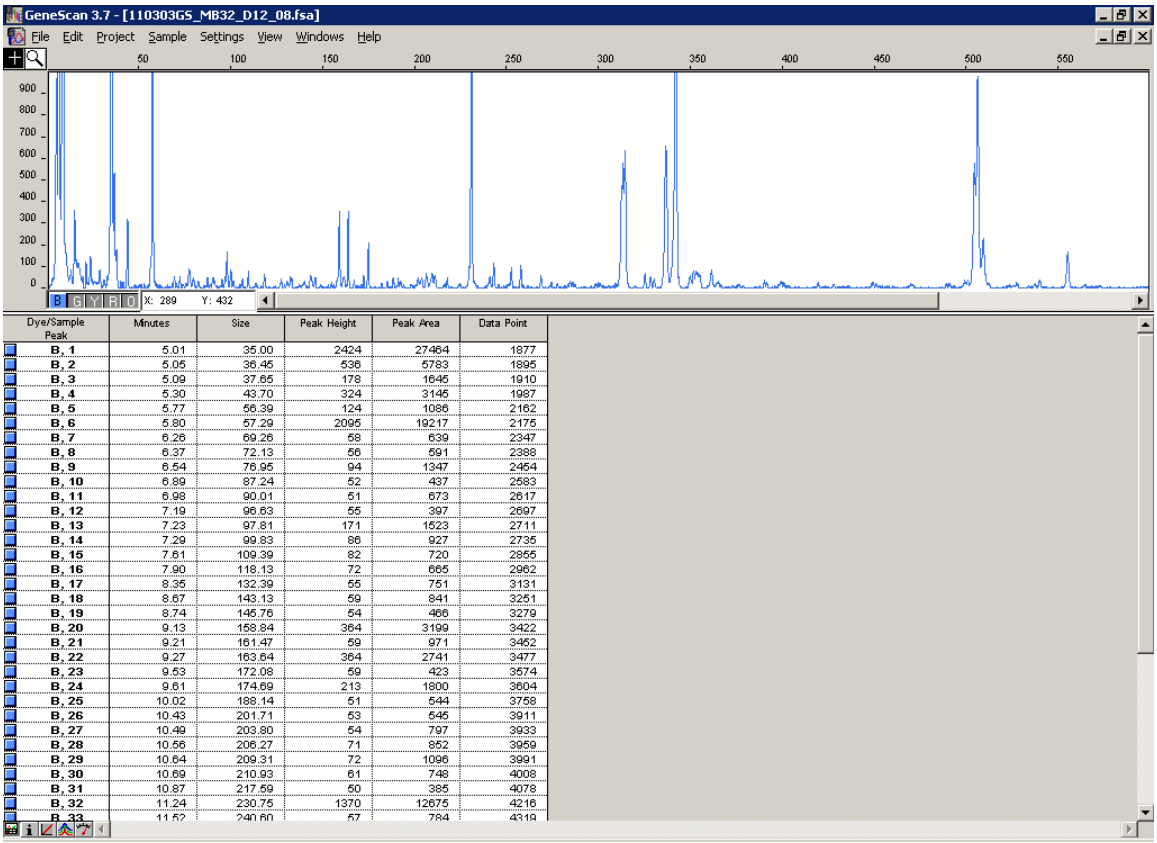
4.6 Enzyme Digests

Fluorescently labeled PCR products were cut with restriction enzymes *Alu*I and *Hha*I. The fragment size between primer sites and restriction enzyme in the environmental DNA is known and can be verified by gel electrophoresis. The PCR products in this study were digested with *Alu*I and *Hha*I at 37^{o}C for 2 hours. After the reaction was complete, enzyme activity was halted by heat shocking at 65^{o}C for 15 minutes.

Test digests were conducted on the publicly available Ribosomal Database Project (RDP; Cole et al. 2003). The *in silico* TAP-TRFLP program from the RDP was used to digest the 16S rRNA genes of all known bacterial sequences. The primer type 2b sequence was entered and multiple, single enzyme digests were run with both *Alu*I and *Hha*I.

4.7 Terminal Fragment Analysis

Triplicate digests were loaded onto Applied Biosystems Microamp Optical 96-well reaction plates and loaded into an Applied Biosystems 3100 Fragment Analyzer. Terminal fragment patterns were analyzed with Genescan 3.7. Electropherograms showed peaks where certain size DNA fragments were detected (Fig. 4.4). Terminal fragment analysis procedures were adapted from Dunbar et al. (2001). In Genescan Analysis Parameters menu, the local southern size calling method was used, relative fluorescence units (rfu) were cut-off at 50, and no smoothing was used. Tables were exported to Excel for further analysis. Replicate profiles were aligned by grouping TRFs greater than 50 bp into 1 bp bins, looking 0.5 bp in each direction. DNA quantity was normalized between replicate profiles by summing peak heights, creating correction factors by dividing the smallest sum by larger sums, and multiplying larger replicate peak heights by the correction factor. A derivative sample profile was created by identifying the subset of TRFs that appeared in all replicate profiles. A binary presence/absence worksheet was created by assigning 0 to a fragment if it did not appear and 1 if it was present. Cluster analyses, a powerful way to describe community dynamics (Dollhopf et al. 2001; Purkhold et al. 2000), were performed in Statistica 6.0 on the binary matrix of each enzyme digest. The resulting binary matrix produced clustered tree diagrams showing the relationships between fragment patterns.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
B, 1	5.01	35.00	2424	27464	1877
B, 2	5.05	36.45	536	5783	1895
B, 3	5.09	37.65	178	1645	1910
B, 4	5.30	43.70	324	3145	1987
B, 5	5.77	56.39	124	1086	2162
B, 6	5.80	57.29	2095	19217	2175
B, 7	6.26	69.26	58	639	2347
B, 8	6.37	72.13	56	591	2388
B, 9	6.54	76.95	94	1347	2454
B, 10	6.89	87.24	52	437	2583
B, 11	6.98	90.01	51	673	2617
B, 12	7.19	96.63	55	397	2697
B, 13	7.23	97.81	171	1523	2711
B, 14	7.29	99.83	86	927	2735
B, 15	7.61	109.39	82	720	2855
B, 16	7.90	118.13	72	665	2962
B, 17	8.35	132.39	55	751	3131
B, 18	8.67	143.13	59	841	3251
B, 19	8.74	145.76	54	466	3279
B, 20	9.13	158.84	364	3199	3422
B, 21	9.21	161.47	59	971	3452
B, 22	9.27	163.64	364	2741	3477
B, 23	9.53	172.08	59	423	3574
B, 24	9.61	174.69	213	1800	3604
B, 25	10.02	188.14	51	544	3758
B, 26	10.43	201.71	53	546	3911
B, 27	10.49	203.80	54	797	3933
B, 28	10.56	206.27	71	852	3959
B, 29	10.64	209.31	72	1096	3991
B, 30	10.69	210.93	61	748	4008
B, 31	10.87	217.59	50	385	4078
B, 32	11.24	230.75	1370	12675	4216
B, 33	11.52	240.80	57	784	4319

Figure 4.4. Electropherogram of terminal restriction fragments from *Hha*I digest of the Hubbard Brook Experimental Forest low elevation Oi/Oe soil sample.

4.8 Results

Out of 75 samples collected, 51 were able to be amplified with the methanotroph specific primer set f27-type 2b. Twenty-nine amplified samples were successfully digested with 2 restriction enzymes and fragment analysis provided useful data for 25 *Alu*I digested samples and 23 *Hha*I digested samples. Possible sources of error or uncertainty, such as high concentration of inhibitor compounds or poor sample recovery, were present at each step of the multi-step process, which reduced the ability for the successful analysis of all 75 samples.

Gene fragment patterns digested *in silico* matched 19 known methanotroph strains, including 9 type II *Methylosinus*, 7 type II *Methylocystis*, and 3 unclassified strains. Based on these known sequences in the database, it was predicted that digested PCR products from the primer set f27-type 2b would produce diagnostic fragments in the sizes of approximately 50, 145, and 500. All 25 samples analyzed showed at least one fragment within 5 bp of one of these predicted terminal fragments, 20 have two or more, and 4 show all three. These data suggest there are some important similarities in these methanotroph communities to known strains of methanotrophs, but that inherent differences exist.

Restriction digest of 25 samples with *Alu*I produced 246 distinct terminal fragments ranging from 50.0 to 499.7 bp. Clustered tree diagrams showed the methanotrophs at the Willowemoc Creek (WC) study site were the most dissimilar to the other samples (Fig. 4.5), producing dominant TRFs not appearing elsewhere (Table 4.3). Methanotrophs in the summer 2002 low elevation Oa/A and mid elevation Oi/Oe horizons at WC were fairly similar to each other (32%), but showed no terminal fragment

Table 4.2. List of 51 successfully amplified samples from the methanotroph specific primer set f27-type 2b (+) and 25 successfully analyzed fragments digested with *Alu*I and *Hha*I (grey). Samples from the Bear Brook Watershed (BBWM); Hubbard Brook Experimental Forest (HBEF); Lye Brook Wilderness (LBW); and Willowemoc Creek (WC) were taken from elevations, soil horizons, and dates indicated.

Sample Location	Date Collected	Elevation (m)	Soil Horizon	f27-FAM-type2b	Sample Location	Date Collected	Elevation (m)	Soil Horizon	f27-FAM-type2b
HBEF	22-May-2002	550	Oi/Oe	+	HBEF	23-Jan-2003	550	Oi/Oe	+
HBEF	22-May-2002	550	Oa/A	+	HBEF	23-Jan-2003	550	Oa/A	+
HBEF	22-May-2002	550	E/B	+	HBEF	23-Jan-2003	550	E/B	-
HBEF	22-May-2002	640	Oi/Oe	+	HBEF	23-Jan-2003	640	Oi/Oe	-
HBEF	22-May-2002	640	Oa/A	+	HBEF	23-Jan-2003	640	Oa/A	+
HBEF	22-May-2002	640	E/B	+	HBEF	23-Jan-2003	640	E/B	+
HBEF	22-May-2002	730	Oi/Oe	+	HBEF	23-Jan-2003	730	Oi/Oe	+
HBEF	22-May-2002	730	Oa/A	-	HBEF	23-Jan-2003	730	Oa/A	+
HBEF	22-May-2002	730	E/B	+	HBEF	23-Jan-2003	730	E/B	+
HBEF	22-May-2002	790	Oi/Oe	+	HBEF	23-Jan-2003	790	Oi/Oe	-
HBEF	22-May-2002	790	Oa/A	+	HBEF	23-Jan-2003	790	Oa/A	+
HBEF	22-May-2002	790	E/B	+	HBEF	23-Jan-2003	790	E/B	+
BBWM	28-May-2002	265	Oi/Oe	-	LBW	23-Feb-2003	580	Oi/Oe	+
BBWM	28-May-2002	265	Oa/A	-	LBW	23-Feb-2003	580	Oa/A	-
BBWM	28-May-2002	265	E/B	+	LBW	23-Feb-2003	580	E/B	-
BBWM	28-May-2002	365	Oi/Oe	+	LBW	23-Feb-2003	670	Oi/Oe	-
BBWM	28-May-2002	365	Oa/A	-	LBW	23-Feb-2003	670	Oa/A	+
BBWM	28-May-2002	365	E/B	+	LBW	23-Feb-2003	670	E/B	-
BBWM	28-May-2002	465	Oi/Oe	-	HBEF	10-Jul-2003	550	Oi/Oe	+
BBWM	28-May-2002	465	Oa/A	-	HBEF	10-Jul-2003	550	Oa/A	+
BBWM	28-May-2002	465	E/B	-	HBEF	10-Jul-2003	550	E/B	-
LBW	14-Jun-2002	580	Oi/Oe	+	HBEF	10-Jul-2003	640	Oi/Oe	+
LBW	14-Jun-2002	580	Oa/A	-	HBEF	10-Jul-2003	640	Oa/A	+
LBW	14-Jun-2002	580	E/B	-	HBEF	10-Jul-2003	640	E/B	+
LBW	14-Jun-2002	670	Oi/Oe	-	HBEF	10-Jul-2003	730	Oi/Oe	+
LBW	14-Jun-2002	670	Oa/A	+	HBEF	10-Jul-2003	730	Oa/A	+
LBW	14-Jun-2002	670	E/B	-	HBEF	10-Jul-2003	730	E/B	-
LBW	14-Jun-2002	760	Oi/Oe	+	HBEF	10-Jul-2003	790	Oi/Oe	+
LBW	14-Jun-2002	760	Oa/A	+	HBEF	10-Jul-2003	790	Oa/A	+
LBW	14-Jun-2002	760	E/B	+	HBEF	10-Jul-2003	790	E/B	+
WC	17-Jun-2002	520	Oi/Oe	-	BBWM	12-Jul-2003	265	Oi/Oe	+
WC	17-Jun-2002	520	Oa/A	+	BBWM	12-Jul-2003	265	Oa/A	+
WC	17-Jun-2002	520	E/B	+	BBWM	12-Jul-2003	265	E/B	+
WC	17-Jun-2002	610	Oi/Oe	+	BBWM	12-Jul-2003	365	Oi/Oe	+
WC	17-Jun-2002	610	Oa/A	+	BBWM	12-Jul-2003	365	Oa/A	+
WC	17-Jun-2002	610	E/B	-	BBWM	12-Jul-2003	365	E/B	+
WC	17-Jun-2002	700	Oi/Oe	-	BBWM	12-Jul-2003	465	Oi/Oe	+
WC	17-Jun-2002	700	Oa/A	+	BBWM	12-Jul-2003	465	Oa/A	-
WC	17-Jun-2002	700	E/B	-	BBWM	12-Jul-2003	465	E/B	-

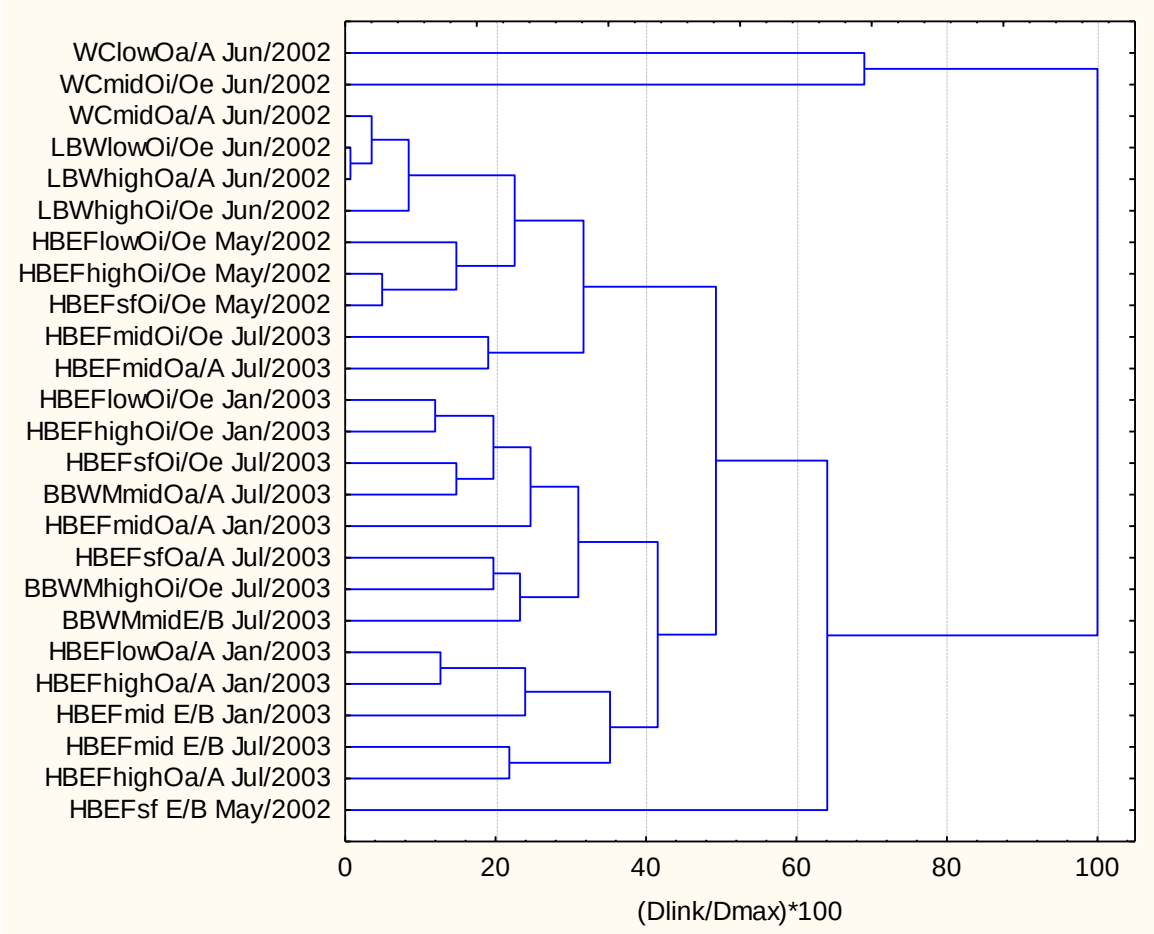


Figure 4.5. Clustered tree diagram for 25 environmental samples digested with *Alu*I with complete linkage analysis and showing percent disagreement. Samples were from the Bear Brook Watershed (BBWM); Hubbard Brook Experimental Forest (HBEF); Lye Brook Wilderness (LBW); and Willowemoc Creek (WC). Samples were taken from different elevations: low, mid, high, and sf (spruce-fir or highest); and soil horizons: Oi/Oe, Oa/A, E/B.

Table 4.3. Unique dominant terminal restriction fragments (base pairs) discovered at the Willowemoc Creek study site. Environmental 16S rRNA genes were amplified with labeled primer set f27-type 2b and digested with restriction enzymes *Alu*I and *Hha*I. Dominant fragments are listed in order of descending signal strength.

WC low Oa/A	WC mid Oi/Oe
*Alu*I	*Alu*I
98	316
102	252
63	456
154	74
*Hha*I	*Hha*I
103	455
79	98
99	57
207	79

pattern relationship to any other sample analyzed (0%). Methanotrophs in the summer 2002 mid elevation Oa/A horizon at WC were highly similar to sample patterns in the low elevation Oi/Oe horizon and high elevation Oa/A horizon at the 2002 summer Lye Brook (LB) samples (96%). Those two terminal fragment patterns from LB were nearly identical to each other (99%) and very similar to patterns in the high elevation Oi/Oe horizon at LB (92%). Summer LB patterns were similar to 2002 summer samples from the Hubbard Brook Experimental Forest (HBEF) (77%) and 2003 summer samples from the HBEF (69%). Summer 2003 mid elevation Oi/Oe and Oa/A HBEF samples were very similar to each other (82%) but only moderately (52%) similar to patterns samples from other elevations and horizons in the summer 2003 HBEF and the Bear Brook Watershed in Maine (BBWM). Low and high elevation winter 2003 Oi/Oe HBEF samples showed very high similarities to each other (88%) and high similarity to summer 2003 highest elevation Oi/Oe HBEF and mid elevation Oa/A BBWM samples (80%).

Summer 2003 highest elevation Oa/A samples from HBEF were also highly similar to high elevation Oi/Oe patterns at BBWM from the same season (80%), and even slightly more similar than from mid elevation E/B patterns at the BBWM (77%). Low and high elevation winter 2003 Oi/Oe and summer 2003 highest elevation Oi/Oe patterns from the HBEF showed moderate relationship (70%) to patterns in samples from summer 2003 highest elevation Oa/A samples from HBEF and high elevation summer 2003 Oi/Oe patterns at BBWM. Winter 2003 low Oa/A and high Oa/A samples from the HBEF were (87%) similar to each other and similar (77%) to the mid E/B pattern. However, the winter samples were only moderately similar (65%) to summer samples at two of the three locations. Winter and summer 2003 HBEF sample patterns were only moderately similar (58%) to five other winter and summer 2003 HBEF samples and three other summer 2003 BBWM samples. The summer 2002 highest elevation E/B horizon pattern at the HBEF showed only fair similarity to any of the above mentioned patterns (36%).

Restriction digest of 23 samples with *Hha*I produced 229 distinct terminal fragments ranging from 50.0 to 499.9 bp. Clustered tree diagrams support the findings that the methanotroph fragment pattern at the Willowemoc Creek (WC) study site were the most dissimilar to the other samples (Fig. 4.6),. Methanotrophs in the summer 2002 low elevation Oa/A and mid elevation Oi/Oe horizons at WC were fairly similar to each other (32%), but showed no terminal fragment pattern relationship to any other sample analyzed (0%) and showed dominant TRFs not present in other samples (Table 4.3). Methanotrophs samples in the summer 2002 mid elevation Oa/A horizon at WC were most similar to patterns in the high and highest elevation Oi/Oe horizon at the 2002 summer HBEF samples and 2003 summer mid Oi/Oe sample (96%). The terminal

fragment patterns from summer 2002 high Oi/Oe HBEF and summer 2003 mid Oi/Oe were nearly identical to each other (99%) and very similar to patterns in the high elevation Oi/Oe horizon in the HBEF summer 2003 sample (97%). Summer 2002 LB patterns were highly similar to each other (>92%) and similar to 2002 and 2003 summer samples from the HBEF (85%). Winter 2003 mid elevation E/B samples at the HBEF were similar to 1 summer 2002 WC, 3 summer 2002 LB, and 4 summer 2002 and 2003 HBEF samples (80%), but only moderately (62%) similar to patterns from other winter and summer samples at the HBEF. High elevation winter 2003 Oi/Oe and Oa/A HBEF samples showed very high similarities to each other (92%) and high similarity to the low elevation winter 2003 Oa/A pattern (90%). Summer 2003 high elevation Oa/A sample from HBEF were also highly similar to mid elevation Oa/A and E/B patterns at BBWM from the same season (85%). Highest elevation summer 2003 Oi/Oe and Oa/A patterns from the HBEF showed a relationship (82%) to each other, but a lower similarity (<69%) to all other patterns.

4.9 Discussion

It is important to note the wide variety of terminal fragment sizes and strength of similarities to one another. The TRF method was capable of detecting substantial diversity in methanotroph populations using methanotroph specific primers. The results showed many within-site similarities, as would be expected. However, what was particularly striking is that samples from the HBEF and BBWM had more similarities than the other two sites and that some samples from the WC seemed to be completely isolated. The fact that two of the WC samples produced unique dominant TRF patterns

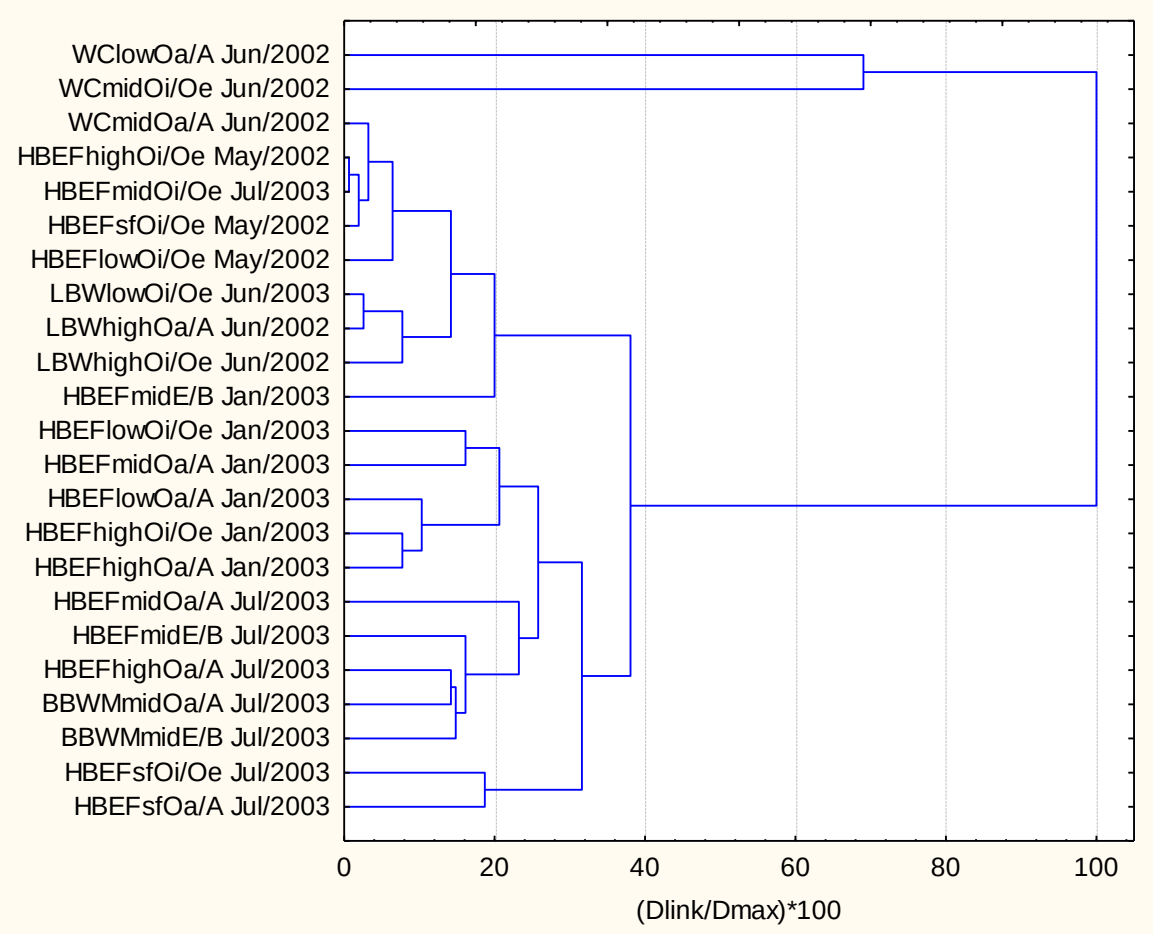


Figure 4.6. Clustered tree diagram for 23 environmental samples digested with HhaI with complete linkage analysis and showing percent disagreement. Samples were from the Bear Brook Watershed (BBWM); Hubbard Brook Experimental Forest (HBEF); Lye Brook Wilderness (LBW); and Willowemoc Creek (WC). Samples were taken from different elevations: low, mid, high, and sf (spruce-fir or highest); and soil horizons: Oi/Oe, Oa/A, E/B.

leads me to believe a novel lineage was detected at the WC, which branched distinctly from all currently known methanotrophs (Fig. 4.5, Fig 4.6). It appears that the chemical differences among the sites discussed in Chapter 2, like the high base saturation at the WC site and the fact that acid deposition is greater at the WC and LBW sites, had a substantial influence on methanotroph diversity. WC was the site that stood out the most, chemically, with high inputs of acid deposition and high soil exchangeable H^+. This environment apparently produced special conditions at the low elevation WC site that allowed completely different terminal fragments patterns in the methanotroph population. Perhaps these patterns came from acid sensitive species that are not able to exist in the acid-stressed environments at the other sites. This conclusion supports a major theory of this thesis that acid stress causes a shift in methane oxidizing bacterial communities to favor acid tolerant species. Spatial variation of similarity values within sites were less than between sites and can be explained by the heterogeneous nature of soil nutrient and moisture availability.

Dunbar et al. (2000) noted a range of distinct 16S rRNA gene TRF patterns in southwestern pinyon-juniper forest soils. In their study, the broad-based 16S gene primers f27-1492r and multiple enzymes were used to characterize the bacterial communities at four sites. They found that TRF profiles of a single sample varied both in the number of TRFs in a profile and in the frequency of distribution of TRFs, and concluded that combining data from different enzyme profiles in valid ways that yield statistically informative results was difficult. In this study, TRF analysis was not used to examine phylotype richness or community evenness.

Temperate soils typically exhibit wide fluctuations in microbial activity and biomass due to temperature, moisture, and nutrient availability. Mummey et al. (2003) showed no patterns toward dissimilarity over time, but the results of this study suggest a change beyond natural seasonal variability. Digestion with the second enzyme avoided underestimation of community compositional changes, which are a common problem with single-enzyme digest analyses, and confirmed the differences observed by the first. Prominent changes were observed among the summer 2002, winter 2002, and summer 2003 samples at the HBEF. The summer 2003 fragment patterns were different than the previous summer's samples and showed none of the same dominant TRFs (Table 4.4) In addition, summer 2002 samples were dissimilar to the winter 2003 and summer 2003 samples, showing shifts in the dominant TRF patterns (Table 4.4). The community did not maintain its 2002 condition or return to its summer state, but changed its composition to favor those organisms that would grow best under the environmental conditions in winter and summer 2003. The TRF fingerprint approach captured these changes and showed the differences in the communities between seasons.

Horz et al. (2001) detected highly diverse methanotroph community TRFs in rice field bulk soil and concluded that type II methanotrophs were more abundant in environments where growth rates are periodically restricted. Similar to those observations, this study found a very diverse and complex assemblage of type II methanotroph TRFs present in forest soil samples and attributes those results to environmental restrictions. The environment of a temperate forest soil is that of competition for limited resources and adaptation to fluctuations in environmental

Table 4.4. Dominant terminal restriction fragments (base pairs) in soil samples collected in May, January, and June at low, mid, and spruce-fir elevations at the Hubbard Brook Experimental Forest. Environmental 16S rRNA genes were amplified with labeled primer set f27-type 2b and digested with restriction enzymes *Alu*I and *Hha*I. Dominant fragments are listed in order of descending signal strength.

HBEF sf Oi/Oe		HBEF low Oi/Oe		HBEF mid Oa/A	
May-02	Jul-03	May-02	Jan-03	Jan-03	Jul-03
*Alu*I	*Alu*I	*Alu*I	*Alu*I	*Alu*I	*Alu*I
125	59	125	59	59	98
119	78	141	78	79	60
	91	201		90	59
		119			
*Hha*I	*Hha*I	*Hha*I	*Hha*I	*Hha*I	*Hha*I
57	78	342	79	79	185
342	111	57	58	111	60
	58	230	111		
	60				

conditions. These factors create a large number of ecological niches for the diverse microbial community to grow into. In this study, as Horz et al. (2001) results suggested, type II methanotrophs were found in great abundance and diversity in soils where growth may be restricted for several different reasons. I would suggest that acid deposition was an important environmental stress that limited the growth of some acid-sensitive methanotrophs and altered the methanotroph community. These conclusions are supported by the data presented in Chapter 2. Acid deposition was highest at the WC and LBW, sites that exhibited the most dissimilarity in methanotroph TRF patterns. Soil depth was greatest at the LBW followed closely by the WC watershed. The dissolved organic carbon – acid neutralizing capacity relationships were strongest at the WC and LBW. For these reasons and others, the environment and chemistry of the sites supports the general conclusions.

In conclusion, the results of this study suggest that environmental factors strongly influence methanotroph TRF patterns. This study represents one of the first attempts to characterize methanotroph 16S rRNA gene TRF patterns in a heterogeneous northern forest soil ecosystem. Analyses like these could help determine the true effects of environmental stressors, such as acid deposition, on natural forest soil microbiological processes.

Chapter 5: References

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Chapter 6: Conclusions and Future Work

6.1 Fulfillment of study objectives

The purpose of this study was to determine if methane oxidizing bacterial diversity in northeastern forest soils was different among sites along an acid deposition gradient. Two culture-independent microbial techniques were successfully used to directly characterize the methanotroph communities of acid forest soils and soil and water chemical analyses were performed.

It was proposed that methane oxidizing microbial populations were decreasing in number due to acid deposition. It was determined through fluorescent *in situ* hybridization (FISH) using rRNA-targeted oligonucleotide probes in combination with confocal scanning laser microscopy that type I and type II methanotrophs occurred in relatively similar numbers regardless of the amount of acid deposition. Methanotrophs contributed up to 0.45% of total cell counts in forest soils. The proportion of type II methanotrophs was affected, but not significantly, by soil pH at the Hubbard Brook Experimental Forest (HBEF). Type I and type II methanotroph populations were not statistically different within or among the study sites.

It was further proposed that methane oxidizing microbial communities were changing community structure due to acid deposition. It was revealed through terminal restriction fragment (TRF) screening with group specific 16S rRNA gene primers that methanotroph communities were different in areas with higher acid deposition and more similar in areas with lower acid deposition. The methanotroph communities were found to be very diverse. The concept that acidification of forest soils altered microbial communities was reinforced by findings that unique TRF patterns were discovered in

methanotroph communities under stress from acid deposition. The evidence showed a potential shift from acid-sensitive species to acid-tolerant species at sites with higher acid deposition.

The soil and water chemistry of the study sites, which were themselves significantly impacted by acid deposition, played important roles in explaining the differences in methanotroph communities among the study sites. In addition, it was revealed that dissolved organic acids play a significant role in the acid-base chemistry of headwater streams. Other factors such as soil horizon depth, soil moisture, soil temperature, elevation, and climate were noted as factors potentially influencing methane oxidizing communities.

A relationship does exist between methanotroph community ecology and the acid-base status of soils and differences in belowground microbial diversity can be seen, at least in part, as a result of differences in acid stress. Methanotrophs are the most important bacteria that oxidize methane and are the only bacteria that use methane as a sole carbon and energy source. This study provides a preliminary analysis of the effects of acid deposition on methanotrophs. Implications of the results of this study are potentially far-reaching because of the suggested high diversity of natural methane oxidizing communities, seemingly ubiquitous nature of methanotrophs, wide distribution of temperate forest ecosystems, and continued increase in concentration of atmospheric methane.

6.2 Suggestions for future work

This study raises additional questions that warrant further research. Long-term monitoring of methane oxidizing communities would provide more insight into seasonal differences and perhaps changes due to regional and global climate change.

Measuring methane oxidation rates at sites with different amounts of acid deposition would give insight into how methanotroph function is affected by that environmental stress. Along those lines, using functional *pmo*A gene probes and primers with FISH and TRF analysis could provide additional information about abundance and diversity of methane oxidizing communities.

More fields of view in FISH and more samples successfully amplified and digested in TRF analysis would give more statistically viable results. More chemical analyses of the soils and soil solutions would help explain the observed trends and perhaps yield statistically significant relationships.

Enrichment cultures and DNA sequencing should be used to determine the diversity of species of methane oxidizers actually present at the sites. In addition, the role of other methane oxidizers, such as the nitrifiers, should be thoroughly investigated to get a more complete picture of the methane oxidizing community.

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Publications in Preparation:

- Bremer, M.A., C.T. Driscoll, and A.M. Costello. Role of dissolved organic anions in the long-term acid-base status of stream water. In preparation.
- Bremer, M.A. and A.M. Costello. Assessment of methanotroph diversity in four northeastern United States forest soils by 16S rRNA terminal restriction fragment analysis. In preparation.