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THE DIVERSITY OF DISSIMILATORY NITRATE REDUCERS IN AN AGROECOSYSTEM

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THE DIVERSITY OF DISSIMILATORY NITRATE REDUCERS IN AN AGROECOSYSTEM

by

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ABSTRACT

The Diversity of Dissimilatory Nitrate Reducers in an Agroecosystem

by

Kristin Michelle Huizinga

Microbial communities are essential to nutrient cycles in agricultural soils, since their activity helps regulate the availability of nutrients to crops. Despite this important role, factors affecting microbial community structure are just beginning to be uncovered. As well as identifying factors affecting microbial communities, it is important to determine the links between particular communities and their ecosystem functions. Therefore, the focus of the work presented is on microbial communities known to be involved in soil nitrogen cycling (denitrifiers) or with the potential for involvement (*Planctomycetales*).

Statistical modeling determined how much of the variability in N_2O and two other greenhouse gas fluxes (CO_2 and CH_4) could be explained from treatments varying in history of agriculture. The amount of explained variation was generally low, with CO_2 flux having the greatest amount of explainable variation. It was concluded that the high amount of explainable variability in CO_2 flux was due to the fact that it is a byproduct of heterotrophic metabolism in soil, making its production responsive to any factors affecting microbial metabolism. Nitrous oxide and CH_4, conversely, are produced or consumed by microorganisms with specialized metabolisms, causing environmental variables alone to be insufficient for explaining amounts of flux from these gases.

Denitrifier community composition and diversity was assessed in three soil treatments varying in average annual N_2O flux and history of agriculture. Comparisons of *nir*K sequence libraries indicated that community composition of successional sites abandoned from agriculture 15 years ago still showed impacts from agriculture. Productivity did not have a significant relationship with measures of denitrifier diversity, but there was a weak, positive relationship between relative disturbance and diversity. In addition, a significant, positive relationship between ribosomal RNA (rrn) operon copy number and growth rate of dissimilatory nitrate reducers was found, indicating that denitrifiers with low and high rrn operon copy numbers differ in ecological strategies. There was a significant effect of soil treatment on evenness, and there are *nir*K OTU's in the never tilled and historically tilled treatments that may play an important role in minimizing the amount of N_2O flux from sites currently not used for agriculture.

Since members of the order *Planctomycetales* may play important roles in nitrogen cycling, a phylogenetic survey of 16S rRNA gene sequences was undertaken to assess soil planctomycete diversity in soils differing in history of agriculture. Sequences clustered with the four recognized genera of planctomycetes as well as in clusters outside those genera. Three sequences were similar to 16S rDNA sequences from organisms capable of anaerobic ammonia oxidation. Comparative analyses indicated that two soil treatments that have been used for agriculture are more similar in community composition and diversity to each other than to a treatment never used for agriculture. However, there were no significant differences between the three communities, suggesting that *Planctomycetales* are marginally affected by agricultural practices.

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When I first started graduate school, my only experience with microbiology was through the job I had held previously in a lab at a pharmaceutical packaging company. It was intimidating at first to jump into research in an area I was unfamiliar with, and I owe a debt of gratitude to all those who helped me initially. From the Schmeznak lab, Joseph Graber, Joel Klappenbach, Bradley Stevenson, Joel Hashimoto, and Daniel Buckley showed me the ropes of life in a research lab. Dan especially was incredibly patient during my rotation in the lab and was the first to teach me about using molecular techniques. Later members of the lab were also an incredible help. Brendan Keough worked with me on my BAC library project and as well as being a great technician was a great friend and gave me a lot of moral support through a tough time during my graduate experience. I would also like to thank Jorge Rodriguez who was also a great help on the BAC library project. Stephanie Eichorst was always willing to lend me a helping hand with experiments and kept lab fun.

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CHAPTER 1

MICROBIAL ECOLOGY OF ORGANISMS CONTRIBUTING TO LOSS OF NITROGEN FROM SOILS

Introduction:

Since the Kellogg Biological Station Long-Term Ecological Research (KBS LTER) site was first set up in 1989, there have been many studies involving soil microorganisms. The majority have sought to determine what impact Midwestern agricultural practices have had on bacterial communities, with the ultimate goal of linking communities with their function. Studies have included those investigating the effects of isolated or combined agricultural practices on either specific microbial groups or the microbial community as a whole. The majority of studies concerning specific groups have focused on microorganisms involved in nitrogen cycling, specifically those that contribute to the loss of nitrogen from soil ecosystems.

The KBS LTER is composed of eleven treatments that vary in type and degree of agricultural management as well as successional status (Table 1.1). Some treatments also contain microplots in which variations on the normally prescribed additions can be investigated. Treatments are replicated between three and six times and there is a publicly available database of KBS LTER environmental measurements going back to 1989. This combination of replication and well-documented historical data for the site has made KBS an ideal setting for studies involving microbes' role in nitrogen cycling.

In this chapter, I summarize work previously completed at the KBS LTER that has implications for the work presented in my thesis, discuss microbial pathways involved in nitrogen cycling, and present an overview of following chapters.

Table 1.1: Treatments used for Microbial Studies at the Kellogg Biological Station Long-Term Ecological Research Site

KBS LTER Designation	Designations Used in This Thesis	Description
T1	CT	Conventional agriculture (corn-soybean-wheat annual rotation)
T2	NOTILL	No-till (corn-soybean-wheat annual rotation)
T3	LI	Low input (corn-soybean-wheat annual rotation)
T4	ORG	Low input organic (corn-soybean-wheat annual rotation with no chemical inputs)
T5	POP	Poplar trees (harvested every 6-7 years)
T6	ALF	Continuous alfalfa
T7	HT	Historically tilled successional plots (abandoned from agriculture in 1989)
T8	NT	Never-tilled successional plots (never used for agriculture)
DF	DF	Deciduous forest (never cut or cleared for agriculture)
SF	SF	Late-successional forest (abandoned from agriculture 40-60 years ago)
CF	CF	Coniferous forest (40-60 years old)

KBS LTER Studies on Microorganisms Involved in Loss of Nitrogen from Soil:

The majority of microbiological studies at the KBS LTER have involved those organisms that participate in the nitrifying and denitrifying portion of the nitrogen cycle (Fig. 1.1). These two groups are particularly important because their combined activity can be responsible for losses of nitrogen from agricultural soil as high as 60% [1].

Nitrifiers: Nitrification is performed by two different groups of bacteria, those capable of ammonia oxidation to nitrite and those that oxidize nitrite to nitrate (Fig. 1.2A). Until recently, it was thought that only certain members of the γ- and β-proteobacteria were capable of nitrification. However, members of the kingdom Chrenarchaeota also are capable of ammonia oxidation [2]. Expression of Chrenarchaeota amoA-like genes was found to occur in soil [3], indicating that this group may also be important in nitrogen cycling [4].

Nitrite and nitrate are highly mobile forms of nitrogen due to their negative charge, and will leach out of agricultural soil and into water resources. Nitrite can be mutagenic when it forms nitrous acid in the environment and small amounts have antimicrobial effects in acidic soils [5]. Nitrification also causes direct losses of nitrogen from soil by production of the greenhouse gas N_2O and indirect losses by creating nitrogen species subsequently used in denitrification. Of the nitrifiers, ammonia oxidizing bacteria (AOB) have been the focus of KBS studies.

The rate of nitrification is affected by ammonium concentration, temperature, moisture, and oxygen concentration [6, 7]. While many studies have focused on nitrification rates, there are few that focus on how nitrifier diversity and community

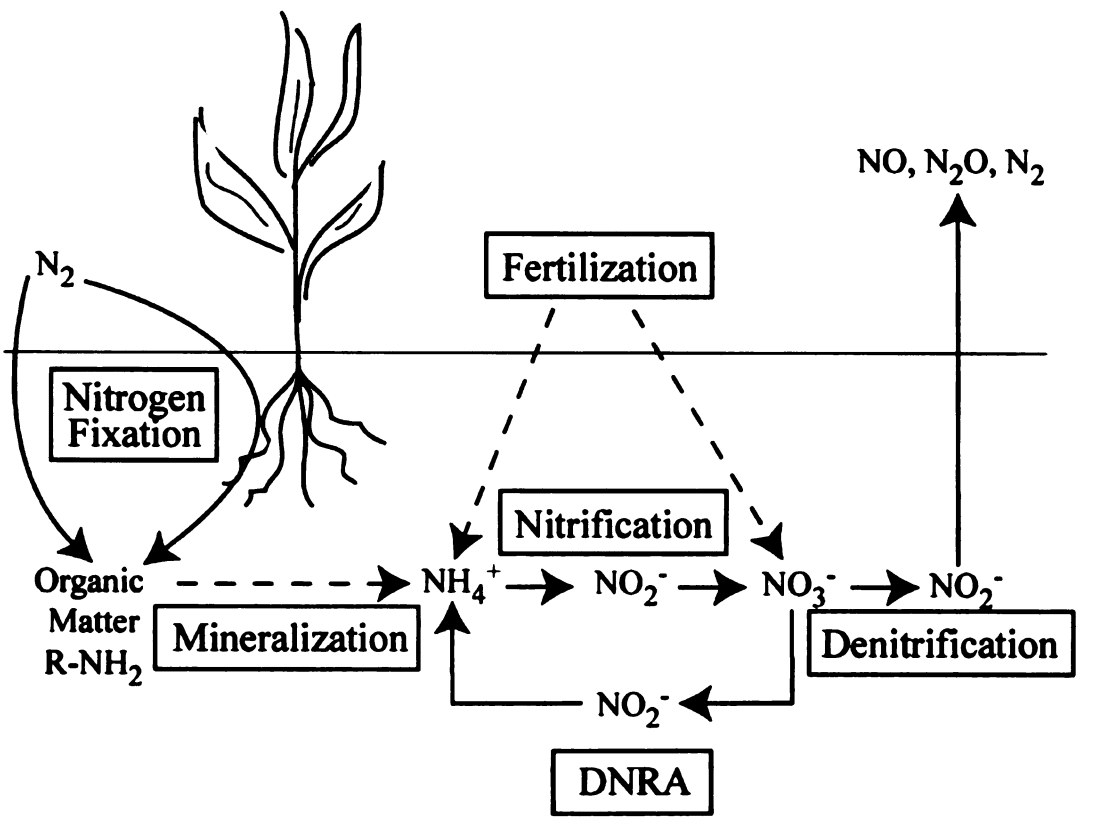

Fig. 1.1: The soil nitrogen cycle. Solid arrows indicate microbial transformations and dashed lines indicate abiotic processes. DNRA = dissimilatory nitrate reduction to ammonia.

A. Nitrification

$$NH_3 \xrightarrow[\text{Amo}]{} NH_2OH \xrightarrow[\text{Hao}]{} NO_2^- \xrightarrow[\text{Nor}]{} NO_3^-$$

B. Denitrification

$$NO_3^- \xrightarrow[\substack{\text{Nar}\\\text{Nap}}]{} NO_2^- \xrightarrow[\text{Nir}]{} NO \xrightarrow[\text{Nor}]{} N_2O \xrightarrow[\text{Nos}]{} N_2$$

Fig 1.2: Nitrogen cycling pathways studied at the KBS LTER. Enzymes responsible for each step in the pathways are named as follows: A) Amo = ammonia monooxygenase, Hao = hydroxylamine oxidoreductase, and Nor = nitrite oxidoreductase. B) Nar and Nap = nitrate reductase, Nir = nitrite reductase (specific to denitrification), Nor = nitric oxide reductase, and Nos = nitrous oxide reductase.

structure affect those rates or how agricultural treatments affect community structure. Therefore the roles of fertilization and tillage as well as seasonal effects on the nitrifier community have been investigated at KBS since these are the primary factors in agricultural systems that may influence nitrifiers.

Fertilization has an effect on the abundance of nitrifiers. Initial most probable number (MPN) experiments with media containing different concentrations of ammonium sulfate indicated that AOB communities in agricultural fields subjected to regular ammonia-containing, nitrogenous fertilizer applications are more tolerant of high concentrations of ammonium and are higher in number compared to communities present in soil that do not receive regular nitrogen inputs [8]. Later studies sought to confirm these results using both MPN counts and competitive PCR (cPCR) [9, 10]. MPN underestimated AOB numbers as compared to cPCR, and in these studies no difference between population numbers was found with MPN. However with cPCR, fertilization again increased nitrifier numbers. Despite the increase, no correlation between potential nitrification rate and AOB numbers was found. This was also the case in a study on non-KBS soils in which AOB community size and nitrification were monitored before and after fertilizer addition. Fertilization caused an increase in nitrification rate within 3 days of fertilization, but the AOB community size did not increase significantly until 6 weeks had passed and nitrification rates had decreased [11]. This indicates that the members of the AOB community or physical factors in the field are more important in determining nitrification rates than the sheer number of organisms. Indeed, a study conducted using plots from the KBS LTER and Living Field Laboratory (LFL) aimed specifically at investigating physical factors influencing nitrification rates found a seasonal pattern to

nitrification potentials, but that the cropping system had no effect [12]. This suggests that seasonal effects, such as temperature or soil moisture, are important drivers of nitrification rates.

The results of KBS studies on the effect of agriculture on nitrifier diversity and community composition indicate that there is a long-lasting effect of agricultural use. Bruns and colleagues [8] found AOB in a conventionally treated agricultural field were less diverse than those from a never-tilled field based on DGGE and sequencing of AOB 16S rRNA gene clones. Sequences from *Nitrosospira* cluster 3 were the only ones found in the agricultural field while sequences from an additional three AOB groups were found in the never-tilled field. Phillips and colleagues [10] found no detectable differences in AOB communities from the same agricultural fields or a site abandoned from agriculture in 1989, using the same techniques. Sequences from *Nitrosospira* cluster 3 dominated at both sites in this study. The treatment abandoned from agriculture was studied in 1994 and 1995; between 5 and 6 years after abandonment. The fact that the AOB community after this amount of time was still no different from that in the agricultural treatment suggests that the community had not recovered from the impact of agricultural practices.

Later studies from outside the KBS LTER supported these findings. Fertilization causes changes in AOB community composition, but these changes occur at a slow rate, most likely due to the inherently slow growth of the microorganisms [13-15]. After fertilizer was added to soil microcosms, significant changes in the DGGE patterns of the *amo*A gene were not detectable until 16 to 20 weeks of incubation had occurred [14], and in an earlier study by the same group there was no difference between communities in soils that received fertilizer and did not receive fertilizer after 4 weeks [13]. Changes in

microcosm communities receiving a concentrated pulse of ammonium would be expected to occur more quickly than under field conditions. Therefore, it is possible that 5 to 6 years after abandonment from agriculture was not enough time to see a shift in KBS nitrifier communities in the field.

In summary, there is a trend of increasing AOB numbers with fertilization, and fertilization selects for AOB that are tolerant to high concentrations of ammonium. Also, nitrification potentials are higher in agricultural treatments, and this cannot be explained solely by the difference in nitrifier numbers. This may be due to the combination of physical factors differing between fields and differences in the diversity and composition of the nitrifier community. In fact, in a recent study physiological differences between members of *Nitrosospira* cluster 3 accounted for differences in the time needed to initiate nitrification [16]. In natural soils, certain members of the group were sensitive to high ammonium concentrations, and others were tolerant, causing a delay in nitrification until tolerant organisms could grow to sufficient population size. KBS LTER studies also demonstrated that agriculture can induce shifts in AOB community structure and that these changes are long-lasting.

Denitrifiers: Denitrification is a form of dissimilatory nitrate reduction performed by a phylogenetically diverse group of microbes that includes Proteobacteria, Gram positive bacteria, Archaea, and fungi. Denitrifiers are typically heterotrophic, facultatively anaerobic organisms that are capable of using nitrate as an electron acceptor under anaerobic conditions. During denitrification, nitrate is reduced in stepwise manner to nitrite, nitric oxide, nitrous oxide and dinitrogen gas (Fig. 1.2B). This process serves

as a source and sink of nitrous oxide (N_2O), a potent greenhouse gas. As well as contributing to gas emissions, denitrification removes nitrogen from soil by converting it to a readily diffusible gaseous product. An average of 20-30% of nitrogenous fertilizer added to an agricultural field will be lost to denitrification [17]. Nitrifiers also carry out denitrification under low oxygen concentrations [18-21]. Studies at the KBS LTER have not focused on nitrifier denitrification and there is some question as to its importance, since measurements of N_2O production by nitrifier denitrification range from insignificant amounts [22] to 30% of the total N_2O produced [23].

As with nitrifiers, studies on denitrifiers at the KBS LTER have sought to link community composition and diversity with ecosystem processes, most notably, that of N_2O flux. Denitrification rates as well as the proportion of denitrification resulting in production of N_2O, or N_2O mole fraction, are influenced by pH, soil moisture, C/N ratio, oxygen concentration, and temperature [7, 24, 25]. KBS LTER studies have supported the hypothesis that in addition to physical factors, denitrifier community composition influences N_2O production.

Experiments at the KBS LTER investigating the response of denitrifiers to the environmental factors of pH, O_2 concentration, and moisture history found differences that help to explain differences seen in N_2O flux between a conventional agriculture treatment, a treatment abandoned from agriculture since 1989, and a never-tilled treatment. The site subjected to conventional agricultural practices has an annual average N_2O flux about three times higher than the two sites not currently used for agriculture [26]. The agricultural treatment was found to have denitrifiers with N_2O production enzymes (Nar, Nir and Nor) that were more sensitive to O_2 concentration than those of

the never-tilled treatment. Conversely, the never-tilled treatment community had N_2O production enzymes that were more sensitive to pH as compared to the agricultural treatment. In both communities, Nos, which destroys N_2O, was sensitive to O_2 but not pH. Nos from the agricultural treatment was significantly more sensitive to O_2 than that from the never-tilled treatment. To follow up this study, denitrifiers were cultivated from both soil treatments, and the sensitivity of Nos from each isolate to O_2 was tested [27]. Nos of the isolates had a wide range of sensitivities to O_2, but there was no difference between activities at the community level. However, this survey of denitrifiers was limited by the fact that only cultivated denitrifiers were examined. In a later comparison of the N_2O mole fraction ($[N_2O]/[N_2O + N_2]$) produced by the agricultural treatment and the treatment abandoned from agriculture, there was a significant difference in N_2O mole fraction, but not in total denitrification [25]. There was also a strong influence of soil moisture history. The agricultural treatment had a significantly higher N_2O mole fraction with no pre-wetting. Pre-wetted soil from both treatments did not differ in N_2O mole fraction, suggesting that the denitrifiers in the site not currently used for agriculture had Nos that was able to persist for a longer time under dry conditions than that from the agricultural treatment.

The diversity and composition of denitrifier communities at the KBS LTER have also been assessed and used to investigate further the link between diversity and ecosystem function as well as factors that impact community structure. Treatments investigated included some of the same used to test differences in response to environmental factors; the conventional agriculture treatment and plots that have never been tilled or used for agriculture.

There are distinct differences between the denitrifier communities of the agricultural and successional treatments. Two studies focusing solely on comparing the agricultural and never-tilled plots found that the never-tilled plot denitrifiers were less diverse than those of the agricultural plot and that their composition differed [27, 28]. These studies employed both cultivation and molecular methods. Of cultivated denitrifiers, a total of 27 taxa were found, but only 12 were shared between treatments. The number of cultivated isolates from each treatment indicated that there were a higher number of denitrifiers in the agricultural treatment. Organisms from the agricultural treatment tended to be members of the α- and β-proteobacteria while organisms from the never-tilled treatment were mostly γ-proteobacteria. The diversity of isolates was higher in the agricultural treatment as compared to those from the never-tilled treatment [27].

A molecular survey of the same treatments confirmed these results. Restriction Fragment Length Polymorphism (RFLP) of *nosZ* was used to assess diversity, and 182 distinct RFLP patterns were identified. As in the previous study, few groups (only 8 RFLP patterns) were shared between communities and the diversity of the agricultural treatment was higher than that of the never-tilled. This was due to the richness and evenness of the agricultural field being higher. Evenness is a measure of the degree a community is dominated by particular species. The higher evenness of the agricultural treatment indicated that few RFLP patterns dominated the population. Indeed, the group with the highest frequency made up less than 5% of the total while the never-tilled field had one group that made up 32% of the RFLP patterns found [28].

The KBS LTER studies on links between the denitrifier community and ecosystem function were among the first to show that communities can differ in their

physiological potentials and response to their environment, which in turn affects functions such as N_2O flux. Other studies on denitrifiers have confirmed these results [29-32]. For instance, not only are there differences in denitrifier communities between soil treatments, but there are also differences within treatments that contribute to differences in ecosystem function. An example of this are the differences noted by Chèneby and colleagues [32] between communities in maize rhizosphere and non-rhizosphere soil. Denitrifiers isolated from the rhizosphere were less diverse than those from the surrounding soil, and the dominant rhizosphere denitrifiers were not able to reduce N_2O to N_2, whereas bulk soil isolates produced N_2 as their denitrification product.

Summary:

KBS LTER studies on nitrifying and denitrifying bacterial communities have demonstrated that they will respond to changes in physical factors caused by agricultural practices, causing them to differ from communities in non-agricultural soils in composition, diversity, and abundance. In turn, microbial communities can differ in their physiological potential and response to environmental conditions. This ultimately results in differences in various ecosystem functions. Published work suggests that microbial communities as well as environmental conditions should be taken into account when investigating ecosystem functions such as gas flux or nutrient cycling.

Thesis Overview:

Nitrous oxide flux from soil tends to be spatially and temporally variable, making predictions of total flux difficult. Therefore, in Chapter 2, a statistical modeling approach

was taken to determine how much of the variability in flux of N_2O and of two other greenhouse gases (CO_2 and CH_4) could be explained from four treatments varying in history of agriculture at the KBS LTER. The amount of explained variation was generally low, with CO_2 flux having the greatest amount of explainable variation. It was concluded that the high amount of explainable variability in CO_2 flux was due to the fact that this gas is a byproduct of all types of heterotrophic metabolism in soil, making its production responsive to any factors affecting microbial metabolism in general. Nitrous oxide and CH_4, conversely, are produced or consumed by microorganisms with specialized metabolisms, causing environmental variables alone to be insufficient to explain amounts of flux from these gases.

The findings presented in Chapter 2 suggest that differences in the composition and diversity of microorganisms responsible for greenhouse gas fluxes are important for understanding variation in this ecosystem function. Therefore, in Chapter 3, denitrifier community composition and diversity was assessed in three soil treatments that varied in their average annual N_2O flux and history of agriculture. Differences in community composition and diversity were found; therefore the effect of disturbance and productivity as well as a possible ecological strategy of these organisms was investigated.

In Chapter 4, the community composition and diversity of microorganisms belonging to the order *Planctomycetales* was determined. There have been numerous studies on planctomycetes from aquatic habitats, and recently isolates from soil have been cultivated. Despite this, little is known about the ecological role of this diverse group. One intriguing recent development was the discovery that organisms capable of anaerobic oxidation of ammonia (anammox) belong to the *Planctomycetales* [33]. If

anammox were occurring in soil, it would have implications for the nitrogen cycle in soil as the end product of this reaction is dinitrogen gas; making this another microbial pathway resulting in loss of nitrogen from soils.

In Chapter 5, the conclusions from this thesis are presented along with proposed experiments that would continue to further our knowledge regarding the ecology of microorganisms involved in the soil nitrogen cycle.

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CHAPTER 2

ASSESSING THE INFLUENCE OF CROPS AND ENVIRONMENTAL FACTORS ON THE FLUX OF GREENHOUSE GASES IN AGROECOSYSTEMS

These results were submitted for publication in the article: Huizinga K.M., U.Y. Levine, J.P. Hoehn, and T.M. Schmidt. 2006. Assessing the Influence of Crops and Environmental Factors on the Flux of Greenhouse Gases in Agroecosystems. Biogeochemistry.

ABSTRACT:

In an effort to better understand variation in the production and consumption of greenhouse gases by microbial communities in soils of agroecosystems, data from four soil ecosystems at the Kellogg Biological Station Long Term Ecological Research Site were analyzed. The data spanned 6 to 9 years for soils that varied from a standard crop rotation of corn, soybeans, and wheat to fallow land and deciduous forest. Gas fluxes were modeled using a production function approach where gas flux is a function of current year management, historical management, and environmental factors. Linear production function parameters were estimated using ordinary least squares with robust standard errors. Independent variables explained between 8 and 50% of the variation in gas flux, with carbon dioxide flux consistently having the greatest amount of explainable variation (29 to 50%). We conclude that since CO_2 is a byproduct of all types of heterotrophic respiration in soil, its flux will be responsive to factors that enhance microbial metabolism in general, and that this phenomenon explains the high coefficients of determination for explaining variation in CO_2 flux at the KBS LTER. Conversely,

since the consumption of CH_4 and production of N_2O require the activity of microbes with specialized metabolic pathways, environmental parameters alone explain a smaller percentage of the flux of these gases. There were also significant effects on gas flux associated with specific crops, with fluxes roughly following the pattern wheat > soybean > corn. In addition, the treatment with the highest level of variation accounted for by environmental factors alone was the late-successional, deciduous forest (19 to 50%). Future modeling efforts will likely be enhanced by including measures of microbial diversity in soil, particularly those microbes involved in methane consumption and nitrous oxide production.

INTRODUCTION:

In terrestrial ecosystems, the exchange of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) between the soil and atmosphere has important effects on ecosystem services such as climate regulation and nutrient cycling. The production and consumption, or flux, of these greenhouse gases varies seasonally and spatially across landscapes, and is influenced directly by land use, particularly agriculture. For instance, the majority of labile organic matter in soil is oxidized to CO_2 and lost to the atmosphere during the first few years after native lands are converted to agriculture [1, 2]. Managing land for agriculture also decreases the amount of CH_4 that is consumed by soil microorganisms [3], and increases the emission of N_2O from soil [4, 5]. Identifying environmental factors that influence the exchange of these greenhouse gases between soils and the atmosphere would enhance predictive models of gas flux, which are important in evaluating ecosystem services provided by agriculture. In addition, it may

also suggest strategies for mitigating the detrimental impact of greenhouse gases produced by agriculture.

Inventories of agricultural gas fluxes used in climate change assessments and policy analysis are typically developed using biogeochemical process models. Process models such as DAYCENT [6, 7] or DNDC [8], are generally composed of several submodels for predicting the effects of many interrelated biological and physical processes on gas flux. These models depend on accurate physical measurements of environmental variables to calculate a predicted amount of gas production or consumption. While techniques for measuring environmental data have become more accurate over time, models are still limited in their predictive power due to the many parameter estimates and assumptions required to construct them. For instance, in terms of assumptions, IPCC guidelines [9] consider all agricultural systems to be equivalent, which does not take into account spatial and temporal variability, or differences in the soil microbial communities that are ultimately responsible for the production and consumption of greenhouse gases.

In this chapter, a production function approach was used to evaluate the effects of environmental and crop management variables on the flux of CO_2, N_2O, and CH_4. An empirical assessment of the importance of these variables prior to their use in process models will serve to improve gas flux predictions. Estimation of the production function parameters is based on data covering approximately 9 years at the Kellogg Biological Station Long Term Ecological Research Site (KBS LTER) in Michigan. The production function approach posits that gas flux is a function of environmental factors, crop management values, and unmeasured factors represented by a stochastic error. This

approach allows us to assess the impact of the environmental and management factors on gas flux, both individually and as a group of measured variables. The variation in flux that is unexplained by the measured variables also provides an upper bound assessment of the effect of unmeasured variables, such as those describing the microbial community. Results indicate that the included measured variables explain 50% or less of the variation in gas flux. The amount of explained variation varies by agroecosystem type and by type of flux and are greatest for CO_2 and least for N_2O. The results suggest that while environmental and management factors are helpful in understanding a minor portion of gas flux, smaller error predictions are only likely to come with a better understanding and measurement of extant unmeasured variables, such as the composition, diversity, and structure of soil microbial communities.

MATERIALS & METHODS:

Study Site

Four treatments were the focus of this study: a conventional agriculture site that receives amounts of fertilizer, herbicide, and conventional tillage (CT) that are typical for the Midwest region and is on an annual rotation of corn (*Zea mays* L.) - soybean (*Glycine max* L.) - wheat (*Triticum aestivum* L.); an agriculture site with a history of tillage (HT) that was abandoned from agricultural use in 1989; a never-tilled site (NT) maintained in a mid-successional plant community by annual mowing and a late-successional deciduous forest (DF). The KBS LTER is set up in a randomized block design and data from four replicates of the CT, HT, and NT treatments was retrieved, as was data from the three DF replicates. Additional details on the KBS LTER can be found at http://lter.kbs.msu.edu/.

The Production Function for Gas Flux

The production function approach posits that gas flux is a function of environmental factors, crop management, and a stochastic term. This allows one to quantitatively estimate the partial effect of each measured environmental and management factor on the ecological service of interest, in this case, gas flux. In addition, the approach indicates the importance of the measured factors in explaining the variation in greenhouse gases relative to the influence of the unmeasured factors represented by the stochastic error term.

The estimated production function is specified as a linear model:

(1) $$g = \alpha + \beta x + \varepsilon$$

where g is gas flux and x is a K-element vector of measured environmental and management variables. The quantities α and β are parameters to be estimated and ε is the stochastic term. The α represents the mean value of g when the environmental and management variables are equal to zero. The β_i, $i = (1,...,K)$ represents the partial effect of the ith environmental or management variable on gas flux; it describes how gas flux changes for a one unit change in x_i. α and β are reported as CO_2 equivalents, or global warming potential (GWP), using IPCC conversion factors for a 20-year time span [9]. Conversion to CO_2 equivalents allows for comparisons of the contribution of a particular factor between modeled fluxes. The formulas used to calculate CO_2 equivalents were those reported in the supplemental material of a study by Robertson and colleagues [5].

Each factor (x_i) was selected based on its known propensity to influence gas fluxes from soil. Variables fall into one of three categories: current year management, historical management, and environmental factors. Current year management factors

apply only to the conventional agricultural treatment and include the effect of different crop types and fertilization (assuming a 30 day residence time). Crops vary in the nature and amount of organic compounds released through root exudates and in their chemical composition: both have the potential to influence the structure and function of microbial communities in soil. Fertilization provides nitrogen not only to plants, but also to microbes in soil and so has the potential to stimulate microbial metabolism and influence the production of CO_2 and N_2O. Historical management factors were considered to be those characteristics of soil that reflect past land use. These factors were percent soil moisture, water filled pore space (WFPS), nitrate concentration, ammonium concentration, percent total carbon, and carbon/nitrogen (C/N) ratio. Water filled pore space was calculated using the bulk density of each soil treatment replicate, percent soil moisture, and assuming an average soil particle density of 2.65 $g \cdot cm^{-3}$ [10]. Moisture affects microbial activity by influencing oxygen concentration and availability of nutrients. In particular, high soil moisture will result in anoxic conditions that favor fermentation and denitrification, but not methane oxidation or aerobic respiration. Nitrogen and carbon will stimulate bacterial growth as mentioned above, and different microorganisms will be favored as the relative amounts of each differ. The environmental factors used in the models were cumulative precipitation from three days before measurement of gas fluxes, cumulative precipitation from seven days before measurement of gas fluxes, maximum air temperature, and minimum air temperature. Precipitation will affect soil moisture and temperature will affect microbial activity. Microbial metabolism increases as temperature rises which increases the rate of gas production or consumption. Records of CO_2, CH_4, and N_2O fluxes along with

management and environmental variables from the KBS LTER were downloaded from the KBS LTER website (http://lter.kbs.msu.edu /Data/DataCatalog.html). The units for each variable and method of collection are recorded in Table 2.1.

If the measurement of any factor was not available on the day that gas fluxes were measured, data from measurements closest to that date were used as follows: if a gas sampling date fell exactly between two field measurements, they were averaged. In addition, for the conventional agriculture treatment measures of C/N ratio, the time of fertilization was used to determine which C/N data point to assign to a particular gas flux measurement. For example, if C/N measurements were taken 5 days before and 2 days after gas measurements, but fertilization took place one day after, the C/N measurement from 5 days before the gas measurement was used to avoid falsely biasing the data due to fertilization.

Data sets for each soil treatment were assembled to maximize the number of data points to be included in the analyses. As such, data sets did not all contain data from the exact same days. The conventional agriculture data set was comprised of 408 days of observations collected from 1992 - 2001 (one outlying N_2O flux measurement was removed), the historical agriculture data set contained data from 351 days between 1992 - 2000, the never tilled treatment consisted of 275 days of data collected from 1992 - 1998, and the data set for the deciduous forest consisted of 223 days of data collection from 1993 - 2000. Gas measurements at the forest treatment were performed so that two samples per replicate were taken on each day of sampling. The two flux measurements were averaged as the corresponding physical data was performed once for each forest replicate.

Table 2.1: Methods Used or Location of Data Analyzed with Statistical Models

Factor Category	Factor	Units	Method Used or Location of Record
Current Year Management	Crop	None	Recorded in KBS LTER agronomic log
	Fertilization	g N/ha	
Historical Management	% Soil Moisture	(g H_2O/g dry soil) * 100	Gravimetric soil water content [11]
	Nitrate Concentration	µg N/g dry soil	KCl extraction and colorimetric analysis [12]
	Ammonium Concentration	µg N/g dry soil	
	% Carbon	(g C/g dry soil) * 100	Dry combustion [13]
	C:N	None	
Environmental	Precipitation (3 and 7 days)	mm	Recorded from KBS LTER weather station
	Air temperature (maximum and minimum)	degrees Celsius	

To determine which factors had significant effects on greenhouse gas fluxes, models were set up using a linear production function in the Stata program (StataCorp, College Station, TX). Separate analyses were performed for each treatment and greenhouse gas. In each analysis, gas flux was the dependent variable (g) and the previously listed environmental measures were independent variables (x_i). The amount of variation in gas flux explained by the environmental and management factors relative to the stochastic term was measured by R^2. R^2 is equal to 1 - σ_ε / σ_g where σ_ε is the variance of ε and σ_g is the variance of g.

RESULTS:

Seasonal Greenhouse Gas Fluxes

Of the four treatments, soils of the conventional agriculture treatment had the highest N_2O flux throughout the year, with peak production in April and May (Fig 2.1A). There was a seasonal peak of flux occurring in May and June in the deciduous forest, but as previously reported, the annual average flux of N_2O from the historical agriculture, never tilled soil, and deciduous forest treatments was similar [5]. The never tilled and forest treatments both had the highest rate of CH_4 consumption during the summer and early fall; the conventional agriculture and historical agriculture treatments had the lowest capacity for CH_4 consumption throughout the year with little variation in flux (Fig. 2.1B). The flux of CO_2 from the historical agriculture and never tilled treatments was highest in late spring, and then peaked again in August (Fig. 2.1C). The CO_2 flux from both the conventional agriculture and forest treatments was lower overall and also peaked during the summer.

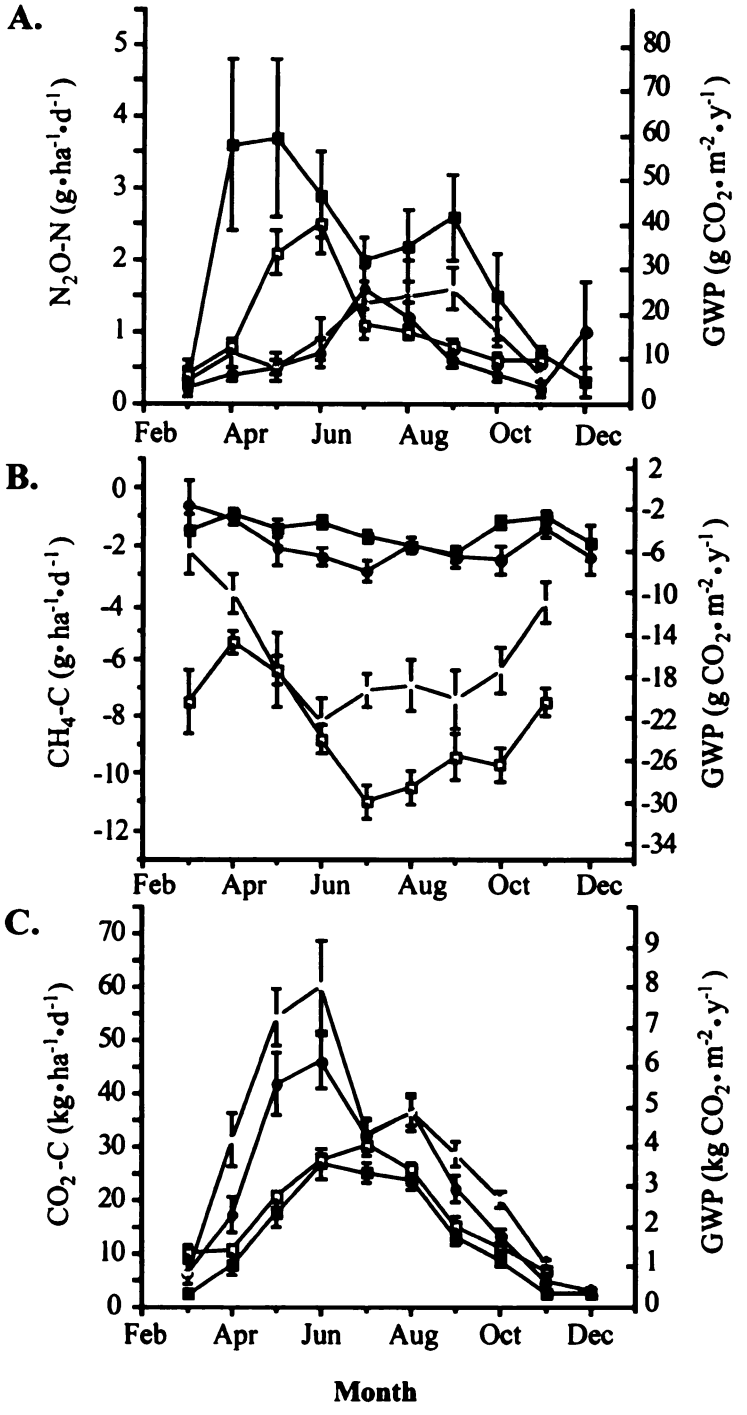

Fig. 2.1: Monthly average greenhouse gas fluxes and associated global warming potential (GWP) for four treatments at the KBS LTER (from data collected from 1992 - 2002). CT (■), HT (●), NT ( ), and DF (□). A) Nitrous oxide flux, B) Methane flux, and C) Carbon dioxide flux. Error bars represent standard errors.

Current Year Management Factors

As the conventional agriculture site was the only treatment in this study currently used for agriculture, it is the only one to which current year management factors applied. Modeling of all gases for this treatment was performed using the corn crop as the baseline, causing the effect of this crop to be absorbed into the intercept coefficient. Both soybean and wheat increased CO_2 and CH_4 flux relative to corn but were not significantly different from each other (Tables 2.2 and 2.3). The effects of corn and soybean crops on N_2O flux were not significantly different from each other, but wheat increased N_2O flux in comparison to corn (Table 2.4). The coefficients for the effect of crops reveal a consistent order of effect such that each crop increases gas flux in the order wheat > soybean > corn. Timing of fertilization did not have a significant effect on any of the gases modeled.

Historical Management Factors

Of the five historical management factors tested, no single factor in the CO_2 and CH_4 models had a significant effect in all four soil treatments. However, C/N ratio had a significant effect in all but the historical agriculture treatment in the CO_2 models and the same was true of percent soil moisture in the CH_4 models. Percent soil moisture was also the factor with a significant effect in the most treatments for the N_2O models, with significant effects in all four treatments. The N_2O and CH_4 models were similar in that the deciduous forest had the greatest number of significant historical management factors of all treatments, while the never tilled treatment had the most significant factors in the CO_2 model.

Table 2.2: Multiple Regression Analysis of Factors Influencing CO_2 Flux

		Estimated Coefficients [a,b]			
Factor Category	Factor	Conventional Agriculture [c]	Historical Agriculture	Never Tilled	Deciduous Forest
Current Year Management	Soybean crop	0.761 (0.19) *** [d]	-	-	-
	Wheat crop	1.55 (0.52) *** [d]	-	-	-
	Fertilization	-0.001 (0.00)	-	-	-
Historical Management	% Soil Moisture	0.024 (0.03)	0.061 (0.04)	0.091 (0.05) *	0.071 (0.02) ***
	Nitrate Concentration	-0.012 (0.02)	-0.245 (0.12) *	0.915 (0.35) ***	-0.049 (0.03)
	Ammonium Concentration	0.016 (0.03)	-0.145 (0.16)	-0.143 (0.05) ***	0.058 (0.06)
	% Carbon	0.316 (0.45)	2.69 (0.74) ***	0.380 (0.62)	-0.269 (0.17)
	C/N	-0.235 (0.13) *	0.036 (0.16)	-0.646 (0.15) ***	-0.111 (0.04) ***
Environmental	Precipitation (3 days)	-0.017 (0.01)	0.010 (0.02)	-0.093 (0.05) *	0.016 (0.01) *
	Precipitation (7 days)	0.013 (0.01)	0.039 (0.02) **	0.117 (0.05) ***	-0.001 (0.01)
	Maximum Air Temperature	0.097 (0.02) ***	0.124 (0.04) ***	0.125 (0.05) ***	0.036 (0.02) *
	Minimum Air Temperature	0.090 (0.03) ***	0.110 (0.04) ***	0.178 (0.07) ***	0.122 (0.02) ***
Other	Intercept	0.392 (1.3)	-3.97 (2.3) *	4.92 (2.3) **	1.34 (0.63) **
	Coeff. Of Determination (R^2)	0.38	0.29	0.32	0.50
	Prob F > 0	0.00	0.00	0.00	0.00
	Number of Observations	408	351	275	223

[a] Coefficients measure the change in CO_2 equivalents (kg CO_2 m^{-2} y^{-1}) for each one unit change in a factor. Standard errors for coefficients are given in parentheses.

[b] Significance levels for the CT data were evaluated with 12 degrees of freedom. HT, NT, and DF were evaluated with 9 degrees of freedom. A "*" indicates significance at $p \leq 0.10$, "**" significance at $p \leq 0.05$, and "***" significance at $p \leq 0.01$.

[c] CT is cropped on an annual rotation between corn, soybeans, and wheat. The corn crop was used as the baseline for this analysis.

[d] The soybean and wheat crop coefficients were not significantly different ($p \geq 0.10$).

Table 2.3: Multiple Regression Analysis of Factors Influencing CH_4 Flux

		Estimated Coefficients [a,b]			
Factor Category	Factor	Conventional Agriculture [c]	Historical Agriculture	Never Tilled	Deciduous Forest
Current Year Management	Soybean crop	1.83 (0.71) ** [d]	-	-	-
	Wheat crop	3.57 (1.3) *** [d]	-	-	-
	Fertilization	0.011 (0.01)	-	-	-
Historical Management	% Soil Moisture	0.142 (0.08) *	0.016 (0.08)	0.510 (0.16) ***	0.534 (0.16) ***
	Nitrate Concentration	0.019 (0.05)	-0.779 (0.30) **	1.90 (1.4)	0.782 (0.35) **
	Ammonium Concentration	0.011 (0.05)	0.240 (0.33)	0.011 (0.25)	-1.15 (0.55) **
	% Carbon	-4.55 (1.6) ***	7.00 (1.9) ***	1.07 (3.5)	-0.035 (1.5)
	C/N	-0.452 (0.30)	0.357 (0.25)	0.210 (0.44)	0.684 (0.30) **
Environmental	Precipitation (3 days)	0.011 (0.03)	-0.057 (0.05)	0.049 (0.08)	0.215 (0.05) ***
	Precipitation (7 days)	0.030 (0.03) *	0.060 (0.03) **	0.052 (0.08)	0.128 (0.03) ***
	Maximum Air Temperature	-0.079 (0.08)	0.093 (0.08)	-0.229 (0.22)	0.123 (0.16)
	Minimum Air Temperature	0.003 (0.08)	-0.174 (0.08) **	-0.035 (0.25)	-0.567 (0.16) ***
Other	Intercept	1.19 (3.3)	-18.5 (4.4) ***	-28.3 (9.8) ***	-40.3 (5.5) ***
	Coeff. Of Determination (R^2)	0.10	0.09	0.10	0.33
	Prob F > 0	0.00	0.00	0.00	0.00
	Number of Observations	408	351	275	223

[a] Coefficients measure the change in CO_2 equivalents (g CO_2 m^{-2} y^{-1}) for each one unit change in a factor. Standard errors for coefficients are given in parentheses.

[b] Significance levels for the CT data were evaluated with 12 degrees of freedom. HT, NT, and DF were evaluated with 9 degrees of freedom. A "*" indicates significance at $p \leq 0.10$, "**" significance at $p \leq 0.05$, and "***" significance at $p \leq 0.01$.

[c] CT is cropped on an annual rotation between corn, soybeans, and wheat. The corn crop was used as the baseline for this analysis.

[d] The soybean and wheat crop coefficients were not significantly different ($p \geq 0.10$).

Table 2.4: Multiple Regression Analysis of Factors Influencing N_2O Flux

		Estimated Coefficients [a,b]			
Factor Category	Factor	Conventional Agriculture [c]	Historical Agriculture	Never Tilled	Deciduous Forest
Current Year Management	Soybean crop	1.08 (9.5) [d]	-	-	-
	Wheat crop	58.9 (24) ** [d]	-	-	-
	Fertilization	0.014 (0.16)	-	-	-
Historical Management	% Soil Moisture	2.47 (0.96) ***	0.675 (0.16) ***	0.658 (0.32) **	1.35 (0.32) ***
	Nitrate Concentration	1.03 (0.48) *	-0.819 (0.80)	5.17 (3.7)	1.77 (0.80) **
	Ammonium Concentration	-0.707 (0.80)	1.16 (0.96)	0.498 (0.48)	2.02 (1.4)
	% Carbon	-38.2 (30)	-6.73 (5.1)	-1.32 (2.9)	-0.530 (3.5)
	C/N	0.418 (4.8)	-0.321 (0.64)	0.514 (0.64)	1.86 (0.80) **
Environmental	Precipitation (3 days)	1.32 (0.64) **	-0.064 (0.16)	-0.658 (0.32) **	0.321 (0.16) **
	Precipitation (7 days)	0.498 (0.32) *	0.048 (0.08)	0.562 (0.32) **	-0.064 (0.16)
	Maximum Air Temperature	2.47 (0.80) ***	0.128 (0.16)	0.032 (0.32)	0.498 (0.32)
	Minimum Air Temperature	-2.33 (1.1) *	0.883 (0.32) ***	0.771 (0.48) *	0.402 (0.32)
Other	Intercept	-31.5 (49)	0.006 (11.4)	-17.2 (12)	-51.7 (14) ***
	Coeff. Of Determination (R^2)	0.13	0.08	0.09	0.19
	Prob F > 0	0.00	0.00	0.09	0.00
	Number of Observations	408	351	275	223

[a] Coefficients measure the change in CO_2 equivalents (g CO_2 m^{-2} y^{-1}) for each one unit change in a factor. Standard errors for coefficients are given in parentheses.

[b] Significance levels for the CT data were evaluated with 12 degrees of freedom. HT, NT, and DF were evaluated with 9 degrees of freedom. A "*" indicates significance at $p \leq 0.10$, "**" significance at $p \leq 0.05$, and "***" significance at $p \leq 0.01$.

[c] CT is cropped on an annual rotation between corn, soybeans, and wheat. The corn crop was used as the baseline for this analysis.

[d] The soybean and wheat crop coefficients were significantly different ($p \leq 0.05$).

Environmental Factors

There were four environmental factors tested and in the CO_2 model, maximum and minimum temperatures were significant in all soil treatments. CO_2 flux increased as either air temperature measurement increased. Each treatment had a minimum of two factors with a significant effect on CO_2, and all four factors were significant in the never tilled treatment. No single environmental factor was significant in the N_2O or CH_4 models for all soil treatments. However, in the N_2O model, cumulative precipitation after three days and minimum temperature were significant in three out of four treatments making them the factors with the most consistent impact. In the CH_4 model, this was true of cumulative precipitation after seven days. The conventional agriculture site had the most significant factors of the N_2O models, while the deciduous forest had the most in the CH_4 models.

Recovery from Agriculture

The historical agriculture treatment was compared to the other successional treatments (NT and DF) and the conventional agriculture treatment to determine if factors affecting gas flux from plots abandoned from agriculture were more similar to those affecting gas fluxes in mid- to late-successional or conventional agricultural treatments. In the case of CH_4 and CO_2 flux, the historical agriculture site had more variables in common with the successional treatments than with the conventional agricultural treatment. This may indicate that the microbial communities in the sites abandoned from agriculture are more similar to those in the successional treatments than those in sites currently used for agriculture. In the N_2O models, the historical agriculture treatment had

only two variables that were significant, and these were also significant in the successional and agricultural treatments.

Overall Modeling Results

Of the three gas fluxes, CO_2 consistently had the highest coefficient of determination for each of the four treatments (R^2 = 0.29 to 0.50). In addition, when looking at all factors, all except fertilization had a significant effect in at least one soil treatment, resulting in CO_2 being the gas flux with the largest number of significant variables. The N_2O and CH_4 models each could only explain a small proportion of the variation in the data (R^2 = 0.08 to 0.19 and R^2 = 0.09 to 0.33, respectively). The deciduous forest had the highest R^2 value for each of the three gases when compared to the other treatments (R^2 = 0.19 to 0.50). The intercept was commonly a significant variable in the historical agriculture, never tilled and deciduous forest treatments. This indicates that there are unmeasured parameters in these treatments that are responsible for explaining some of the gas flux from these sites.

The impact of measuring moisture by percent soil moisture or WFPS was also investigated (data not shown). In most cases, percent soil moisture was significantly different from zero, whereas WFPS was not, indicating percent soil moisture is a better variable to depict soil moisture in our models. Use of percent soil moisture instead of WFPS was found to sharpen and clarify the relationship found between soil moisture and gas fluxes, therefore all subsequent analyses included only percent soil moisture.

DISCUSSION:

The extensive worldwide acreage converted to agricultural uses has contributed to an increased concentration of greenhouse gases in Earth's atmosphere. The annual net release of CO_2 from agriculture is estimated to contribute ca. 14% of current fossil fuel emissions [1]. Agricultural practices also increase the flux of N_2O relative to non-agricultural soils [4, 5], and decrease the capacity of soil to serve as a methane sink. Conversion of tropical, subtropical and temperate soils to agricultural use is estimated to have decreased the methane sink by 3 to 9% worldwide [14, 15]. To identify trends in the variability of greenhouse gas fluxes from soils in agroecosystems associated with different crops and under different management regimes at the KBS LTER we used a linear production function approach.

The coefficients of determination for these analyses were generally lower than those obtained in previously published studies (Table 2.5), which is due to experimental design and length of the studies. Many of the higher coefficients of determination (R^2) were obtained from experiments in which a single independent variable was altered, and so the likelihood of explaining that data is greater. Also contributing to their increased predictive power is the fact that the majority of the studies took place over a shorter time period, eliminating annual variations from the model. In a meta-analysis of studies on N_2O flux published between 1980 and 1997, Sozanska and colleagues [16] found that only 3% of all studies were longer than one year and had measurements taken on at least a weekly basis. While process modeling is the usual approach to predict gas fluxes, empirical models such as ours are useful for identifying variables important in describing flux and calculating how well those variables account for the flux observed.

Table 2.5: Studies Using Multiple Regression Models to Determine Factors Affecting Greenhouse Gas Fluxes

Gas Measured	Soil Type/Location	Coefficient of Determination (%)	Duration of Study	Variables Experimentally Manipulated	Reference
N_2O	Boreal clay and loamy sand soils/Finland	80	3 years	None	[17]
	Agricultural & non-agricultural soils/England	40	Meta-analysis of data published from 1980 - 1997	Fertilization, manure application, freeze-thaw, plowing	[16]
CH_4	Loamy forest soil/ MA, USA	61	6 months	Temperature	[18]
	Temperate deciduous forest/England	78 [a]	13 months	None	[19]
	Spruce forest/Germany	79 [a]	27 weeks (measurements taken over 3 years)	None	[20]
CO_2	Loamy forest soil/ MA, USA	94	6 months	Temperature	[18]
	Spruce-fir forest/ME, USA	74-76	21 months (measurements taken over 3 years)	Temperature	[21]
	Sandy grassland/Minnesota, USA	55	1 year (100 min after vegetation was clipped)	Plant diversity, ambient CO_2, fertilization level, and nitrogen supply	[22]

[a] For control plot only.

In our analysis, there was consistently a greater ability to explain the variability in CO_2 flux than that of CH_4 or N_2O, and the same factors that explained variability in CO_2 flux were consistently less effective in accounting for variability in N_2O or CH_4 flux. Carbon dioxide flux had a greater proportion of environmental factors that were significant than the N_2O or CH_4 models. The differences in R^2 values are interpreted to be due to differences in the microbial metabolisms involved in the production of each gas. Carbon dioxide is produced by most microbes in soil: it is a product of fermentation as well as both aerobic and anaerobic respiration. However, CH_4 consumption and N_2O production are the result of specialized forms of microbial metabolism that are favored under specific conditions, and the microbes that carry out these reactions are less abundant in soil. Methane consumption is carried out under aerobic conditions by methanotrophic bacteria, while N_2O production is favored under anaerobic conditions and is likely produced primarily by denitrifying bacteria. The organisms carrying out these specialized metabolisms make up a very small proportion of the bacterial soil population. Estimates of nitrifier abundance place them at a maximum of 0.01% of the total bacterial population in soils from the KBS LTER [23]. Soil denitrifier and methanotroph abundance has been shown to be approximately 0.01% [24, 25] and 0.3% [26] respectively, of the total bacterial population. Therefore, changes in variables that have a general effect on cellular metabolism would be expected to have a greater effect on rates of CO_2 production.

In the agricultural treatment, there were consistent differences in the effect of each crop on gas flux. Wheat was associated with the highest average flux of N_2O and CO_2 and the lowest ability to oxidize CH_4. The three crops grown at KBS vary in their

amount and type of root exudate [27], plant carbon content and timing of carbon availability [28], as well as the timing of fertilization. These differences affect the quantity and quality of nutrients microbes require; therefore it is possible that the annual rotation of crops at KBS results in annual changes in microbial communities that vary in their metabolic capabilities, resulting in differences in gas fluxes. Recent studies have shown that different species of plants will influence the composition of microbial communities, thereby changing the metabolic capabilities of the community [29-33]. This result suggests that a possible mitigation option for greenhouse gases would be through the growth of only certain crops.

Another notable observation in the agricultural treatment was the lack of a significant effect of the timing of fertilization on fluxes of N_2O and CH_4. Nitrogenous fertilizer would be expected to cause an increase in N_2O flux due to an increase in nitrogen available to those organisms producing it, and a decrease in CH_4 consumption due to the inhibitory effects of ammonium on CH_4 oxidation. Despite the lack of a significant effect from the timing of fertilization, nitrate concentration was found to have an effect on N_2O flux, which has been found in other studies. Syvalso and colleagues [17] found no correlation between N_2O flux and the amount of fertilizer applied, but did identify a correlation with the total amount of nitrogen in soil. In addition, a previous study at the KBS LTER concluded that it is nitrogen availability and not fertilizer specifically that leads to increased N_2O flux [5]. Nitrogen from fertilizer will not become available to N_2O-producing organisms until the proper conditions develop. This results in a disconnect between the timing of N_2O production and fertilizer application. Future modeling efforts, especially for N_2O, may be better served by including measures on

nitrogen availability at the time gas measurements are taken in addition to amounts of fertilizer applied.

The insignificant effect of fertilization on CH_4 flux is likely indicative of a long-term response of the microbial community to fertilization. After years of fertilization, CH_4 flux does not respond to subsequent N input, and the lack of response is likely due to the selection of a methanotroph community that is unaffected by fertilization and other agricultural perturbations [34]. The possibility of such a community shift has rarely been addressed, but a study characterizing the methanotroph communities in paired agricultural, successional and forest soils found that agricultural soil only had a subset of the methanotroph groups in the forest soil [35]. Other characterizations of agricultural soil without paired sites have also found that they lack sometime dominant forest methanotrophs [36]. Therefore, a methanotroph community shift driven by long-term agricultural perturbations is likely to have occurred at KBS.

Our data suggests that this methanotroph community that is unaffected by agricultural perturbations is also largely unresponsive to changes in the environmental variables we have modeled, and can be thought of as being perturbation resistant. The lack of significant variables and low CH_4 R^2 values observed in the conventional agriculture, historical agriculture and never tilled sites imply those treatments are likely dominated by these seemingly perturbation resistant methanotrophs. The comparatively high R^2 value and multiple significant variables found for the deciduous forest indicates that perturbation sensitive methanotrophs are likely present at significant levels. We hypothesize that it is these perturbation sensitive methanotrophs that lead to the model's greater explanatory power in the deciduous forest.

It is not obvious if similar arguments apply to the flux of N_2O and CO_2, but studies suggest that the transition from native lands to agriculture causes a shift in the CO_2 producing microbial community [37, 38]. The authors of these studies hypothesize that changes in labile organic matter that accompany the land-use transition is the driving force behind the differences in the CO_2 producing microbial community. In addition, Stevenson and colleagues [39] found distinct functional patterns in the CO_2 producing microbial community on a landscape scale between pasture and forest soil. Presumably the differences were caused by variation in litter input quality and management practices. Such explanations would be supported by our data since a CO_2 producing microbial community with a comparatively high R^2 value is found in the forest, perhaps due to the presence of more labile organic matter, land management practices, and different litter input.

While the above explanations of variability in gas flux are consistent with expectations based on microbial physiology and ecology, it is of note that there are other differences between the treatments that may also influence the flux of greenhouse gases. As discussed previously, the above-ground plant community differs among treatments and is likely to impact the microbial community. In addition, primary productivity and pH also change between treatments. None of these factors were accounted for in our model, but like labile organic matter and the CO_2 producing microbial community, may impact the composition and activity of the microbial community and the associated gas fluxes.

The linear production approach implemented in this study provided the important insight that, especially for N_2O and CH_4, the microbial community needs to be assessed

in order to build an accurate process model. Process models typically assume that environmental and land management factors are the primary determinants of soil gas flux. Process models tend to treat microbial communities in soil as a "black box," with associated inputs and outputs that are influenced by a collection of environmental parameters. Measures of the composition or diversity of microbial communities are generally not included in models, both because of the difficulty in collecting this data and the general expectation that measurements of the precise composition of the community are not necessary due to metabolic redundancy. Recent studies however, suggest that the diversity and composition of microbial communities may help to explain differences in ecosystem functions [40-44].

The findings presented in this study are relevant to both current year and historical management plans given the economic costs associated with loss of fertilizer and climate change brought about by increased concentrations of greenhouse gases. Climate change has been implicated in contributing to increased temperatures, altered precipitation patterns and the increased frequency of extreme weather events. These factors all serve to depress crop yields and increase production risks so that the gap between food production and consumption between developed and developing nations is predicted to increase [45]. The recent increase in weather-related catastrophes has also had effects on regional and national economies, with windstorms from 1983 to 1992 alone accounting for $88.1 billion in economic losses [46]. Given the impact of greenhouse gas emissions, it is important to begin to treat microbial communities, especially methanotrophs and denitrifiers, as important factors in modeling variations in gas flux.

CONCLUSION:

Land management practices and environmental factors influence the exchange of greenhouse gases between soils and the atmosphere by altering the metabolism of microbial communities in soil. It is concluded that since CO_2 is a byproduct of all types of heterotrophic respiration in soil, its flux will be responsive to factors that enhance microbial metabolism in general, and that this phenomenon explains the high coefficients of determination for explaining variation in CO_2 flux at the KBS LTER. Conversely, since the consumption of CH_4 and production of N_2O require the activity of microbes with specialized metabolic pathways, environmental parameters alone explained a smaller percentage of the flux of these gases. Advances in the modeling of CH_4 consumption and N_2O production will likely require estimates of the diversity and activity of the microbial populations that oxidize CH_4 and emit N_2O.

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CHAPTER 3

COMPOSITION, DIVERSITY, AND N_2O FLUX OF DENITRIFIERS IN AN AGROECOSYSTEM

ABSTRACT:

Denitrifying microorganisms in soil act as a source and sink of nitrous oxide and contribute to losses of nitrogen from agricultural fields. The denitrifier community composition and diversity of three soil treatments varying in level of agricultural management and N_2O flux were compared. Comparisons of proteobacterial *nir*K sequence libraries indicated that community composition of successional sites abandoned from agriculture 15 years ago still showed impacts from agriculture, and that the community composition of the 0-7 cm portion of this site had diverged from that found at 13-20 cm. Productivity (%C) did not have a significant relationship with measures of denitrifier diversity, but an analysis using Spearman's Correlation Coefficient indicated a weak, positive relationship between relative disturbance and diversity ($P = 0.1$ to 0.2). In addition, a significant, positive relationship between ribosomal RNA (rrn) operon copy number and growth rate of dissimilatory nitrate reducers was found, indicating that denitrifiers with low and high rrn operon copy numbers differ in their ecological strategies. There was a significant effect of agricultural treatment on evenness ($P = 0.0476$), with the conventional agriculture treatment at 0-7 cm having the highest evenness. This, along with rank/abundance curves, indicates that there are *nir*K OTU's in the never tilled and historically tilled treatments that may play an important role in minimizing the amount of N_2O emitted from sites currently not used for agriculture.

INTRODUCTION:

Denitrification is a dissimilatory process carried out by a phylogenetically diverse group of bacteria and fungi that are predominantly heterotrophic and facultatively anaerobic [1]. During denitrification, nitrate is reduced in a step-wise manner to nitrite, nitric oxide, nitrous oxide, and finally dinitrogen gas [1, 2]. Typically, denitrification results in the release of inorganic nitrogen as either N_2O or N_2, and the N_2O mole ratio and N_2O flux produced by soil denitrifiers varies based on physical properties such as soil pH, oxygen status, available carbon, or soil moisture history [3-5]. Knowledge of the factors influencing N_2O mole ratio and flux is important, as N_2O is a greenhouse gas that contributes to global warming and destroys ozone. Natural sources of N_2O contribute approximately 10 Tg $N \cdot yr^{-1}$ to the global budget, and of this, soil contributes 65% [6]. In addition, in agroecosystems loss of nitrogenous fertilizer as gaseous products decrease farm profit margins as greater amounts of fertilizer are used to balance the loss of inorganic nitrogen from the field [7].

While the physical factors affecting N_2O flux have received much attention, there is also evidence that differences in denitrifier communities can influence N_2O flux [8-12]. Traditionally, microbial diversity especially within groups carrying out a specific biogeochemical process, was assumed to be functionally redundant. This has led to the creation of many process models that predict N_2O flux, but do not account for functional differences in community composition. For this reason, many models estimating N_2O flux from agricultural soils remain limited [13]. For example, IPCC guidelines [14] consider all agricultural systems to be equivalent, which does not take into account

environmental factors affecting the microbial communities that produce and remove N_2O from the soil environment.

Determining factors that influence or maintain diversity have been long-time goals of ecological studies. Two factors found to be important in both macro- and microbiological communities are disturbance and productivity [15-18]. Most studies on disturbance find that communities follow the predictions of the Intermediate Disturbance Hypothesis (IDH), which states that species diversity will be highest in environments having an intermediate level of disturbance [19]. The proposed differences in diversity are due to a balance between reduced richness from competitive exclusion at low disturbance levels and from increased mortality at high disturbance levels [19, 20]. Productivity of an environment also has effects on diversity; however this relationship is less predictable than that found with disturbance. It can vary with the directness of the productivity measure, trophic level of the organism(s) being studied, or the scale of the experiment [21, 22].

In addition to understanding factors that influence community composition and diversity, it is important to understand the underlying ecological strategies employed by organisms. It has been demonstrated that the ribosomal RNA (rrn) operon copy number of aerobic, heterotrophic bacteria reflects their ecological strategy [23]. In experiments, high copy number organisms were able to respond quickly to new resources while low copy number organisms grew more slowly. This is thought to be the result of a trade-off occurring between benefits incurred from being able to produce ribosomes quickly when resources are available versus the high energetic cost of maintaining many ribosomes in a low nutrient environment. Agricultural treatments at the KBS LTER vary in the

availability of nutrients and frequency of nutrient inputs, therefore different ecological strategies may be favored in different treatments. To determine specifically whether the rrn operon copy number of organisms might reflect an ecological strategy, the relationship between growth rate, phylogeny and rrn operon copy number was explored. Dissimilatory nitrate reducers capable of either denitrification or reduction of nitrate to ammonia were included in this portion of the study in order to determine how widespread the potential trade-off was amongst organisms that carrying out this type of metabolism.

Many studies assessing microbial diversity or community composition rely on the analysis of 16S rDNA genes. Denitrifying microorganisms belong to a phylogenetically diverse group; therefore it is not possible to design 16S rDNA primers that would capture sequences only from organisms with denitrifying capability. Instead, a functional gene in the denitrification pathway was used to assess the denitrifier community. Nitrite reductase (Nir) catalyzes the key step in denitrification since it produces the first gaseous product in the pathway and is responsible for the first step in the pathway that distinguishes between nitrate respirers and denitrifiers [1]. There are two types of nitrite reductase genes that encode for enzymes that are structurally different, but functionally the same. *NirK* encodes for a copper containing nitrite reductase and *nir*S encodes for a cytochrome cd_1 containing enzyme. Known denitrifiers possess either one of these genes, but not both [24, 25]. The work presented here indicated that *nir*S was either not present or in low abundance in the soil treatments studied, therefore diversity was assessed using *nir*K nucleotide sequences.

One of the primary goals of this study was to determine whether proteobacterial denitrifier community composition differed between sites that differ in annual average

N_2O flux and history of agriculture. Sampling was performed at the Kellogg Biological Station Long-Term Ecological Research (KBS LTER) site where average N_2O flux from a conventional agricultural treatment (3.22 ± 0.45 g N_2O-N·ha^{-1}·d^{-1}) is approximately three times higher than from either a historically tilled treatment (0.92 ± 0.08 g N_2O-N·ha^{-1}·d^{-1}) or a mid-successional, never-tilled treatment (1.13 ± 0.11 g N_2O-N·ha^{-1}·d^{-1}) [26]. Measurements of greenhouse gases taken at the KBS LTER indicate that N_2O production from the annually cropped treatments is the major contributor to global warming potential (GWP) [27]. Other primary goals were to determine whether there were differences in the diversity of denitrifier communities, to identify factors contributing to differences in diversity and composition, and finally, to determine whether differences were associated with changes in N_2O flux. Secondary goals included an exploration of dissimilatory nitrate reducer ecological strategy, comparison of replicate clone libraries, and an assessment of the impact of agriculture on denitrifier communities.

MATERIALS & METHODS:

Study Site & Soil Collection

Soil samples were collected from the Kellogg Biological Station Long-Term Ecological Research Site (KBS LTER) located in Hickory Corners, MI. The dominant soil series at the station are the Kalamazoo and Oshtemo series, which are fine-loamy and coarse-loamy mesic Typic Hapludalfs, respectively. These soils have accumulated clay and have a medium to high supply of plant nutrients and water with moist surface layers that are slightly leached [28]. The three treatments focused on in this study were: an

agriculture site that receives amounts of fertilizer, herbicide, and conventional tillage (CT) that are typical for the Midwest region and is on an annual corn (*Zea mays* L.) - soybean (*Glycine max* L.) - wheat (*Triticum aestivum* L.) rotation; a historically tilled site (HT) abandoned from agriculture in 1989; and a never-tilled site (NT) maintained with a mid-successional plant community by annual mowing. A more detailed description of treatments at the KBS LTER can be accessed on the World Wide Web at http://lter.kbs.msu.edu/.

Initial studies on the presence of *nir*S and *nir*K in the CT and NT treatments were conducted with soil collected in December 2003. Cores of 2.5 cm diameter and 10 cm depth were collected from all five sampling sites within each treatment replicate. Soil from the same treatment was pooled, homogenized by sieving (2 mm mesh), and stored at 4°C until DNA was extracted using the method of Zhou and colleagues [29].

Soil was collected again in December 2004 for an investigation of denitrifier community composition and diversity. At the time of sampling, the CT treatment was fallow and only wheat stubble remained. Soil was collected at depths of 0-7 cm and 13-20 cm from two replicate plots of the CT, HT, and NT treatments. At each treatment plot, a total of five cores of a 2.5 cm diameter were taken, the soil was divided into 0-7 cm and 13-20 cm portions, and portions from the same depth and replicate treatment were pooled in a Whirl-Pak bag (Nasco, Modesto, CA). The soil was kept on ice until it was brought back to the laboratory and stored at 4°C overnight. The following day, the soil was sieved (2 mm mesh) to remove stones, plant biomass, and for homogenization. Portions of soil were frozen in liquid nitrogen and stored at -80°C for later DNA

extraction. The remaining soil was stored at 4°C for pH and percent gravimetric soil moisture determination.

Disturbance Experiment

In June 2005 microplots were constructed within the two previously sampled CT replicate plots. In each replicate plot two 3 x 3 m microplots were set up side-by-side in the Northeast corner. During the time of the experiment the main CT treatment had been planted to corn. Prior to manipulation, all corn was removed from the microplots. One microplot was a control in which no further disturbances were applied to the soil and the other microplot was disturbed at intervals of once per week for a total of three weeks. Weekly disturbances to the control microplot consisted of the following: rototilling the entire microplot to a depth of 7 cm to simulate tillage and addition of water and fertilizer to the center 1 m^2 of the microplot. Water addition simulated an average rainfall of 0.5 in per cm^2 and fertilizer application was equivalent to 40 gal per acre with a 28% nitrogen solution containing ammonium nitrate and urea. Each individual water and fertilizer addition was a typical amount for the region. Five soil cores 2.5 cm in diameter and 7 cm deep were taken from the center 1 m^2 of each plot before and after each round of disturbances, one week after the final disturbance, and five weeks after the final disturbance. The center 1 m^2 of the plots was sampled to avoid edge effects from the surrounding corn. Soil was sieved and processed in the same manner as the December 2004 samples.

Relative Disturbance Scale

In order to investigate the relationship between disturbance and proteobacterial denitrifier diversity, a relative disturbance scale was set up for the denitrifier

communities in the December 2004 soil samples taken at 0-7 cm and 13-20 cm depths. Disturbances were considered to be activities that could result in altered niche opportunities for denitrifiers [30]. The scale was based on documented treatment manipulations taking place at the KBS LTER including, tillage, herbicide/insecticide application, fertilization, harvest/mowing, burning, and liming. Precipitation was also considered a disturbance. Different treatments and depths were graded on a presence/absence scale for each category of disturbance (Table 3.1).

pH and Percent Gravimetric Soil Moisture

To characterize physical aspects of the soils that have been shown to affect denitrification, soil pH and percent gravimetric soil moisture were measured using standard methods for LTER sites [31]. Soil pH was measured after 15 g of sieved soil was mixed with 30 mL deionized water. The soil solution was allowed to equilibrate with the atmosphere for a minimum of 30 minutes followed by pH measurement with a pH meter (Orion Research Inc., Beverly, MA). Percent gravimetric soil moisture was measured after 15 g of field moist soil was weighed and dried at approximately 50°C for a minimum of 2 days. Soil moisture was calculated on a dry weight (dw) basis as 100 * ((field moist weight - dry weight)/dry weight). Three replicate measurements of both pH and percent gravimetric soil moisture were performed for each soil treatment and depth (Table 3.2).

Carbon and Nitrogen Measurements

Total percent carbon and nitrogen of soil were determined by dry combustion. Dried soil samples from December 2004 and August 2005 were ground using a mortar and pestle. Three replicate 15 - 20 mg samples of soil were weighed out on a

Table 3.1: Specific Disturbances and Presence (+) or Absence (-) at KBS LTER Sampling Sites

	Sampling Date	December 2004						August 2005	
Disturbance	Effect	CT [a] 0-7 cm	CT [a] 13-20 cm	HT [b] 0-7 cm	HT [b] 13-20 cm	NT [c] 0-7 cm	NT [c] 13-20 cm	CT Control [d] 0-7 cm	CT Ultra [d] 0-7 cm
Precipitation	Increase in anaerobic sites, C and N from cell lysis and osmoprotectants	+	+/-	+	-	+	-	+	++
History of Agriculture	Decrease in aggregate size, increase in C and N availability	+	+	+/-	+/-	-	-	+	+
Tillage and Planting	Same as history of agriculture	+	+	-	-	-	-	+	++
Plant Diversity	Source of C and N	-	-	+	-	+	-	-	-
Fertilization	Increase in inorganic P, N and K	+	+/-	-	-	-	-	+	++
Herbicide	Possible C source, incorporation of plant material	+	+/-	-	-	-	-	+	+
Liming	Increase in pH	+	+/-	-	-	-	-	+	+
Burning	Removal of plant biomass; change in plant exudates	-	-	+	-	-	-	-	-
Harvest or Mowing	Same as burning	+	+/-	-	-	+	-	+	+
	Relative Disturbance Score [e]	7	4.5	3.5	0.5	3	0	7	10
	Ordinal Ranking [f]	1	2	3	5	4	6	-	-
	Categorical Ranking	High	Inter.	Inter.	Low	Inter.	Low	High	High

[a] CT = Conventionally tilled agricultural plot

[b] HT = Historically tilled plot; abandoned from agriculture in 1989

[c] NT = Never-tilled plot

[d] These plots were part of an experiment in T1 in which increased amounts of fertilization, tillage and water amendment were performed.

[e] The relative disturbance score was determined by adding up the total number of disturbances (++ = 2, + = 1, and +/- = 0.5).

[f] Used in Spearman's Rank Correlation Coefficient calculations.

Table 3.2: pH, Percent Soil Moisture, and N_2O Flux of Sampling Sites

Date Sampled	Sampling Site	pH [a]	% Soil Moisture [a]	N_2O Flux [b] (N_2O-N g ha^{-1} d^{-1})
December 2004	CT 0-7 cm	6.54 (0.07) ab	19.06 (0.52) a	3.22 (0.45) a
	CT 13-20 cm	6.78 (0.05) a	18.66 (0.22) a	
	HT 0-7 cm	6.60 (0.08) a	26.56 (1.04) b	0.92 (0.08) b
	HT 13-20 cm	6.59 (0.06) ab	19.56 (1.05) a	
	NT 0-7 cm	6.07 (0.09) bc	37.07 (0.89) c	1.13 (0.11) b
	NT 13-20 cm	5.96 (0.19) c	20.54 (0.30) a	
August 2005	CT Control 0-7 cm	5.80 (0.10) A	8.16 (0.58) A	No measurements
	CT Ultra 0-7 cm	4.81 (0.02) B	9.15 (0.31) A	

[a] December 2004 data is reported as the average of 4 measurements; one measurement from each of 4 treatment replicates. August 2005 data is reported as the average of 6 measurements; two measurements at each of 3 treatment replicates. Data followed by the same letter are not significantly different using Scheffé's test. Data collected in December 2004 and August 2005 were analyzed separately. Standard errors are in parentheses.

[b] Average and standard error from measurements taken from 1991-1999. Measurements represent the flux of the whole soil environment and are not separated into depths. Data followed by the same letter are not significantly different at the α=0.05 level using analysis of variance [26].

microbalance (Sartorius, Edgewood, NY) and packaged in tin capsules (Costech Analytical Technologies, Valencia, CA). Combustion with a Costech Elemental Combustion System was performed to calculate percent carbon, percent nitrogen, and carbon/nitrogen ratio (Table 3.3). The standard for carbon and nitrogen content was Phenacetin (Costech) and a combustion standard of Cyclohexanone-2,4 dinitrophenyl-hydrazone (Costech) was also used.

Available carbon was measured from 1989 to 1996 as the short-term respiration potential for soil collected from 0-25 cm. This was a routine measurement taken at the KBS LTER, and as such the protocol and raw data are available on the KBS LTER website. The average respiration potentials were: 115 ± 5 μg C · g soil^{-1}, 158 ± 5 μg C · g soil^{-1}, and 261 ± 14 μg C · g soil^{-1} for the CT, HT, and NT treatments respectively.

_nir_S Detection Limit

In order to obtain a general calculation of how much template DNA with *nir*S would have to be present for detection with the PCR, various reactions were set up, each containing a total of 50 ng DNA. Genomic DNA from *Pseudomonas stutzeri* JM300, which has one copy of the *nir*S gene and a genome size of 4.03 Mb (ca. 2.3×10^5 copies *nir*S/ng DNA) [32], was added to DNA extracted from the CT or NT treatments. The primers used (nirS1F and nirS6R) and PCR protocol followed were from Braker and colleagues [25].

_nir_K Library Construction

Libraries were made from two replicate agricultural treatments and soil depths. In one case, two libraries were made from the same treatment replicate and depth (HT 0-7 cm, Rep. 2). For each library, DNA was extracted from 0.25 to 1.0 g soil using an

Table 3.3: Percent Carbon, Percent Nitrogen and C:N of Sampling Sites

Date Sampled	Sampling Site	%C [a]	%N [a]	C:N [a]
December 2004	CT 0-7 cm (Rep. 1)	0.65 (0.02) a	0.07 (0.00) a	9.29 (0.22) a
	CT 0-7 cm (Rep. 2)	0.74 (0.02) a	0.06 (0.00) a	12.12 (0.47) cd
	CT 13-20 cm (Rep. 1)	0.62 (0.03) a	0.06 (0.01) a	9.95 (0.41) ab
	CT 13-20 cm (Rep. 2)	0.68 (0.03) a	0.06 (0.00) a	12.36 (0.10) cd
	HT 0-7 cm (Rep. 1)	1.62 (0.06) b	0.14 (0.01) b	11.78 (0.10) bcd
	HT 0-7 cm (Rep. 2)	1.73 (0.01) b	0.14 (0.00) b	12.49 (0.32) cd
	HT 13-20 cm (Rep. 1)	0.47 (0.01) a	0.05 (0.00) a	9.18 (0.33) a
	HT 13-20 cm (Rep. 2)	0.76 (0.04) a	0.06 (0.00) a	13.17 (0.15) c
	NT 0-7 cm (Rep. 1)	3.01 (0.23) c	0.25 (0.02) c	12.14 (0.08) cd
	NT 0-7 cm (Rep. 2)	3.65 (0.28) c	0.27 (0.02) c	13.38 (0.09) c
	NT 13-20 cm (Rep. 1)	0.50 (0.00) a	0.05 (0.00) a	11.01 (0.43) abd
	NT 13-20 cm (Rep. 2)	0.78 (0.03) a	0.06 (0.00) a	13.77 (0.48) c
August 2005	CT Control 0-7 cm (Rep. 1)	0.73 (0.05) A	0.05 (0.00) A	14.20 (0.94) A
	CT Control 0-7 cm (Rep. 2)	1.14 (0.05) B	0.10 (0.00) B	11.89 (0.39) AB
	CT Ultra 0-7 cm (Rep. 1)	0.84 (0.11) AB	0.08 (0.01) AB	10.94 (0.26) BC
	CT Ultra 0-7 cm (Rep. 2)	1.14 (0.08) B	0.13 (0.01) C	8.86 (0.15) C

[a] Reported as the average of 3 measurements, standard error in parentheses. Data followed by the same letter are not significantly different using Scheffé's test. Data collected in December 2004 and August 2005 were analyzed separately.

UltraClean Soil DNA Kit (MoBio, Carlsbad, CA). The primers F1aCu and R3Cu [33] were used to amplify *nir*K genes. Amplification of DNA from soil was performed with 1 U *Taq* polymerase (Invitrogen, Carlsbad, CA), a 1X concentration of the manufacturer's PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture (Invitrogen), 0.01% Triton X-100, 0.02% BSA, 50 pmoles of each primer, and 10 to 50 ng of template DNA. PCR mixtures were incubated in a PTC-200 DNA Engine gradient thermocycler (MJ Research, South San Francisco, CA) with the following touchdown PCR protocol: (i) 3 min initial denaturation at 94.0°C; (ii) 10 cycles, with each cycle lasting 30 s at 94.0°C, 40s starting at 60.0°C and decreasing by 0.5°C each cycle to end at 55.5°C, and 40 s at 72.0°C; (iii) 15 cycles, with each lasting 30 s at 94.0°C, 40 s at 57.0°C, and 40 s at 72.0°C; and (iv) 7 min at 72.0°C.

A low number of PCR cycles were used during library construction to reduce PCR bias from PCR drift. Twenty-five cycles were used as this was the minimum number of cycles needed to produce enough PCR product to be visible when run on an agarose gel and stained with ethidium bromide. Each *nir*K library was created using six 50 µL PCR reactions which were pooled and concentrated using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The PCR product was electrophoresed through a 1.0% agarose gel and extracted from the gel with a QIAEX II kit (Qiagen) to eliminate non-specific PCR products that might compete in the cloning reaction. Purified product was cloned with a TOPO TA Cloning Kit for Sequencing with pCR 4 and One Shot® TOP10 competent cells (Invitrogen) according to the manufacturer's instructions. Transformants were plated on LB agar containing 50 µg/mL kanamycin and clones screened for the correct insert size using modified M13 primers F2 and R4* [34]. Prior

to sequencing, PCR product was cleaned up with ExoSAP-IT® (USB Corporation, Cleveland, OH) to remove unused primers and dNTP's. 1.3 μL of PCR product and 0.25 μL ExoSAP-IT® were incubated at 37°C for 30 min and the reaction inactivated at 80°C for 15 min. Product was sequenced using a capillary sequencer (Applied Biosystems, Foster City, CA) with dye-terminator fluorescent cycle sequencing technology at the Michigan State University Research Technology Support Facility (MSU RTSF).

Diversity Data

*Nir*K sequences of ca. 473 base pairs were aligned using the program ARB [35], the primer sequence masked, and a distance matrix and phylogenetic tree based on neighbor joining constructed that included all sequences. ∫-LIBSHUFF version 1.3 [36], and in some cases the program UniFrac [37], was used to compare pairs of replicate *nir*K libraries to determine whether libraries from replicate agricultural treatments and depths were significantly different. Library comparisons in ∫-LIBSHUFF are performed so that not only is Replicate Library X compared to Replicate Library Y, but Replicate Y is also compared to Replicate X. This can result in cases where in one comparison the libraries are significantly different, but in the other they are not. This indicates that one library is a subset of the other and subsequently the libraries were considered to be not significantly different.

DOTUR version 1.53 [38] was used to analyze the α-diversity of each library. Input into DOTUR consisted of distance matrices based on neighbor joining from each individual library which are used by the program to assign sequences to OTU's at all percent nucleotide similarities. DOTUR output was used to calculate Simpson's diversity indices (-lnD), Simpson's evenness measure ([1/D]/S, where S is equal to the number of

OTU's present in each library rarified to the number of clones in the smallest library), Good's coverage ([1-(n/N)]*100, where n equals the number of singletons and N equals the total number of sequences), and rarefaction curves at three nucleotide similarity cutoffs of 94%, 97%, and 99%. Output from DOTUR was also used to calculate rank/abundance curves in which OTU's from libraries were plotted from most to least abundant versus their relative abundance.

In addition, β-diversity was determined also using both DOTUR and ∫-LIBSHUFF. DOTUR was used to separate sequences from all libraries into OTU's at 94%, 97%, and 99% nucleotide similarities. A program was written (GeneMatrix) by personnel at the Ribosomal Database Project (RDP) to convert the DOTUR .list file into a matrix that could be used as input in the program EstimateS version 7.00 [39]. Jaccard dissimilarity matrices created with EstimateS data were converted to dendrograms using the program MEGA version 3.1 [40].

rrn Copy Number & Growth Rate

Dissimilatory nitrate reducing microorganisms with known ribosomal RNA (rrn) operon copy numbers were chosen to investigate whether there was a relationship between rrn operon copy number and growth rate. Nine organisms were used in the study, and of these, the growth rate of six (*Escherichia coli* K12, *Pseudomonas fluorescens* ATCC 33512, *Pseudomonas stutzeri* JM300, *Ralstonia metallidurans, Serratia marcescens,* and *Shewanella putrefaciens* ATCC 8071) were determined in the laboratory. The growth rates of the remaining three organisms (*Chromobacterium violaceum* CS-1, *Magnetospirillum magnetotacticum* MS-1, and *Rhodopseudomonas palustris* BK1) were obtained from the literature.

Strains were grown on Tryptic Soy Broth (TSB) with 5 mM KNO_3 that was prepared anaerobically (Helium headspace) for growth of organisms capable of denitrification or dissimilatory nitrate reduction to ammonia (DNRA). Growth rates were obtained by monitoring the OD_{600} of replicate cultures (n = 3 to 6) during incubation at 25°C and 180 rpm. Doubling times (d) were calculated during the exponential phase of growth and maximum growth rate (μ_{max}) calculated as ln(2)/d. The impact of phylogeny on the results was assessed using the computer program CONTINUOUS (http:// www.rubic.rdg.ac.uk/meade/Mark/).

Nucleotide Sequence Accession Numbers

Partial *nir*K gene sequences were deposited in the EMBL, GenBank, and DDBJ sequence databases under accession numbers DQ782971 - DQ783217, DQ783219 - DQ783225, DQ783227 - DQ783279, DQ783281 - DQ783813, DQ783815 - DQ783894, and DQ783896 - DQ784090.

RESULTS:

Percent Nucleotide Similarity Cutoffs

Nucleotide similarity cutoffs of 94%, 97%, and 99% were used to assess whether *nir*K sequences belonged to the same denitrifier species. A range of cutoffs was used to confirm that the trends found did not depend on a particular cutoff. 94% was chosen as the lowest cutoff due to the fact that in a recent survey of conserved genes from 70 fully sequenced genomes, an average nucleotide identity of ca. 94% corresponded to the traditional 70% DNA-DNA reassociation standard currently used to define microbial species [41]. Higher percentage cutoffs were used as some microbial species (e.g., *E.*

coli) have been shown to have ca. 99% nucleotide similarity between strains analyzed with multi-locus sequence tagging (MLST). Additionally, higher percent cutoffs may be needed since there is evidence for lateral gene transfer of *nir*K between species. In order to test whether lateral gene transfer would cause an underestimation of the number of species, 40 *nir*K sequences from 36 different species of known denitrifiers were analyzed with the computer program DOTUR which assigns sequences to OTU's [38] (data not shown). At 94%, 97%, and 99% nucleotide similarity cutoffs, the number of species was underestimated indicating that if lateral gene transfer were to occur amongst denitrifiers in soil, the number of denitrifier species predicted using *nir*K may be an underestimate of the true number of species.

Detection Limit of *nir*S

Using two different sets of *nir*S primers and protocols [25, 33], little to no detection of the correctly sized product was obtained in PCRs with environmental DNA from the CT and NT treatments. Reactions with CT DNA consistently resulted in a very low amount of product being produced, while *nir*S was never detected in reactions with NT DNA. To investigate, a series of PCRs were performed with DNA from *P. stutzeri* JM300, which possesses the *nir*S gene. Genomic DNA from *P. stutzeri* was added in various amounts to CT and NT treatment DNA. As a low amount of PCR product was found in CT reactions with no added *P. stutzeri* DNA, the lower limit of detection was determined to be 1 pg of *P. stutzeri* DNA as this amount was the lowest causing an increase in band intensity on an agarose gel. The lowest amount of *P. stutzeri* DNA resulting in a visible band when mixed with NT DNA was 4 pg (Fig. 3.1). Therefore the primers should be capable of amplifying *nir*S genes even if DNA known to contain the

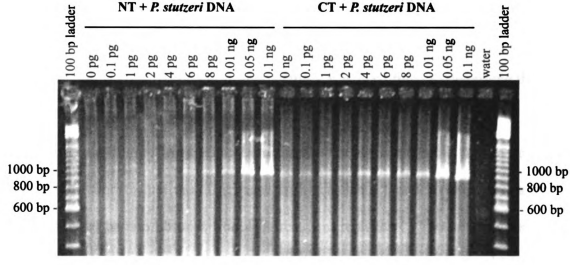


Figure 3.1: Agarose gel of PCRs of genomic DNA from *nir*S-bearing *Pseudomonas stutzeri* JM300 mixed with environmental DNA from either the NT or CT treatments. Picogram and nanogram amounts of DNA listed above lanes refer to the amount of *P. stutzeri* genomic DNA present in a PCR containing a total of 50 ng DNA.

proper template made up only 0.002% to 0.008% of the total DNA in the reaction. Due to the fact that little to no *nir*S product was obtained from environmental DNA, the denitrifier population possessing *nir*K was focused on.

Analysis of *nir*K Replicate Libraries

∫-LIBSHUFF was used to determine whether pairs of replicate *nir*K libraries were significantly different from each other. Initially, eight pairs of libraries were compared and in six cases the libraries were not significantly different, while in two they were (Table 3.4). Libraries from treatment replicates 1 and 2 were significantly different from HT 0-7 cm and NT 13-20 cm and further analysis was performed to determine the cause of the differences.

A second library from HT 0-7 cm replicate 2 was constructed from a new DNA extraction to confirm that the differences in the replicate 1 and 2 libraries was not the result of bias. The two libraries from HT treatment replicate 2 (library replicates 2a and 2b) were not significantly different from each other when subjected to ∫-LIBSHUFF analysis. In addition, a *P* test was performed using the program UniFrac. Both the ∫-LIBSHUFF and *P* test analysis results were in agreement, indicating that HT treatment replicate 1 and 2 communities do differ at a depth of 0-7 cm. It was noted that one OTU dominated in the replicate 2 libraries that was not present in the replicate 1 library. After this OTU was removed and the ∫-LIBSHUFF analysis repeated, the replicate 1 and 2 libraries were not significantly different (data not shown). Therefore, the differences in the HT 0-7 cm libraries of replicate 1 and 2 were due to the presence of one prominent OTU in the replicate 2 community.

Table 3.4: P-values from ∫-LIBSHUFF Analysis of Replicate *nir*K Libraries

	Library Pairs							
Comparison	CT 0-7 cm	CT 13-20 cm	HT [a] 0-7 cm	HT 13-20 cm	NT 0-7 cm	NT 13-20 cm	Disturbance Control 0-7 cm	Disturbance Experiment 0-7 cm
Rep 1 to 2	0.4865	0.6615	N/A	0.1601	0.0061 *	0.0000 *	0.2237	0.1465
Rep 2 to 1	0.0165 *	0.3418	N/A	0.4082	0.4105	0.0006 *	0.0400 *	0.5349
Rep 1 to 2a	N/A	N/A	0.0001 *	N/A	N/A	N/A	N/A	N/A
Rep 2a to 1	N/A	N/A	0.0084 *	N/A	N/A	N/A	N/A	N/A
Rep 1 to 2b	N/A	N/A	0.0010 *	N/A	N/A	N/A	N/A	N/A
Rep 2b to 1	N/A	N/A	0.0046 *	N/A	N/A	N/A	N/A	N/A
Rep 2a to 2b	N/A	N/A	0.4578	N/A	N/A	N/A	N/A	N/A
Rep 2b to 2a	N/A	N/A	0.6915	N/A	N/A	N/A	N/A	N/A
Result	Rep 1 is a subset of Rep 2	ND	Rep 1 is SD than Rep 2; Rep 2a & b are ND	ND	Rep 2 is a subset of Rep 1	SD	Rep 1 is a subset of Rep 2	ND

[a] One library from HT 0-7 cm was made from treatment replicate 1 and two libraries were made from treatment replicate 2. Comparisons of all three libraries results in six pairwise comparisons in ∫-LIBSHUFF.

* = Significantly different at $\alpha = 0.05$

ND = Not different; SD = Significantly different

N/A = Not applicable

The NT 13-20 cm replicate libraries were also analyzed with a *P* test that confirmed the significant difference between them. There were only a few OTU's that were unique to each library and none dominated the library. It was concluded that the libraries reflected a true difference in the communities of both replicates.

Denitrifier Community Analysis

Differences between communities, or β-diversity, were compared by using ∫-LIBSHUFF and cluster analysis with Jaccard dissimilarity matrices on *nir*K libraries from soil collected in December 2004 and August 2005. The ∫-LIBSHUFF analysis showed that there are similarities between the communities of the CT libraries at 0-7 cm and 13-20 cm libraries from December 2004 soil and the CT 0-7 cm libraries from August 2005 soil (Fig. 3.2). The 13-20 cm library from the HT treatment was similar to the CT 13-20 cm library, but not to the CT 0-7 cm library. Only one replicate library from HT 0-7 cm showed a similarity to other libraries.

Jaccard dissimilarity matrices were used to create dendrograms of the relationships between treatments based on OTU composition. Unlike ∫-LIBSHUFF, the Jaccard measure does not take the abundance of OTU's into account and is therefore purely a measure of the differences between community compositions. At all three nucleotide similarity cutoffs, libraries grouped into four clusters (Fig. 3.3). Cluster I consists of 0-7 cm libraries from the CT treatment from both the December and August timepoints. The 13-20 cm libraries from both the CT and HT treatments form Cluster II. The HT 0-7 cm libraries form Cluster III and the 0-7 cm and 13-20 cm libraries from the NT treatment comprise Cluster IV. These results corroborate those found in the

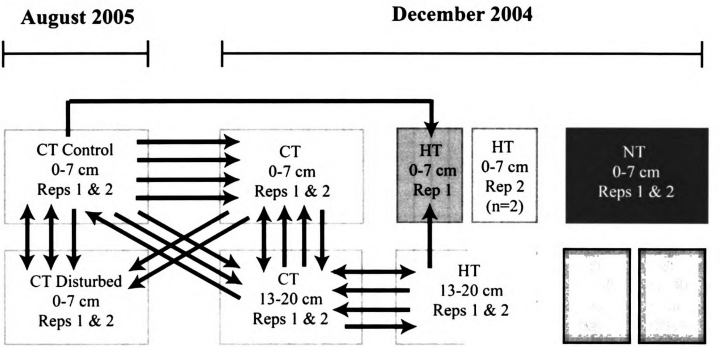


Figure 3.2: A visual representation of the similarities between libraries determined by ∫-LIBSHUFF analysis. Large blocks represent the two replicate libraries from each agricultural treatment and soil depth which were not significantly different. Small blocks represent the libraries from replicate treatments that were significantly different. Comparisons in ∫-LIBSHUFF between two libraries involve two library comparisons. Therefore, depending on the number of libraries represented by each block in the figure, comparisons between treatments and depths can involve a total of 4 or 8 ∫-LIBSHUFF comparisons. Two headed arrows between blocks indicate that two libraries were not significantly different and a one-headed arrow that a library from one treatment is a subset of a library in the treatment that is pointed to. No arrows between blocks indicate that libraries were significantly different from all others.

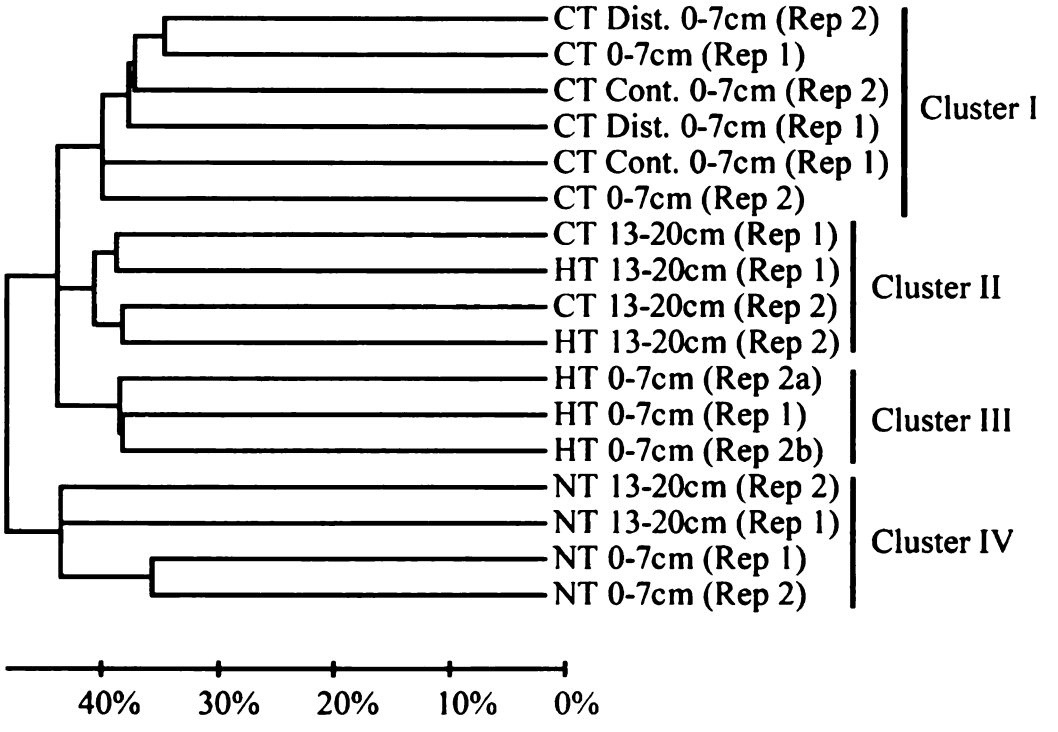


Figure 3.3: Dendrogram based on a Jaccard dissimilarity matrix at a 94% nucleotide similarity cutoff. Dendrograms from the 97% and 99% nucleotide similarity cutoff are not shown as the same clusters were formed. Scale represents percent dissimilarity.

∫-LIBSHUFF analysis and also show that the relationships seen between communities are due to differences in community composition and not just abundance of OTU's.

Diversity, Richness and Evenness

Because differences were seen in community composition, the diversity of the communities was compared. To assess whether differences existed, the overall diversity, richness, and evenness of the *nir*K libraries was calculated at three nucleotide similarity cutoffs (Tables 3.5, 3.6, and 3.7). Simpson's Diversity index was used as an overall diversity index as it incorporates the two aspects of diversity, richness and evenness. Richness and evenness were also calculated separately. Since the number of clones in each library differed, richness was calculated as the number of OTU's observed when the libraries were rarified to the size of the smallest library. Simpson's evenness was calculated from the Simpson's Diversity index and the rarified number of observed OTU's. Good's coverage of each library was compared in an effort to determine whether differences in sampling effort between libraries existed. Coverage varied from 58 to 86%, 53 to 82%, and 37 to 69% at the 94%, 97%, and 99% nucleotide similarity cutoffs, respectively. A 2-way ANOVA assessed whether any differences seen in diversity, richness or evenness were due to agricultural treatment, soil depth, or an interaction between the two. At all nucleotide similarity cutoffs there was no significant effect found for Simpson's Diversity index, rarified number of OTU's or Simpson's evenness due to either depth or a depth * agricultural treatment interaction ($P > 0.05$). However, at the 94% nucleotide similarity cutoff, there was a significant effect from agricultural treatment (Fig 3.4) on both the rarified number of OTU's ($P = 0.0447$) and Simpson's evenness ($P = 0.0476$). Pairwise t-tests indicated that it was the richness and evenness

Table 3.5: Diversity Statistics and Coverage of *nir*K Libraries at a 94% Nucleotide Similarity Cutoff

Sample Date	Treatment, Depth & Replicate	Number of Clones	Good's Coverage	Simpson's Diversity Index (-lnD)	Rarified Number of OTU's	Simpson's Evenness ($E_{1/D}$)
December 2004	CT 0-7 cm (Rep 1)	60	62	4.08	37	1.59
	CT 0-7 cm (Rep 2)	57	67	3.53	32	1.21
	CT 13-20 cm (Rep 1)	60	58	3.12	34	0.67
	CT 13-20 cm (Rep 2)	83	70	3.12	29	0.78
	HT 0-7 cm (Rep 1)	76	74	3.32	31	0.89
	HT 0-7 cm (Rep 2a)	59	78	2.06	22	0.36
	HT 0-7 cm (Rep 2b)	81	77	2.45	25	0.46
	HT 13-20 cm (Rep 1)	84	69	2.80	27	0.61
	HT 13-20 cm (Rep 2)	59	75	2.63	26	0.60
	NT 0-7 cm (Rep 1)	90	80	3.01	27	0.75
	NT 0-7 cm (Rep 2)	62	81	2.74	24	0.70
	NT 13-20 cm (Rep 1)	63	75	3.17	28	0.85
	NT 13-20 cm (Rep 2)	63	76	2.55	27	0.54
August 2005	CT Control 0-7 cm (Rep 1)	55	75	2.71	25	0.65
	CT Control 0-7 cm (Rep 2)	59	64	3.21	31	0.92
	CT Disturbed 0-7 cm (Rep 1)	58	78	2.72	24	0.69
	CT Disturbed 0-7 cm (Rep 2)	57	86	2.00	20	0.41

Table 3.6: Diversity Statistics and Coverage of *nir*K Libraries at a 97% Nucleotide Similarity Cutoff

Sample Date	Treatment, Depth & Replicate	Number of Clones	Good's Coverage	Simpson's Diversity Index (-lnD)	Rarified Number of OTU's	Simpson's Evenness ($E_{1/D}$)
December 2004	CT 0-7 cm (Rep 1)	60	55	4.30	40	1.84
	CT 0-7 cm (Rep 2)	57	63	3.55	33	1.20
	CT 13-20 cm (Rep 1)	60	53	3.74	37	1.14
	CT 13-20 cm (Rep 2)	83	67	3.41	32	0.94
	HT 0-7 cm (Rep 1)	76	68	3.71	34	1.20
	HT 0-7 cm (Rep 2a)	59	75	2.50	24	0.51
	HT 0-7 cm (Rep 2b)	81	72	2.85	28	0.62
	HT 13-20 cm (Rep 1)	84	63	3.00	32	0.63
	HT 13-20 cm (Rep 2)	59	71	3.47	30	1.20
	NT 0-7 cm (Rep 1)	90	77	3.20	30	0.81
	NT 0-7 cm (Rep 2)	62	76	3.14	27	0.96
	NT 13-20 cm (Rep 1)	63	71	3.30	30	0.90
	NT 13-20 cm (Rep 2)	63	70	3.27	32	0.91
August 2005	CT Control 0-7 cm (Rep 1)	55	69	2.79	28	0.65
	CT Control 0-7 cm (Rep 2)	59	59	3.57	34	1.15
	CT Disturbed 0-7 cm (Rep 1)	58	78	2.90	25	0.79
	CT Disturbed 0-7 cm (Rep 2)	57	82	2.38	23	0.52

Table 3.7: Diversity Statistics and Coverage of *nir*K Libraries at a 99% Nucleotide Similarity Cutoff

Sample Date	Treatment, Depth & Replicate	Number of Clones	Good's Coverage	Simpson's Diversity Index (-lnD)	Rarified Number of OTU's	Simpson's Evenness ($E_{1/D}$)
December 2004	CT 0-7 cm (Rep 1)	60	37	4.84	46	2.75
	CT 0-7 cm (Rep 2)	57	56	3.69	36	1.29
	CT 13-20 cm (Rep 1)	60	48	4.04	39	1.46
	CT 13-20 cm (Rep 2)	83	58	3.80	37	1.21
	HT 0-7 cm (Rep 1)	76	59	4.02	39	1.43
	HT 0-7 cm (Rep 2a)	59	61	2.74	29	0.53
	HT 0-7 cm (Rep 2b)	81	62	3.14	33	0.70
	HT 13-20 cm (Rep 1)	84	61	3.22	35	0.72
	HT 13-20 cm (Rep 2)	59	66	3.66	33	1.30
	NT 0-7 cm (Rep 1)	90	67	3.69	36	1.11
	NT 0-7 cm (Rep 2)	62	69	3.27	29	1.01
	NT 13-20 cm (Rep 1)	63	68	3.63	33	1.14
	NT 13-20 cm (Rep 2)	63	57	3.48	36	1.02
August 2005	CT Control 0-7 cm (Rep 1)	55	60	3.59	34	1.17
	CT Control 0-7 cm (Rep 2)	59	49	4.31	40	2.07
	CT Disturbed 0-7 cm (Rep 1)	58	67	3.94	34	1.67
	CT Disturbed 0-7 cm (Rep 2)	57	67	2.68	29	0.56

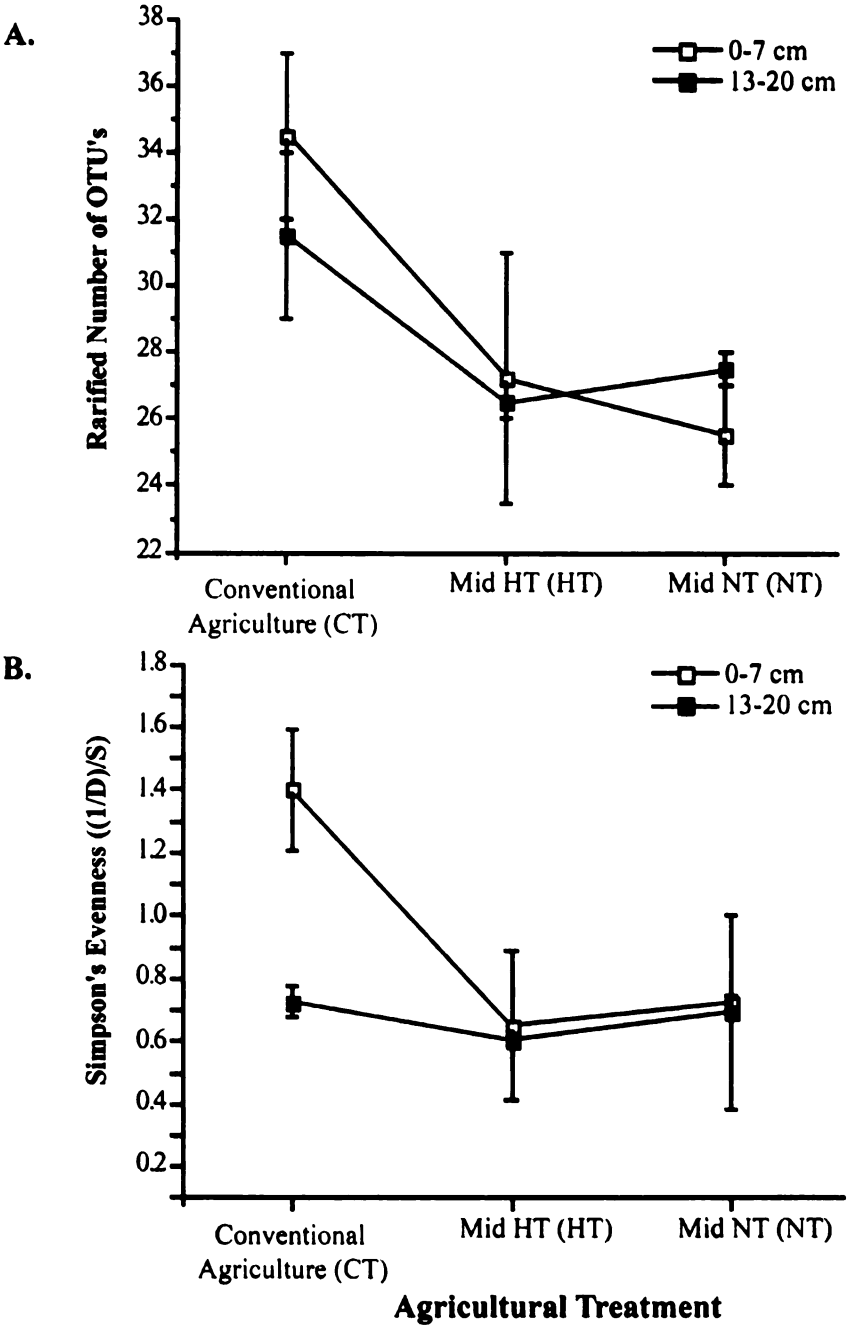


Figure 3.4: Results of a 2-way ANOVA using data from a 94% nucleotide similarity cutoff. Significant effects due to agricultural treatment were found for both richness and evenness at $\alpha = 0.05$. Graphs show averaged data from 2 replicate libraries from the same treatment and depth. A) Richness assessed as the number of OTU's rarified to the number of clones in the smallest *nir*K library. B) Simpson's Evenness. Error bars are standard errors.

measure values from the CT treatment at 0-7 cm that were the most different from those of the other treatments and soil depths.

Another method used to compare species abundance data is to construct rank/abundance curves [42]. These plots highlight communities that differ greatly in evenness by showing whether the community is dominated by one or a few OTU's. Rank/abundance curves were constructed from data at the 94% nucleotide similarity cutoff, with those replicate libraries that were found to not differ significantly in the ∫-LIBSHUFF analysis represented by an averaged curve (Fig. 3.5). The CT 0-7 cm treatment libraries had the flattest curve indicating few, if any dominating groups. All other libraries differed in this regard, some significantly as shown with a Kolmogorov-Smirnov two sample test. Both the HT 0-7 cm replicate 2 and NT 13-20 cm replicate 2 rank/abundance curves differed from the CT 0-7 cm libraries ($P < 0.05$ and $0.05 < P < 0.10$, respectively).

In order to determine whether the same *nir*K OTU's were dominant in the NT and HT clone libraries, the NT OTU's were ranked according to the percentage of the library they comprised and compared with the same OTU's in the HT and CT libraries. The 0-7 cm libraries were concentrated on as this zone of the soil contains the majority of microbes and is the location of most metabolic activity. There were six NT 0-7 cm OTU's (out of a total of 47) that accounted for approximately 50% of the total sequences (Table 3.8). These same OTU's comprised ca. 25% of the HT 0-7 cm clone libraries, but only ca. 1% of the CT 0-7 cm libraries. This has implications for determining which denitrifiers in NT and HT might be responsible for contributing to the low N_2O fluxes found in these treatments.

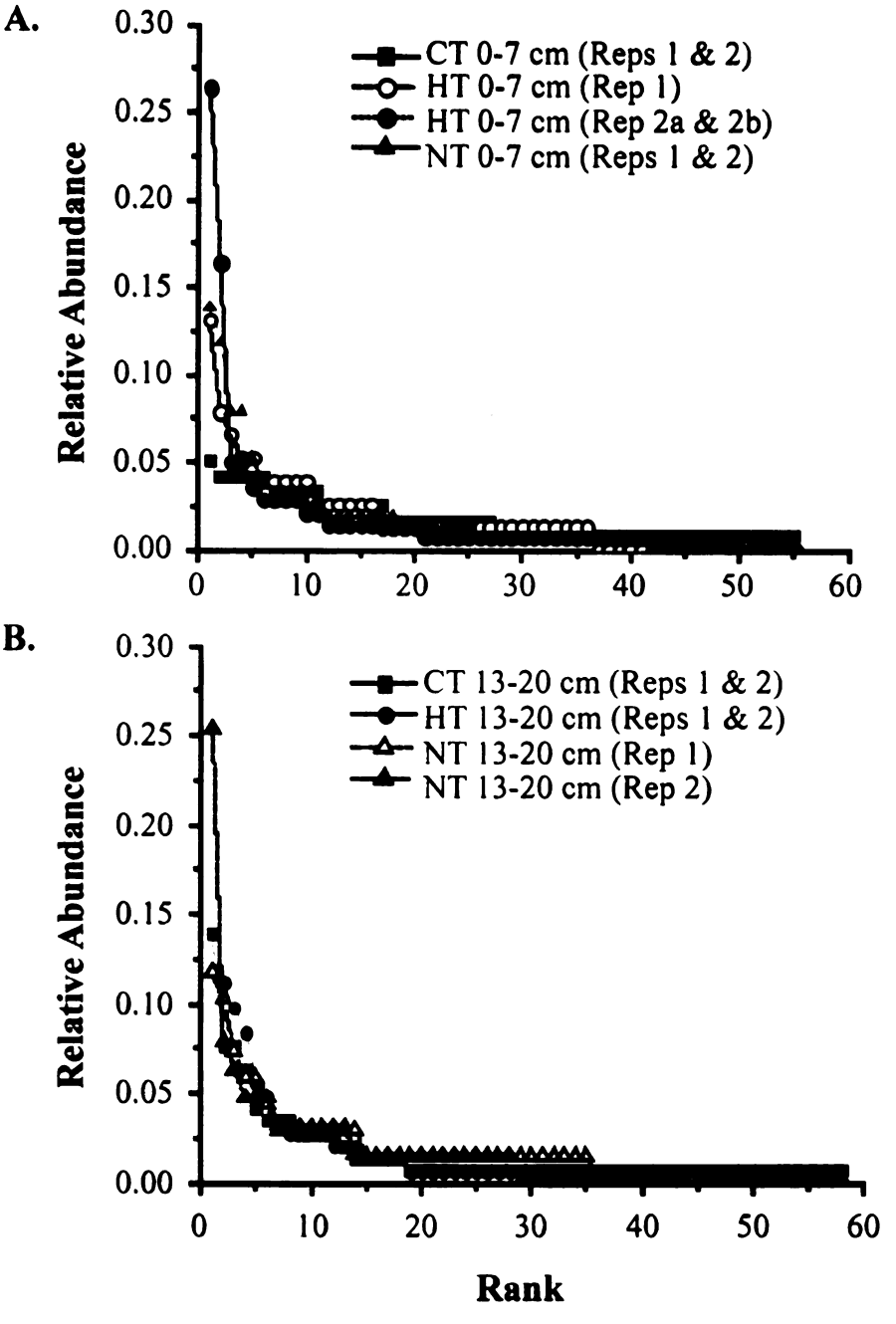


Figure 3.5: Rank/abundance curves of *nir*K libraries at a 94% nucleotide similarity cutoff. Treatment replicates that were not significantly different in a ∫-LIBSHUFF analysis were combined to obtain an average rank/abundance curve. Treatment replicates that were significantly different were kept separate. A) Libraries from a depth of 0-7 cm. B) Libraries from a depth of 13-20 cm.

Table 3.8: Dominant *nir*K OTU's from NT 0-7 cm and their Percent Abundance in HT and CT at 0-7 cm [a]

	Percentage of Library (%)		
NT OTU Rank	NT	HT	CT
1	11.8	0	0
2	9.2	7.9	0.9
3	7.9	1.4	0
4	7.9	0	0
5	5.3	0.5	0
6	4.6	13.9	0
Total	46.7	23.7	0.9

[a] OTU's that represented ≥ 4% of the combined NT 0-7 cm clone libraries were considered dominant OTU's

Relationship Between Diversity and Productivity

Productivity was measured as the total percent carbon (%C) of the soil environment that each library was constructed from (Table 3.3). There was no difference in %C (or %N) between the 0-7 cm and 13-20 cm depths of the CT treatment, the 13-20 cm depths of the HT treatment, and the 13-20 cm depth of the NT treatment. The 0-7 cm HT and NT treatments were significantly different from all other treatments and depths in %C. Linear regression was performed to determine whether there was a relationship between %C and overall diversity, richness, or evenness. No significant relationship was noted at any of the percent nucleotide cutoffs and with all three diversity statistics (all $P > 0.05$).

Relationship Between Diversity and Disturbance

In order to investigate whether there was a relationship between diversity and disturbance, each agricultural treatment and soil depth was rated as to its level of disturbance (Table 3.1). Disturbances were considered to be events that could affect denitrifier niche opportunities [30]. At all percent nucleotide similarity cutoffs an increasing trend in Simpson's Diversity was noted with increasing disturbance (Fig. 3.6). To investigate whether this relationship was significant, Spearman's Rank Correlation Coefficient was calculated. At the 94% and 99% nucleotide cutoffs there were weakly significant, positive relationships between disturbance and Simpson's Diversity index, the rarified number of OTU's, and Simpson's evenness (Table 3.9).

To determine whether the denitrifier diversity and disturbance relationship would follow the predictions of the IDH a disturbance experiment was conducted in the summer of 2005. It was hypothesized that an increase in disturbance level at the most highly

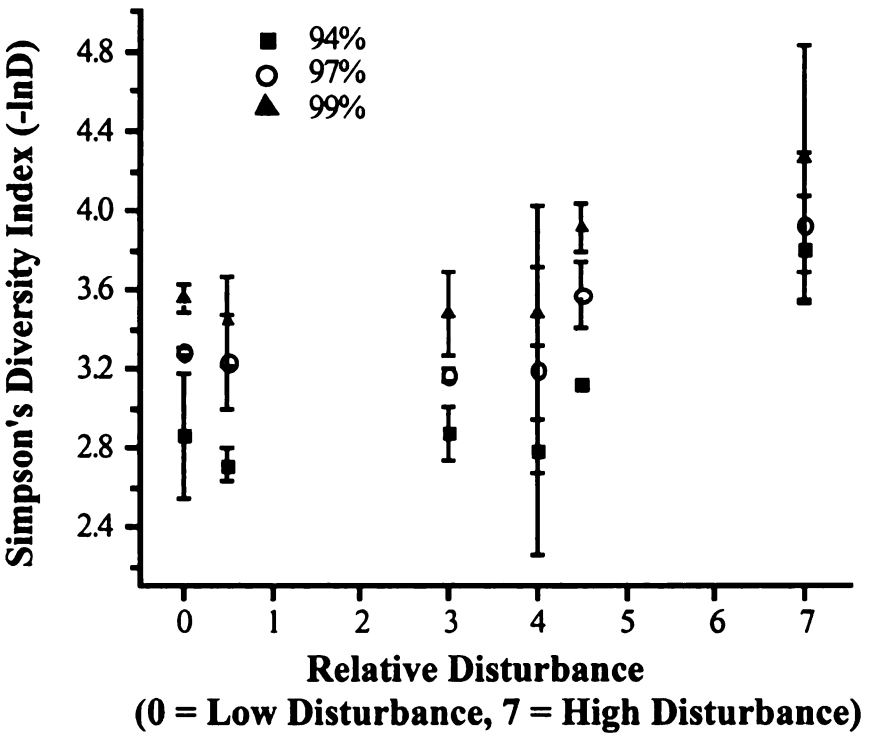


Figure 3.6: Relationship between Simpson's Diversity Index and relative disturbance at 94%, 97% and 99% nucleotide similarity cutoffs. Data is from libraries created from soil collected in December 2004.

Table 3.9: Spearman's Rank Correlation Coefficient for Diversity Measures (December 2004 *nir*K Libraries)

% Nucleotide Similarity Cutoff	Spearman's Rank Correlation Coefficient (ρ) [a]		
	Simpson's Diversity (-lnD)	Rarified Number of OTU's (S)	Simpson's Evenness ($E_{1/D}$)
94%	0.714 [c]	0.600 [c]	0.671 [c]
97%	0.486	0.529	0.529
99%	0.643 [c]	0.771 [b]	0.600 [c]

[a] Spearman's Rank Correlation Coefficients were calculated based on average diversity measure values for replicate soil treatments and depths.
[b] A significant correlation at $\alpha = 0.10$ (N = 6, df = 4).
[c] A significant correlation at $\alpha = 0.20$ (N = 6, dt = 4).

disturbed site would cause a decrease in diversity. *NirK* libraries were created from DNA collected from both control and disturbed microplots located within the CT treatment. A paired, one tailed t-test indicated that there was no significant difference in overall diversity, richness, or evenness between the control and disturbed plots ($P > 0.25$).

rrn Operon Copy Number and Growth Rate

Previous work has shown evidence for a trade-off occurring between rrn operon copy number and microorganism growth rate. To determine whether a relationship existed specifically for dissimilatory nitrate reducers, denitrifiers and organisms capable of dissimilatory nitrate reduction to ammonium (DNRA) with known rrn operon copy numbers were identified. Growth rates under dissimilatory nitrate reducing conditions were obtained either from the literature or were determined in the laboratory (Table 3.10). A significant, positive relationship ($R^2 = 0.8115$, $p = 0.0009$) was found between rrn operon copy number and growth rate (Fig. 3.7). Since species cannot be considered independent units due to their phylogenetic relationships, the impact of phylogeny was investigated [43, 44]. The computer program "Continuous" was used in the investigation to show that phylogeny was independent of the relationship between growth rate and rrn copy number ($\lambda = 0$).

DISCUSSION:

The primary goal of this study was to compare proteobacterial denitrifier community composition between sites differing in N_2O flux, which is an important ecosystem function, and history of agriculture. Analysis showed that the CT treatment communities did not differ from each other at 0-7 cm and 13-20 cm. This is most likely

Table 3.10: Organisms used to determine the relationship between rrn operon copy number and growth rate

Organism	Phylogenetic Group	Denitrifier or DNRA	rrn Operon Copy Number [a]	Growth Rate (hr^{-1}) ± SE [b]	Growth Rate Reference
Magnetospirillum magnetotacticum MS-1	α-proteobacteria	Denitrifier	2	0.04	[45]
Rhodopseudomonas palustris BK1	α-proteobacteria	Denitrifier	2	0.09	[46]
Pseudomonas stutzeri JM300	γ-proteobacteria	Denitrifier	4	0.22 ± 0.01	This study
Ralstonia metallidurans	β-proteobacteria	Denitrifier	4	0.05 ± 0.00	This study
Pseudomonas fluorescens ATCC 33512	γ-proteobacteria	Denitrifier	5	0.24 ± 0.01	This study
Escherichia coli K12	γ-proteobacteria	DNRA	7	0.44 ± 0.01	This study
Serratia marcescens	γ-proteobacteria	DNRA	7	0.55 ± 0.02	This study
Chromobacterium violaceum CS-1	β-proteobacteria	Denitrifier	8	0.84	[47]
Shewanella putrefaciens ATCC 8071	γ-proteobacteria	DNRA	9	0.57 ± 0.01	This study

[a] rrn copy numbers were found using the rrndb Release 2.5 [48] or the Comprehensive Microbial Resources database on the TIGR website (http://www.tigr.org).
[b] Standard errors were not found for growth rates obtained from the literature.

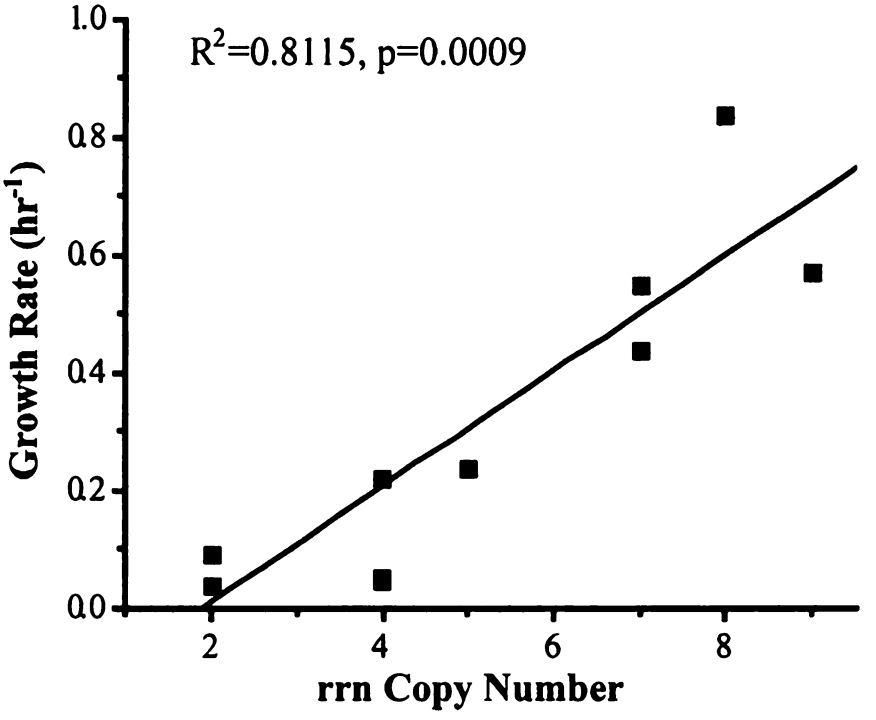


Figure 3.7: Relationship between dissimilatory nitrate reducer growth rate and rrn copy number under anaerobic conditions.

due to tillage, which homogenizes soil, making it a likely cause for the similarities seen between the upper and lower soil communities. The CT 13-20 cm and HT 13-20 cm communities were very similar, however the HT 13-20 cm community differed from that found in CT at 0-7 cm. This is evidence that by December, enough time had passed for the CT community to begin differentiating based on soil depth. In addition, there is evidence for changes in the HT treatment denitrifier community occurring more quickly in the upper portion of soil as compared to the lower portion. At the time of sampling it had been 15 years since HT was used for agriculture. ∫-LIBSHUFF analysis shows that the community in the upper portion of soil bears few similarities to CT, but is also not similar to the NT treatment. Since the community in the lower portion of soil still shares similarity with the lower portion of CT, it seems that community change is taking place at a faster rate in the top 7 cm of soil. Plants are known to influence microbial communities in soil, most likely through carbon inputs [49-53]. Since %C and %N are significantly higher at 0-7 cm than 13-20 cm, selective pressures probably differ at each depth resulting in different communities of denitrifiers. The fact that the HT community still shows a detectable impact from agriculture after 15 years is not surprising. Previous work at the KBS LTER has shown that there is a long-lasting effect on microbial communities after soil is used for agriculture [54, 55]. This is most likely due to the fact that soil carbon and nitrogen can take greater than 60 years to recover to pre-agricultural concentrations and qualities [56, 57].

Community composition and diversity are different concepts in the sense that it is possible for communities that differ in composition to have the same diversity. In

addition, there are many ecological theories regarding the maintenance of diversity, and for these reasons overall diversity and factors that might influence it were investigated.

Differences in diversity measures were not related to the productivity of the environment. This is not surprising as previous studies indicate that relationships between diversity and productivity can vary based on scale, trophic level, or directness of the productivity measure [21, 22]. The use of total %C as a measure of belowground productivity for denitrifiers does not account for the availability or quality of carbon and may not have been a direct enough measure to capture relationships between denitrifier diversity and productivity.

There was a weak, positive relationship between disturbance level and overall diversity, richness and evenness. Subsequent increases in level of disturbance did not result in a significant change in overall diversity, richness, or evenness indicating that the highest disturbance level employed was not high enough to cause increased denitrifier mortality. The lack of a significant change may have been due to the lack of carbon inputs as all plants were removed from the microplots. While the results of these experiments do not support or refute the predictions of the Intermediate Disturbance Hypothesis (IDH), increasing disturbance through agricultural management is related to increases in denitrifier diversity. Supporting this is the fact that there was a significant difference found in richness and evenness based on agricultural treatment. Evenness in particular plays an important role in the difference seen between communities. Dominance of particular OTU's in the HT and NT treatments that are not present or at low number in the CT treatment may be important in explaining differences in N_2O flux seen in these treatments.

The increase in diversity measures with increased agricultural management seen in this study confirms previous results obtained in a study in which denitrifier diversity was measured in 0-25 cm cores from the CT and NT treatments. *NosZ* gene diversity was measured using RFLP with the result that the CT treatment had higher overall diversity, richness and evenness [58]. DNA was extracted from soil at about the same time of year as in this study, but took place in 2001. The consistency in trends seen over the course of three years shows that the diversity measured is a consistent, and not transient, property of these denitrifier populations.

Most studies exploring differences between bacterial communities rely on comparisons between only one replicate of each treatment. To determine the potential impact of this practice, an investigation into whether replicates of agricultural treatments have the same diversity and harbor the same denitrifier communities was performed. In the majority of paired libraries, the communities were the same. However, in two cases there were significant differences in the communities as reflected in the libraries created from them. The different libraries were from HT and NT treatments which have much higher plant diversity than the CT treatment. One possible explanation for the heterogeneity found within replicates is that it is due to differences in local plant life. However, if this were the case, it would be expected that the 0-7 cm libraries from the NT treatments be significantly different as well since the majority of plant roots are found in the 0-7 cm depth of the treatment. As this was not the case, the difference noted in the NT 13-20 cm libraries is not readily explainable. It may be that the soil properties of the NT treatment are more heterogeneous at a lower soil depth causing microbial populations to differ. The differences seen within treatments is consistent with past work in which

the variability of 16S rDNA TRFLP profiles from the total microbial community of the CT, HT, and NT treatments were compared [54]. The CT community was less variable than the HT and NT communities, significantly so in the case of the NT community. Regardless of the underlying causes of differences within treatment replicates, this study demonstrates the need for replication in sequence libraries when molecular surveys of soil communities are performed.

In addition to investigating the differences between communities in treatment replicates, the occurrence and extent of PCR bias was investigated. In any study of diversity based on molecular methods, questions regarding bias must be addressed. PCR bias has been observed to occur due both to PCR drift and PCR selection [59]. In order to minimize the contribution of PCR drift, which is due to random variation in the early cycles of PCR, the minimum number of PCR cycles needed to produce a visible band on an agarose gel was employed and multiple PCR reactions from a particular treatment and depth were combined before cloning occurred. If PCR selection were to occur, this would result in preferential amplification of certain *nir*K OTU's so that their observed number in clone libraries would not reflect their abundance in the soil environment. This preferential amplification should occur in a repeatable manner so that libraries would not differ significantly in the frequency of these OTU's. To address this issue, the libraries from HT 0-7 cm (Replicates 2a and 2b) and NT 0-7 cm (Replicates 1 and 2) were compared. The three OTU's that were found in the highest frequency in both treatments were compared with a χ^2 test to determine whether they were found in a 1:1 relationship as would be expected if PCR selection were occurring. In two out of three cases, the frequency of specific OTU's were significantly different ($\alpha = 0.05$). While this does not

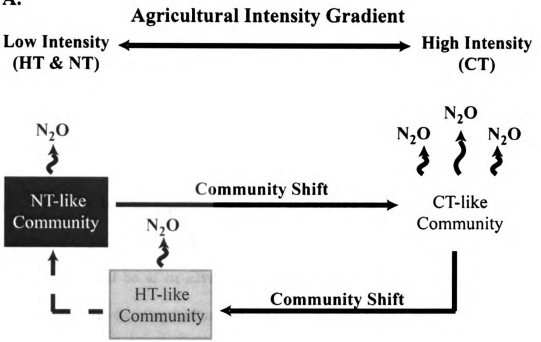
prove that PCR selection is not occurring, it does show that if it was, it was not a consistent process. In a different study performed at KBS the diversity of denitrifiers from the CT and NT treatments was assessed using RFLP on PCR amplified *nosZ* genes [58]. As in the present study, higher overall diversity, richness and evenness were found in the communities from the CT treatment. It is unlikely that the same results would be found using two different genes from the same pathway if PCR bias were occurring to a large extent.

Having determined that agricultural management influenced denitrifier diversity and community composition, a possible ecological strategy employed by denitrifiers was also investigated. There was a significant, positive relationship between dissimilatory nitrate reducer growth rate and rrn operon copy number. The same relationship was previously found for aerobic, heterotrophic soil microorganisms, indicating that rrn operon copy number reflects the ecological strategy of microorganisms [23]. Therefore, there may be a trade-off occurring amongst dissimilatory nitrate reducers between the capability to respond quickly to inputs of nutrients and the energetic cost of maintaining multiple ribosomes during stable conditions. The HT and NT treatments both have significantly higher amounts of total carbon at 0-7 cm (Table 3.3) and available carbon at 0-25 cm than the CT treatment. Due to its higher level of carbon limitation, it could be argued that the CT treatment is then a more competitive environment, which could select for organisms with K-selected traits, such as slower, more efficient growth. In that case, it would be expected that low rrn copy number microorganisms would be favored in the CT treatment, while high rrn copy number organisms would be favored in the less competitive HT and NT treatments.

N_2O flux differs significantly between the treatments in that the CT treatment has an average annual flux that is approximately three times higher than that of the HT and NT treatments. Besides higher N_2O production, other differences have been noted when agricultural soils are compared to uncultivated soils. Upon cultivation, there is generally a loss of carbon and subsequent decrease in the C/N ratio [60, 61], along with an increase in nitrate and decrease in ammonium. These changes result in situations where denitrifiers experience periods when nitrate is in excess of carbon, and NO_3^- is incompletely utilized so that N_2O mole fraction increases relative to situations when carbon is not limiting [62, 63]. Supporting this is the fact that previous work at the KBS LTER indicated activity of *nosZ* in the NT treatment was significantly higher than in the CT treatment [8]. *NosZ* catalyzes the final step in denitrification in which N_2O is reduced to N_2, and a decrease in its activity relative to N_2O forming enzymes would be indicative of an increase in N_2O mole ratio.

In conclusion, a model is proposed to explain the observed differences in denitrifier communities and soil physical factors after cultivation (Fig. 3.8). The model depicts the changes observed in community composition in the three soil treatments at the KBS LTER which vary in their amount of agricultural intensity. It is proposed that increases in agricultural intensity result in a change in physical factors so that previously dominant denitrifiers are lost or decrease in prevalence within the community, causing an increase in denitrifier diversity, richness and evenness. The dominant denitrifiers present before agricultural management are those selected for based on their ability to completely reduce NO_3^- to N_2. By allowing electron flow through nitrous oxide reductase (Nos) instead of stopping at nitric oxide reductase (Nor), a second electron sink is present,

A.



B. **Community Characteristics:**

Low Intensity (HT & NT):

1. **Dominant HT & NT-like OTU's**
2. **Low richness & evenness**
3. Complete reduction of NO_3^- (NO_3^- to N_2) favored
4. High rrn copy # favored

High Intensity (CT):

1. **Loss or decrease in NT & HT-like OTU's**
2. **High richness & evenness**
3. Incomplete reduction of NO_3^- (NO_3^- to N_2O) favored
4. Low rrn copy # favored

Figure 3.8: A) Model describing denitrifier community shifts in response to changes in an agricultural intensity gradient. The dashed line indicates a speculated community shift from an HT-like community to a NT-like community given enough time. B) The community characteristics that were noted in this study and also speculated upon. Points proven in this study are in black; speculated properties of the denitrifier community are in grey.

resulting in faster electron flow and more protons translocated across the cytoplasmic membrane per unit time. This community is also predicted to be dominated by organisms with a high rrn operon copy number. After a site is converted to agriculture, the majority of denitrifiers present will be selected for based on their ability to efficiently utilize carbon. Efficient carbon use results by stopping denitrification at N_2O because at this point in the reaction, the maximum number of protons have been translocated per electron. This model is based on the γ-proteobacteria *Pseudomonas stutzeri* which is capable of translocating a proton when NO is reduced to N_2O, but not when N_2O is reduced to N_2 [1]. This environment should also favor low rrn operon copy number organisms that will be at an advantage in a competitive, carbon limited environment. Removal of a site from agriculture allows another community shift to occur in which denitrifiers capable of complete NO_3^- reduction begin to again dominate the community.

This work, along with that previously conducted at the KBS LTER, demonstrates that a difference in denitrifier communities along with differences in functional properties and ecological strategies of the communities may be responsible for the differences in N_2O flux seen in agricultural and successional soils.

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CHAPTER 4

THE DIVERSITY OF *PLANCTOMYCETALES* IN SOILS DIFFERING IN HISTORY OF AGRICULTURE

ABSTRACT:

The order *Planctomycetales* is comprised of diverse, morphologically unique bacteria that are ubiquitous in nature. Given their potential for important roles in soil nutrient cycles, especially nitrogen cycling, a phylogenetic survey using 16S rRNA gene sequences was undertaken to assess soil planctomycete diversity in three soils differing in their history of agriculture. Sequences clustered with the four genera of planctomycetes as well as in clusters outside the recognized genera. Three sequences were similar to 16S rDNA sequences from organisms capable of anaerobic ammonia oxidation, but formed a separate group. Comparative analyses indicated that two soil treatments that have been used for agriculture are more similar in community composition and diversity measures to each other than to a treatment never used for agriculture. However, there were no significant differences found when comparing the three communities, suggesting that *Planctomycetales* are marginally affected by agricultural practices.

INTRODUCTION:

Members of the order *Planctomycetales* have drawn interest since their discovery in the early 1900's [1, 2]. Traits of interest include the recent discovery of the capacity for anaerobic ammonium oxidation (anammox) by some members of the order and physiological traits that resemble those of eukaryotes. Despite numerous studies on aquatic planctomycetes and the recent cultivation of isolates from soil, not much is

known about their ecological role in either environment. The information that is known about planctomycetes however, suggests that they may play important roles in nutrient cycling.

Historically, it was assumed that planctomycetes grew only in aerobic, aquatic habitats, as this is where they were commonly cultured. Planctomycetes have been observed in fresh, marine, and brackish water [3, 4], as well as in aquatic environments that vary in their trophic status; however they seem to be most prevalent in eutrophic habitats [4]. Besides aquatic habitats, planctomycetes have been shown to make up a measurable portion of soil microbial populations (approximately 2 to 11%) by studies employing rDNA libraries [5-12], rRNA hybridization [13, 14] and FISH [15, 16]. Previously, the order was categorized as aerobic, but there is recent evidence that obligate anaerobes exist. Isolates have been recovered from an anoxic bioreactor [17], anoxic sediment [18], an anaerobic wastewater reactor [19], and anoxic rice microcosms [20]. In addition, *Planctomycetales* have been isolated from the postlarvae of the Giant Tiger Prawn, *Penaeus monodon* [21]. At this time it is not known whether these findings denote a symbiotic or commensal relationship; however it has been found that a species of *Verrucomicrobia*, an order that is one of the closest relations to the *Planctomycetales*, is an endosymbiont of nematodes of the genus *Xiphinema* [22]. The fact that planctomycetes are more ubiquitous, and present in a wider variety of environments than previously thought suggests that they play an important role in those environments.

Known members of the order *Planctomycetales* have distinct morphological and biochemical characteristics that make them unique among the eubacteria and suggest possible ecological roles. *Planctomycetales* are the only known cell-wall containing

eubacteria besides the *Chlamydiae* and mycoplasmas that lack peptidoglycan [23-25]. In a study to identify a medium suitable for the isolation of aquatic *Planctomycetales*, N-acetylglucosamine was used as both a carbon and nitrogen source [4]. N-acetylglucosamine is a component of peptidoglycan, and its consumption by an organism lacking this same compound implies that the *Planctomycetales* have a role in the degradation or mineralization of cell wall material. Also, some planctomycetes produce stalks that can act as hold-fasts, allowing the organisms to attach to substrates in the environment or each other [3]. Manganese and iron oxide encrustations have been found on the stalks of some aquatic *Planctomycetales* leading to the speculation that they may be capable of manganese and iron oxidation [26, 27].

One of the most intriguing characteristics of the *Planctomycetales* is the recent discovery that some members play an important role in global nitrogen cycling. Uncultured representatives have been shown to carry out anaerobic oxidation of ammonia (anammox) [28]. During anammox, ammonia is oxidized and nitrate or nitrite reduced to form dinitrogen gas [29, 30]. This process has been detected in wastewater treatment plants, freshwater and marine sediments, and the anoxic ocean water column. Recently it was estimated that anammox is responsible for up to 50% of the removal of fixed nitrogen from the ocean [31, 32]. There have been no reports of anammox being detected in soil, but a full investigation into its occurrence has not been reported.

Recent work at the Kellogg Biological Station Long-Term Ecological Research (KBS LTER) site has shown that soils differing in inorganic and total nitrogen as well as carbon and history of agricultural use, also differ in their amounts of nitrous oxide flux [33]. This indicates that different microbial communities involved in nitrogen cycling

may be present at each site. Due to the possible involvement of the *Planctomycetales* in nitrogen cycling, an investigation was performed in which the diversity and community composition of planctomycetes from three soil treatments were compared. A phylogenetic analysis was also performed to determine how closely soil organisms were related to anammox organisms.

MATERIALS & METHODS:

Study Site & Soil Collection

Soil samples were collected in October 1996 from the Kellogg Biological Station Long-Term Ecological Research Site (KBS LTER) located in Hickory Corners, MI. The dominant soil series at the station are the Kalamazoo and Oshtemo series. These are fine-loamy and coarse-loamy mesic Typic Hapludalfs, respectively. The KBS LTER has soil treatments that endure a wide range of human impact. Three treatments were focused on in this study; a conventional agriculture (CT) site that receives amounts of fertilizer, herbicide, and tillage that are typical for the Midwest region and is on an annual corn (*Zea mays* L.) - soybean (*Glycine max* L.) - wheat (*Triticum aestivum* L.) rotation, a historically tilled site (HT) abandoned from agriculture in 1989, and a never-tilled site (NT). The KBS LTER is described fully on the World Wide Web at http:// lter.kbs.msu.edu/.

Soil was collected at a depth of 0-10 cm from the CT, HT and NT treatments. At each treatment plot, a total of five cores of a 2.5 cm diameter were taken, and cores from the same treatment were pooled, sieved (2 mm mesh), and frozen in liquid nitrogen in the

field. The soil was kept on dry ice until it was brought back to the laboratory and stored at -80°C.

Library Construction

One library was made from DNA extracted from each of the three soil treatments. Total soil DNA was extracted from 0.25 - 1.0 g soil using an UltraClean Soil DNA Kit (MoBio, Carlsbad, CA). The *Planctomycetales* specific forward primer Pla37F (5' TGG CGG CRT GGA TTA G 3'; modified from Neef and colleagues [34]) and universal reverse primer 1540R (5' AAG GAG GTG ATC CAR CCG CA 3') were used to amplify 16S rRNA genes (both primers modified or designed by Daniel Buckley, personal communication). PCR amplification was performed with 1 U of Amplitaq Gold® *Taq* polymerase (Applied Biosystems, Foster City, CA), a 1X concentration of the manufacturer's PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin), 2.0 mM $MgCl_2$, 0.2 mM dNTP's (Roche, Indianapolis, IN), 0.01% BSA, 50 pmol of each primer, and 10-50 ng of template DNA in a 25 μL volume. PCR mixtures were incubated in a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer, Boston, MA) with the following PCR protocol: (i) 12 min initial denaturation at 95.0°C; (ii) 30 cycles of denaturation for 30 s at 95.0°C, primer annealing for 40s at 64.0°C, extension for 45 s at 72.0°C; and (iii) a final extension for 10 min at 72.0°C. Genomic DNA from *Planctomyces limnophilus* ATCC 43296 was used as a positive control and *Verrucomicrobium spinosum* ATCC 43997 as a negative control for the PCR.

Each 16S rDNA clone library was created using three pooled reactions. Purified product was cloned using a TOPO TA Cloning Kit for Sequencing with cloning vector pCR 2.1 and transformed into *Escherichia coli* One Shot TOP10® competent cells

(Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transformants were plated on LB agar containing 100 μg/mL ampicillin. Clones were screened for the correct insert size and the PCR product sequenced using a model 373A DNA sequencer (Applied Biosystems) with dye-terminator fluorescent cycle sequencing technology. All sequences were checked with the Chimera Check tool [35] to ensure that no chimeric sequences were included in the analysis. A total of 98 sequences were found to be of sufficient quality to be included in phylogenetic analyses, during which three were found to group more closely to sequences from *Chlamydia* and *Verrucomicrobia* than planctomycetes. Therefore, the three sequences were not included in further community composition or diversity analyses. This resulted in there being 43 clones in the CT library, 27 in the HT library, and 25 in the NT library. Full-length sequencing of seven 16S rRNA genes was completed to aid in phylogenetic analyses.

Phylogenetic Analyses

Phylogenetic analyses were performed using the program ARB [36]. Previously published *Planctomycetales* 16S rDNA sequences were obtained from NCBI and aligned along with the sequences obtained in this study. Sequence alignments were performed using the automatic aligner in ARB followed by visual inspection and correction as needed. Sections of the sequence with ambiguous alignments as well as the primer sequences were excluded from the analyses. The phylogenetic tree was constructed using the neighbor joining method.

Diversity Analyses

A nucleotide similarity cutoff of 97% was used to define *Planctomycetales* operational taxonomic units (OTU's). This cutoff was chosen due to the fact that it was

shown to correspond well with the traditional 70% DNA-DNA reassociation standard currently used to define microbial species [37].

∫-LIBSHUFF version 1.3 [38] was used to analyze 16S rDNA libraries to determine whether those created from different treatments were statistically different, while DOTUR version 1.53 [39] was used to analyze the α-diversity of each library. Input into DOTUR consisted of neighbor joining distance matrices from each individual library. DOTUR output was used to calculate Simpson's diversity indices (-lnD), Simpson's evenness measure ([1/D]/S, where S is equal to the number of OTU's present in each library rarified to the number of clones in the smallest library), Good's coverage ([1-(n/N)]*100, where n equals the number of singletons and N equals the total number of sequences), and Chao1 curves at a 97% nucleotide similarity cutoff.

In addition, β-diversity was determined using both DOTUR and ∫-LIBSHUFF. DOTUR was used to separate sequences from all libraries into OTU's and the DOTUR .list file converted into a matrix used as input for the program EstimateS version 7.00 [40]. A Jaccard dissimilarity matrix created with EstimateS data was converted to a dendrogram using the program MEGA version 3.1 [41].

RESULTS:

Phylogenetic Analysis

Phylogenetic analysis revealed that the 95 cloned 16S rRNA genes fell into four recognized genera within the order *Planctomycetales* and also into four groups not associated with the known genera (Fig. 4.1). Ten sequences clustered with the genus *Pirellula*, 11 with the genus *Planctomyces*, 33 with the genus *Gemmata*, and 10 with the

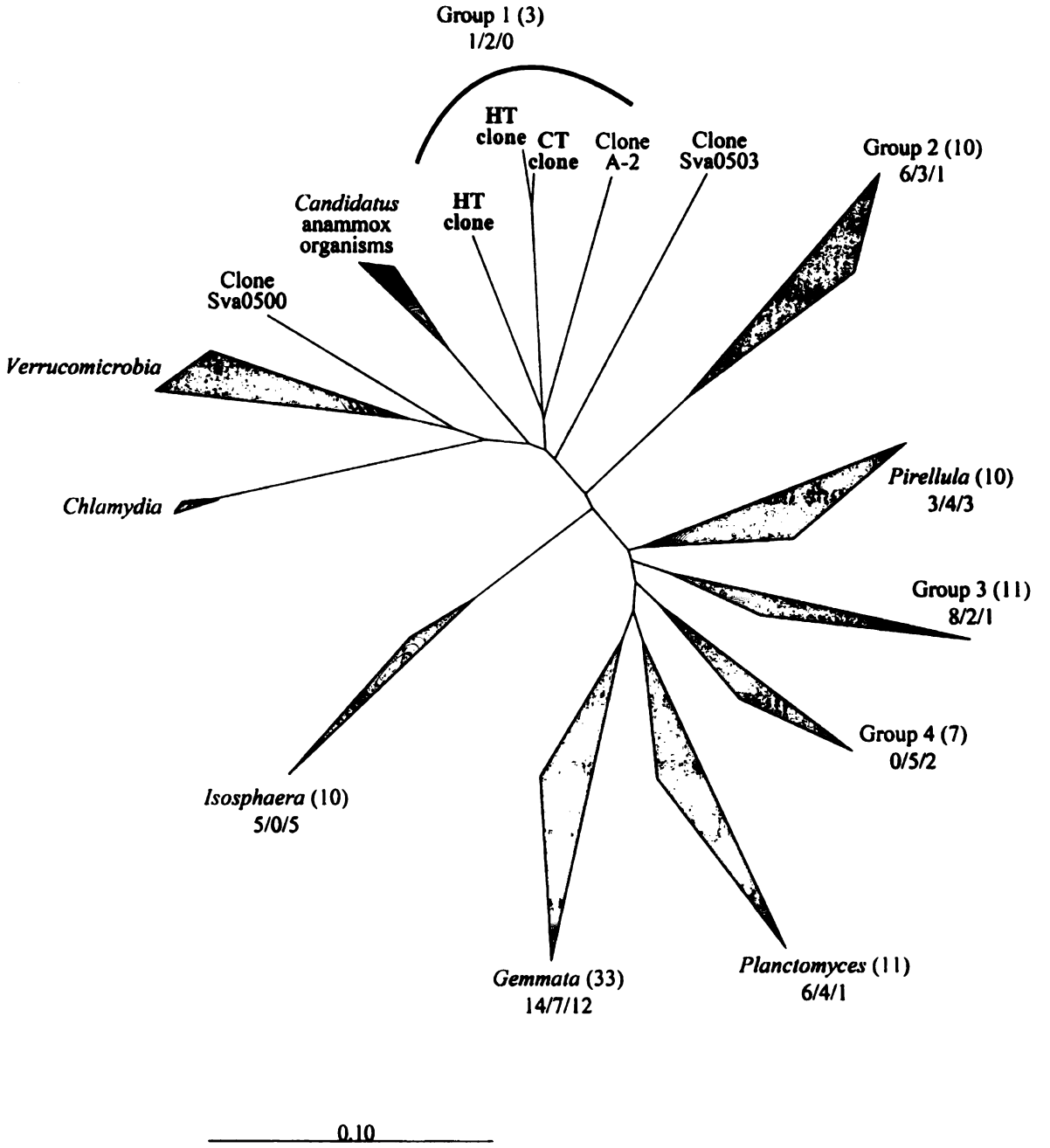

Figure 4.1: Neighbor joining phylogenetic tree constructed from full and partial 16S rDNA sequences. Numbers in parentheses refer to the number of KBS LTER clones within each group. Numbers separated by backslashes indicate the number of clones in each group from a specific soil treatment library (conventional agriculture/abandoned from agriculture/never tilled). Clones in bold are those clustering most closely with planctomycetes capable of anammox. The scale bar represents a 10% difference between nucleotide sequences.

genus *Isosphaera*. The majority of the remaining sequences clustered into four groups which for the purposes of this chapter were designated Groups 1 through 4. Three sequences clustered in Group 1, 10 clustered in Group 2, 11 clustered in Group 3, and 7 sequences clustered in Group 4. Sequences from each of the three treatments were present in most of the genera and groups. The exceptions were that no Group 1 sequences from the never tilled treatment, no *Isosphaera* sequences from the treatment abandoned from agriculture, and no Group 4 sequences from the conventional agriculture treatment were found (Fig. 4.1). The 3 clones in Group 1 clustered near 16S rDNA sequences from *Planctomycetales* known to perform anammox and showed between 78.8 and 84.6% sequence similarity to the anammox planctomycete sequences.

Community Composition

The composition of the three *Planctomycetales* soil communities were compared by statistical comparison of the 16S rRNA gene libraries. Analysis with the program ∫-LIBSHUFF showed that overall, none of the communities were significantly different from each other (Fig. 4.2). Library comparisons in ∫-LIBSHUFF are performed so that not only is Replicate Library X compared to Replicate Library Y, but Replicate Y is also compared to Replicate X. This can result in cases where in one comparison libraries are significantly different, but in the other they are not. This indicates that one library is a subset of the other and subsequently the libraries were considered to not be significantly different. The library from the conventional agriculture treatment was not significantly different from those from the treatment abandoned from agriculture or the treatment that had never been tilled. The never tilled treatment library was a subset of the treatment abandoned from agriculture. In order to ascertain which libraries were more similar to

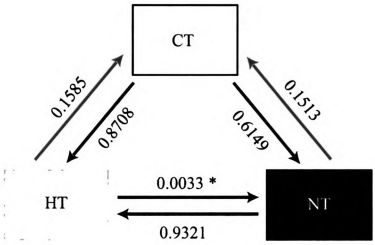

Figure 4.2: Results of ∫-LIBSHUFF analysis for *Planctomycetales* 16S rDNA sequence libraries from three KBS LTER soil treatments (libraries are represented by blocks). Comparisons between two individual libraries with ∫-LIBSHUFF actually entail two statistical comparisons: library X is compared to library Y and Y to X. As such, arrows point to the library that is being compared to the library being pointed from. Numbers are p-values from comparisons between libraries, and an asterisk (*) designates p-values that are significant at $\alpha = 0.05$.

each other, a dendrogram was constructed using a Jaccard dissimilarity matrix (Fig. 4.3). Even though none of the communities differ significantly, those from both treatments that have been used for agriculture (CT and HT) are more similar to each other than the community from the treatment never used for agriculture (NT).

Diversity of Soil *Planctomycetales*

Overall diversity, richness, and evenness were calculated for the three soil treatment libraries (Table 4.1). Simpson's diversity index was highest in the two treatments that have been used for agriculture (CT and HT) and lowest in the never tilled treatment. In order to determine if one of the two components of diversity had a stronger influence than the other, richness and evenness were compared.

The richness of libraries that differ in number of clones they contain can be compared by using rarefaction to estimate how many species would be present if all libraries had as many clones as the smallest library [42]. Rarefaction indicated that the number of species in each treatment were approximately equal (Table 4.1). Richness can also be compared by using nonparametric statistics, such as the Chao1 richness estimator [43]. Chao1 curves were plotted along with 95% confidence intervals to both compare richness and assess whether more sampling would be required to obtain an accurate richness estimate (Fig. 4.4). As with the rarefaction analysis, the Chao1 curves indicated that richness of *Planctomycetales* in the three soil treatments did not differ. One interesting point, however, is that the Chao1 curves for the conventional agriculture treatment and the treatment abandoned from agriculture are asymptotic, indicating that no further sampling is required for an accurate prediction of richness. The curve from the never tilled treatment library is non-asymptotic, indicating that the clone library is too

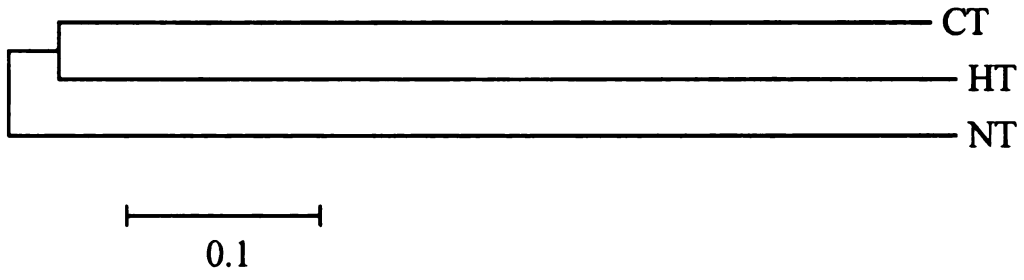

Figure 4.3: Dendrogram based on a Jaccard dissimilarity matrix calculated at a 97% nucleotide similarity cutoff. Scale represents percent dissimilarity.

Table 4.1: Diversity statistics for *Planctomycetales* calculated from 16S rDNA libraries at a 97% sequence similarity cutoff

Treatment [a]	Number of Clones	Chao1 Richness Estimate [95% CI's]	Simpson's Diversity Index [b]	Number of OTU's [c]	Simpson's Evenness [d]	Good's Coverage (%) [e]
CT	43	103 [62, 210]	5.01	23	6.5	28
HT	27	109 [52, 293]	5.17	23	7.6	15
NT	25	117 [50, 343]	4.32	22	3.4	20

[a] CT = conventional agricultural treatment, HT = treatment abandoned from agriculture, and NT = never tilled treatment.
[b] Calculated as –lnD.
[c] Calculated as S = number of OTU's rarified to 25 clones.
[d] Calculated as (1/D)/S.
[e] Calculated as [1-(n/N)]*100; where n = number of singletons and N = total number of sequences.

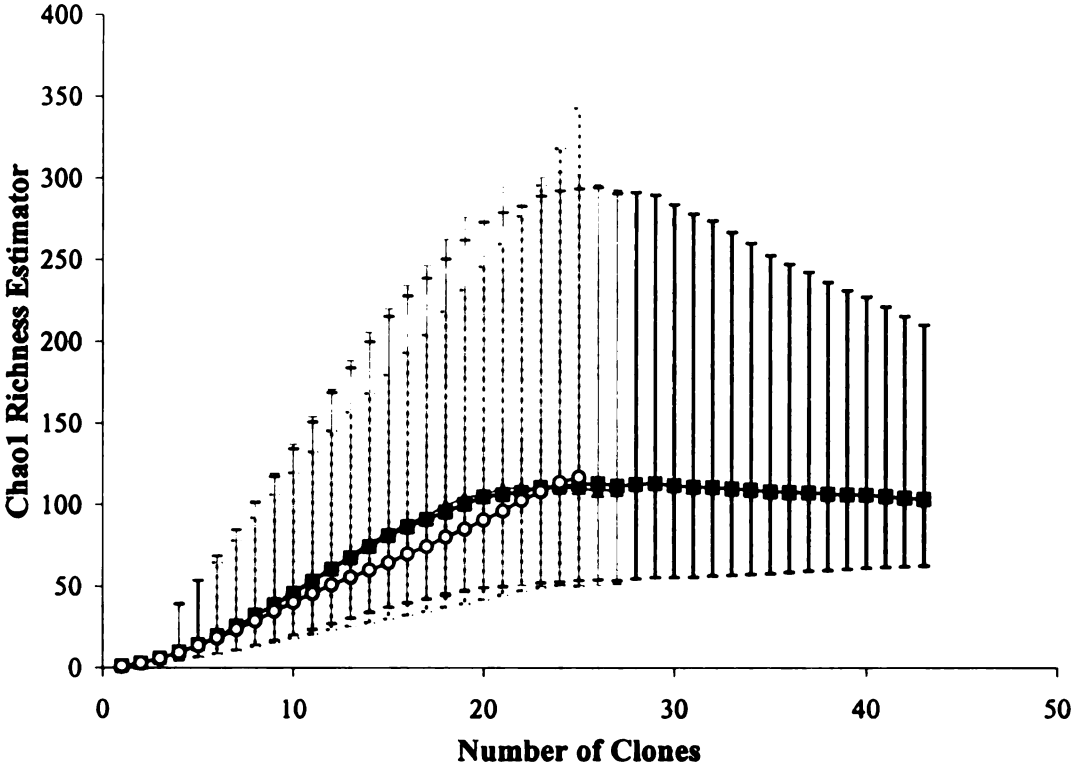

Figure 4.4: Chao1 estimates of *Planctomycetales* richness in three KBS LTER soil treatments at a 97% 16S rDNA sequence similarity cutoff. (■) Conventional agricultural treatment, (▲) treatment abandoned from agriculture, and (○) never tilled treatment. Error bars represent 95% confidence intervals.

small to obtain an accurate prediction of *Planctomycetales* richness in this treatment. At best, the value obtained is a minimum estimate of the richness in this treatment, suggesting that richness in the never tilled treatment may actually be higher than in the other two treatments.

Evenness describes the relative abundance of OTU's in each library and was highest in the conventional agriculture treatment and the treatment abandoned from agriculture. This was further illustrated by plotting rank/abundance curves for each library (Fig. 4.5) and comparing them statistically. Kolomogorov-Smirnov 2-sample tests indicated that the rank/abundance curves of the conventional agriculture and never tilled treatment differed significantly ($P < 0.05$). Therefore, differences in evenness have the largest influence on the *Planctomycetales* diversity measures.

DISCUSSION:

The order *Planctomycetales* encompasses a group of organisms with a high level of diversity as well as unique biochemical and morphological characteristics. Members of the order are ubiquitous in soils, however not much is known regarding potential ecological roles of these organisms. In fact, only recently has a group within the order been identified as being capable of anaerobic oxidation of ammonia, which plays an important role in ocean nitrogen cycling [31, 32]. Due to the potential of *Planctomycetales* to play important, but as yet undiscovered roles in soil nutrient cycles, a phylogenetic analysis and comparison of community structure in three different soil treatments was undertaken. The goal of the study was to gain insights into factors driving

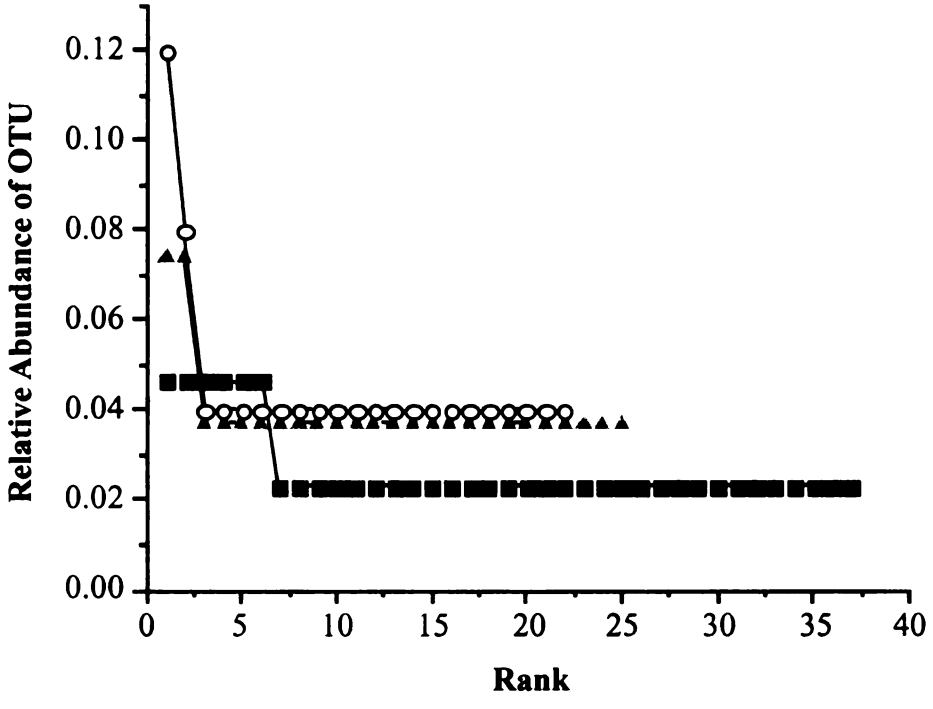

Figure 4.5: Rank/abundance curves of *Planctomycetales* OTU's defined at a 97% sequence similarity cutoff. (■) Conventional agricultural treatment, (▲) treatment abandoned from agriculture, and (○) never tilled treatment. Error bars represent 95% confidence intervals.

planctomycetes community structure and to determine whether members of the soil community have any relation to planctomycetes capable of anammox.

Of the 98 16S rRNA gene clones analyzed, three clustered near the 16S rRNA gene sequences of organisms capable of anammox. The three soil sequences had percent nucleotide similarities to the anammox organism sequences of ca. 79 to 85%, which is intriguing considering that the different genera of anammox capable planctomycetes show <85% 16S rDNA nucleotide sequence similarity to each other. [44] The clone libraries constructed from each soil treatment were small and had coverage values of only 15 to 28% (Table 4.1), indicating that further sequencing may result in discovery of sequences with higher similarity to anammox organism sequences. Anammox planctomycete-specific primers have been developed and used in aquatic environments [45] which if applied to soil could reveal the presence of these organisms. In addition, direct detection of anammox in soil may be possible with the use of stable isotopes as in recent aquatic studies [31, 45-49].

The three soil treatments focused on were chosen due to their different agricultural histories. Both a treatment subjected to conventional agricultural practices and one never used for agriculture were sampled, as well as a treatment that had not been subjected to agriculture in 7 years at the time soil samples were taken. α- as well as β-diversity measures revealed that the two treatments with a history of agriculture were more similar to each other with respect to community composition, diversity, evenness, and estimated richness than to the treatment never used for agriculture. The fact that the two treatments with an agricultural history have more similar communities is not surprising given that previous work has shown that the impact of agricultural practices on

microbial communities can last for decades [13, 14]. This is thought to be due to the long amount of time needed for soil carbon and nitrogen to recover to pre-agricultural concentration and quality [50, 51].

Despite the fact that the communities of two treatments with a history of agriculture were more similar to each other than to a treatment never used for agriculture, there was no significant difference in libraries from the three soils. Initially, this was unexpected considering that Buckley and Schmidt [13, 14] previously reported that microbial community composition of the KBS LTER conventional agriculture and abandoned from agriculture treatments were not significantly different, but did differ significantly from the never tilled treatment community. However, when the composition of the *Planctomycetales* communities were specifically focused on [14], soil treatment did not have a significant effect on community composition which is in agreement with the results presented here. This suggests that *Planctomycetales* are not contributing to differences seen in total microbial community composition, may only be marginally affected by agricultural practices, and/or be capable of faster recovery after cultivation than other microbial groups.

CONCLUSION:

The findings presented in this chapter invite further studies into factors affecting *Planctomycetales* community structure and diversity. In particular, the presence of 16S rDNA sequences clustering near those of anammox organisms, despite the small number of clones analyzed, indicates that further investigation into the presence of anammox organisms and the occurrence of anammox in soil is warranted. In addition, preliminary

results show that treatment effects are not reflected in planctomycete community composition, suggesting that the community is only marginally affected by agricultural practices.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

SIGNIFICANCE:

Microbial communities are essential players in agricultural soil nutrient cycles as their activity helps regulate the availability of nutrients to crops. Despite this important role, the main factors affecting microbial diversity and community structure are just beginning to be uncovered. As well as identifying factors affecting microbial communities, it is important to determine the links between particular communities and the ecosystem functions that they perform. The identification and understanding of microbial communities and their interactions will allow more accurate predictions to be made regarding the effect of agricultural practices on ecosystem functions. For example, the work presented in this dissertation and from recent published studies indicates that greenhouse gas flux cannot be explained solely by physical factors and suggests that differences in microbial populations should be accounted for in flux models in order to improve their accuracy.

The focus of the work presented in this dissertation was on microbial populations known to be involved in soil nitrogen cycling (denitrifiers), or with the potential for involvement (*Planctomycetales*). Denitrification in particular is detrimental in agricultural soils as it leads to loss of added nitrogen and production of the greenhouse gas, N_2O. Therefore, statistical modeling (Chapter 2) and molecular methods (Chapters 3 and 4) were used to investigate how physical aspects of the environment affect microbial

communities and, in turn, how microbial communities may affect the ecosystem function of N_2O flux.

A summary of the work presented in this dissertation is listed below and followed by suggestions for further research.

SUMMARY:

Chapter 2:

1. Field measurements of physical factors affecting microbial populations explained between 8 and 50% of the variation in CO_2, CH_4 and N_2O flux at the KBS LTER, with CO_2 flux consistently having the greatest amount of explainable variation (29 to 50%). Since CO_2 is a byproduct of all types of heterotrophic respiration in soil, its flux should be responsive to factors enhancing microbial metabolism in general, and this explained the high coefficients of determination obtained for CO_2 flux. Conversely, since the consumption of CH_4 and production of N_2O require the activity of microbes with specialized metabolic pathways; environmental parameters alone explain a smaller percentage of the flux of these gases.
2. In plots used for conventional agriculture, there were significant effects on gas flux associated with specific crops, with fluxes roughly following the pattern wheat > soybean > corn. This indicates that the annual rotation of crops at KBS may result in annual changes in microbial communities that vary in their metabolic capabilities and subsequent gas flux.

3. The treatment with the highest level of variation accounted for by environmental factors alone was the late-successional, deciduous forest (19 to 50%). This was hypothesized to be due to the selection of a microbial community, through differences in carbon and nitrogen inputs and land management, which is more responsive to changes in environmental parameters.

Chapter 3:

1. There was a significant effect of agricultural treatment on evenness ($P = 0.0476$), with the conventional agriculture treatment at 0-7 cm having the highest evenness. This, along with rank/abundance curves, indicates that there are *nir*K OTU's in the never tilled and historically tilled treatments that may play an important role in minimizing the amount of N_2O emitted from sites currently not used for agriculture.
2. Productivity (%C) did not have a significant relationship with measures of denitrifier diversity, but an analysis using Spearman's Correlation Coefficient indicated a weak, positive relationship between relative disturbance and diversity.
3. Denitrifier communities show long-term effects from agriculture, as the 0-7 cm and 13-20 cm communities from a site abandoned from agriculture 15 years ago were still more similar to those from a site currently used for agriculture than to those from a site never in agricultural use.
4. Communities in the 0-7 cm portion of the sites abandoned from agriculture and never tilled had diverged from those found at 13-20 cm, indicating that selective

pressures differ at each depth, most likely due to the differential level of influence by plants at both depths.

5. A significant, positive relationship between rrn operon copy number and growth rate of dissimilatory nitrate reducers was found, indicating that denitrifiers with low and high rrn operon copy numbers differ in their ecological strategies.

Chapter 4:

1. Three 16S rDNA sequences from libraries created with planctomycete-specific PCR primers were similar to sequences from a group of planctomycetes capable of anaerobic ammonia oxidation, but not enough to be affiliated with this group. Due to the low coverage of the libraries, further study is warranted to determine whether anammox is occurring or anammox capable planctomycetes are present in soil.

2. Comparative analyses indicated that two soil treatments that have been used for agriculture are more similar in planctomycetes community composition and diversity measures to each other than to a treatment never used for agriculture. A statistical comparison of the three libraries however, indicated no significant differences in communities, suggesting that *Planctomycetales* are not contributing to differences seen in total microbial community composition, may only be marginally affected by agricultural practices, and/or be capable of faster recovery after cultivation than other microbial groups.

DIRECTIONS FOR FURTHER STUDY:

During the course of the work completed for this dissertation, additional intriguing avenues for later study became apparent. More work is needed to identify the denitrifying organisms whose *nir*K sequences dominate the KBS soil treatments never used for agriculture and those abandoned from agriculture. Isolation of representatives of these groups in pure culture would allow for physiological studies to determine whether these organisms produce less N_2O independent of the physical conditions under which they are grown. The question of whether the dominant denitrifiers in the two treatments are responsible for the lower N_2O flux noted in these soil treatments as compared to the flux from conventional agricultural soils could then be answered.

Another interesting avenue of research involving denitrifiers would be an investigation into the significance of those organisms possessing one type of nitrite reductase over the other. There are two known nitrite reductase enzymes, *nir*K and *nir*S, which are mutually exclusive. These enzymes differ in their structure as well as their K_m for nitrite, with the average K_m for *nir*K being higher than that of *nir*S [1]. This could lead to situations in which denitrifiers with one type of Nir gene would be at a selective advantage over those with the other gene in different environments. In molecular surveys, it is not uncommon to find evidence for only one type of nitrite reductase (Table 5.1), as was the case for the experiments presented in Chapter 3. It is difficult to discern a clear trend as to which Nir genes are found where, but in general, *nir*K organisms tend to be found in a more diverse set of environments. Organisms possessing *nir*S are not found in soil very often and seem to favor consistently wet environments, such as activated sludge or water-body sediments. The lack of *nir*S-bearing organisms in soil

Table 5.1: Environmental Surveys for *nir*K and *nir*S

Environment	*nir*K Presence (+/-)	*nir*S Presence (+/-)	Reference
Enrichment culture for denitrifying methylotrophic bacteria	+	+	[2]
Activated sludge	+	+	
Water from Lake Kleiner Ploner See	+	+	
Sediment from Lake Kleiner Ploner See	+	+	
Water from Lake Plusse	+	+	
Activated sludge samples (7 total)	+ (in 7/7)	+ (in 5/7)	[3]
Washington continental margin sediment	+	+	[4, 5]
Puget Sound sediment	-	+	
Activated sludge from a paper mill	-	+	[6]
Paper mill effluent	-	+	
River Colne estuary sediment (presence and expression)	+ [a]	+ [a]	[7]
Forested, upland soil	+	-	[8]
Marsh soil	+	+	
Silt Loam Soil	+	-	[9]
Sediment from an O_2 deficient zone on the western Mexican continental margin	+	+	[10]
Nitrate and uranium contaminated groundwater	+	+	[11]
4-chlorobenzoate degrading denitrifying consortia from estuary sediment and agricultural soil	-	+	[12]
Six soils (agricultural, garden, rainforest, and termite nests)	+	NA [b]	[13]

Table 5.1 (continued):

Environment	*nir*K Presence (+/-)	*nir*S Presence (+/-)	Reference
Soil and stream sediment from Great Smoky Mountains and Oak Ridge National Laboratory	+	NA	[14]
Arable soil from three sites	+	+	[15]
Peat sample from small fen	+	+	
Activated sludge from wastewater treatment plant	+	+	
Denitrifying water column of the coastal Arabian Sea	NA	+	[16]
Rhizosphere soil from putting greens	+	+	[17]
Agricultural soil receiving either mineral fertilizer or cow manure:			[18]
March (before growing season)	+	+	
July (growing season)	+	-	
October (after growing season)	+	-	
Oxygen minimum zone of water column off the coast of Chile	NA	+	[19]
Rhizosphere samples from legume crops (presence and expression)	+	-	[20]
Activated sludge from a sequencing batch reactor	NA	+	[21]
Activated sludge from a municipal wastewater treatment plant	+	+	[22]
Beach aquifer	+	+	[23]
Grassland soil microcosms subjected to freeze-thaw (presence and expression)	+[a]	+[a]	[24]

[a] *nir*K was present but not expressed, *nir*S was both present and expressed.
[b] NA, not tested for.

may be due to a lesser ability to compete in this environment, but could also be due to poor primer design [15] or seasonal effects [18]. Resolution of this issue would serve to increase our knowledge regarding soil denitrifier ecology.

The last few suggested areas of interest deal with processes in the nitrogen cycle besides denitrification by denitrifying microorganisms. Dissimilatory nitrate reduction to ammonium (DNRA) and denitrification by nitrifiers (nitrifier-denitrification) are two microbial processes known to take place in soil that should receive more attention. Under anaerobic conditions, DNRA organisms compete with denitrifiers for nitrate, but unlike denitrification, under acidic conditions the end product of DNRA is not a gas that is lost from the system. It has been hypothesized that DNRA organisms are selected for in high carbon, low electron-acceptor (NO_3^-) environments and that the opposite conditions select for denitrifiers [25, 26]. Therefore, the relative importance of each of these dissimilatory processes may vary depending on the soil environment. For example, at the KBS LTER the conventional agriculture treatment has a relatively high amount of NO_3^- (6.54 ± 0.53 μg NO_3-N g^{-1}) and low amount of organic C (0.94 ± 0.05 kg C m^{-2}) while the opposite is true of the never-tilled mid-successional treatment (0.47 ± 0.03 μg NO_3-N g^{-1} and 2.84 ± 0.22 kg C m^{-2}) [27]. It would be worthwhile to determine whether DNRA dominates in the never-tilled treatment and denitrification dominates in the conventional agriculture treatment, as this may be a contributing factor to the low N_2O flux from the never-tilled treatment.

In addition to determining the role of DNRA in the soil nitrogen cycle, a better understanding is needed of the contribution of nitrifier-denitrification to nitrogen loss from soil. Ammonia oxidizing nitrifiers will denitrify when under stressful conditions,

such as low pH and low oxygen concentration. The end product of nitrifier-denitrification can be N_2O or N_2, which contributes to nitrogen loss. The contribution of nitrifier-denitrification to soil N_2O production is still under debate, with present estimates ranging from insignificant amounts [28] to ~30% of N_2O production [29]. Owing to the influence of environmental factors on whether nitrifier-denitrification occurs and the difference in physical characteristics of soil at different sites, this process may vary in relative importance between treatments, just as with DNRA.

The last suggestion for further study involves a recent discovery that would have interesting repercussions in an agricultural soil environment. This was the observation of anaerobic ammonia oxidation (anammox) occurring in a biofilm from a denitrifying wastewater treatment plant. 16S rDNA analysis indicated that the organism responsible for anammox was a member of the *Planctomycetales* [30]. The net anammox reaction is written as the equation: $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$ and has a favorable Gibb's free energy change of $\Delta G^{\circ\prime} = -297$ kJ/mol NH_4^+ [31]. The occurrence of this reaction in wastewater treatment plants has proved effective in removing nitrogen sources as dinitrogen gas [32]. In an agricultural setting, chemical fertilizers high in nitrogen content are applied, and in most soils there exist anaerobic microsites within soil aggregates [33]. These sites should be capable of supporting anaerobic microorganisms that may be capable of using the anammox reaction as an energy supply. Theoretically, anammox can convert NH_4^+ added as chemical fertilizer and NO_2^- from nitrification or denitrification into dinitrogen gas. This N_2 can then diffuse out of the soil, wasting a significant amount of time, effort, and money on the part of the farmer. Therefore,

determination of whether, and to what extent, anammox occurs in soil would be important so that methods to control anammox could be developed.

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APPENDIX

A STRATEGY FOR CAPTURING PHYLOGENETICALLY USEFUL INFORMATION IN BAC OR FOSMID LIBRARIES

INTRODUCTION:

Current estimates of bacterial numbers in soil are approximately 1 x 10^9 cells per gram dry weight of soil, comprising greater than 10^5 species [1]. Cultivation attempts routinely recover ca. 1% of this soil bacterial population [2, 3], making it difficult to learn about the metabolic capabilities of not-yet-cultured organisms that may play important ecological roles in the soil environment. As a result, studies employing molecular techniques in place of cultivation have become common. These techniques allow an assessment of the diversity of populations based on phylogenetically conserved genes such as 16S rDNA to be performed. Unfortunately, these surveys do not provide information about the metabolic potential of the organisms studied.

Recently, metagenomic experiments employing the use of bacterial artificial chromosome (BAC) or fosmid vectors have been successful in cloning large pieces of environmental DNA [4, 5]. Typically, BAC and fosmid libraries have average insert sizes of 50 to 100 kb and 40 kb respectively, so that when a clone containing a phylogenetically conserved gene is identified, there are many other functional genes captured as well. Sequencing of the clone then provides information not only about the probable identity of the organism, but also about its metabolic capabilities.

One disadvantage of the classical BAC and fosmid library cloning strategies is that extensive screening is required to identify the few clones containing phylogenetically useful genes. For example, in a BAC library from soil made up of 24,400 clones, only 20

(ca. 0.1%) were found to possess rRNA genes [6]. A second disadvantage is that screening of clones for rRNA genes with universal primers or probes is made more difficult due to the presence of host cell DNA. Therefore, the strategy described in this appendix was initiated in an effort to create libraries where all DNA cloned would have rrn operon-bearing ends and hence be in a location on the BAC where end sequencing of the insert would result in easy identification of its source. While the method ultimately was not successfully employed using environmental DNA from soil, progress was made in cloning DNA from a pure culture of *Xanthomonas campestris* pv. *campestris* ATCC 33913. Insights and progress made during the course of the project are reported.

DESCRIPTION OF BAC LIBRARY STRATEGY:

In order to capture rRNA genes in each BAC clone, a vector (SuperPhyloFOS) was created containing a restriction site for I-*Ceu*I (New England Biolabs, Beverly, MA). I-*Ceu*I is a homing endonuclease isolated from a large subunit rRNA gene of a chloroplast in *Chlamydomonas eugametos* [7]. This endonuclease has been demonstrated to recognize and cleave within a recognition site of approximately 26 bp present in most prokaryotic 23S rRNA genes [8]. Cloning DNA that has been cut on one end with I-*Ceu*I will ensure that a portion of the 23S rRNA gene is inserted into the vector along with other genetic information. Also, due to the conserved order of genes within the rRNA operon, there is a high likelihood that the 16S rRNA gene would be captured along with about one-third of the 23S rRNA gene (Figure 1).

The BAC cloning scheme successfully used to clone *X. campestris* DNA is outlined in Figure 2 (see Figure 3 for a map of SuperPhyloFOS). The vector was

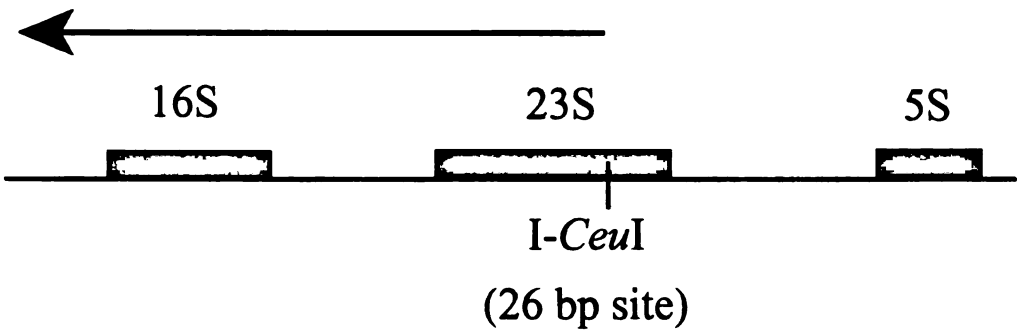

Figure 1: Typical orientation of an rRNA operon. Due to the nonpalindromic nature of the I-*Ceu*I cut site and the orientation of the site in pSuperPhyloBAC, approximately two-thirds of the 23S rRNA gene and the complete 16S rRNA gene (arrow) of insert DNA will be ligated into the vector.

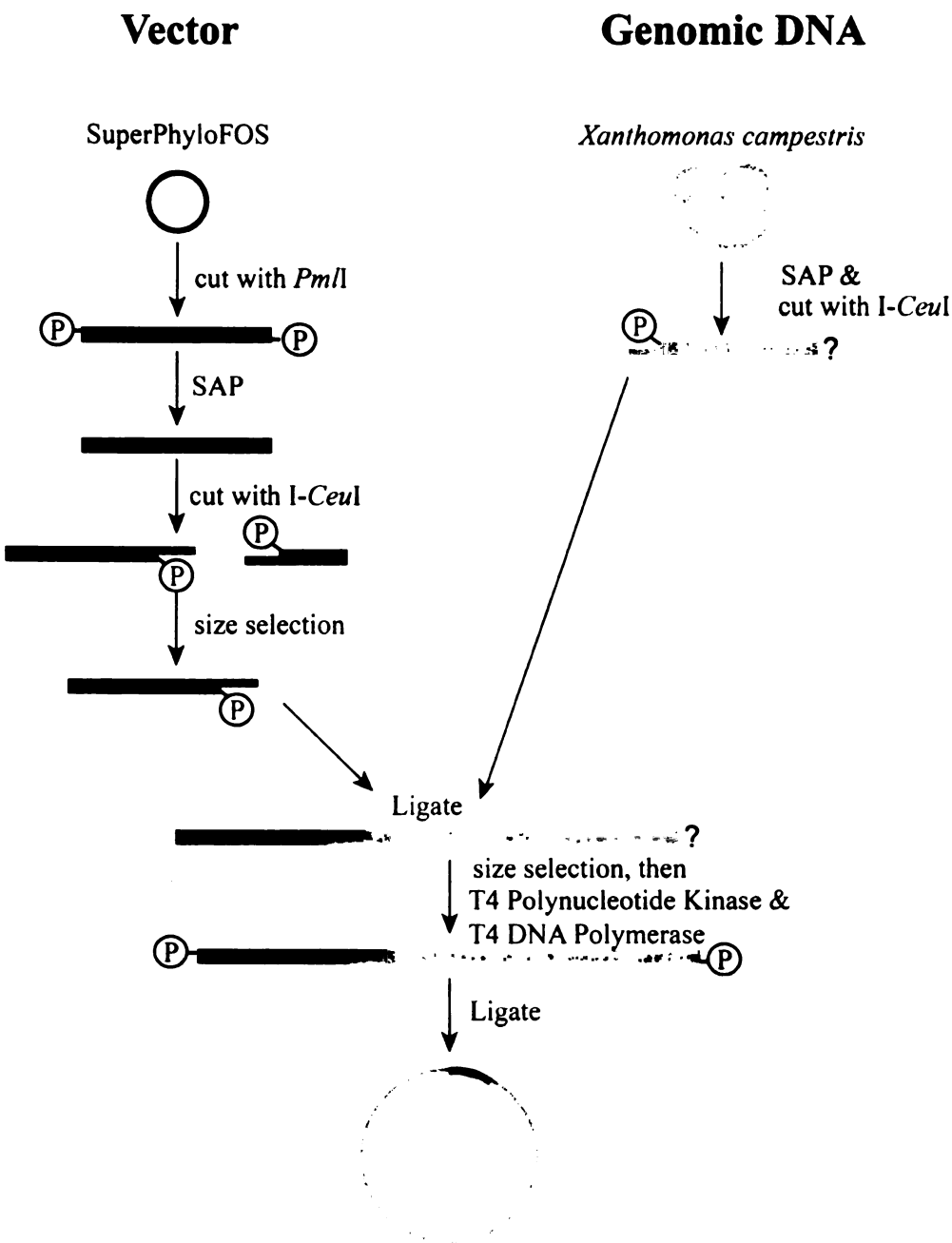

Figure 2: Scheme followed during cloning of *Xanthomonas campestris* pv. *campestris* ATCC 33913 DNA into SuperPhyloFOS. A (?) denotes a DNA end resulting from shearing; therefore it is unknown whether the end has an overhang or is blunt.

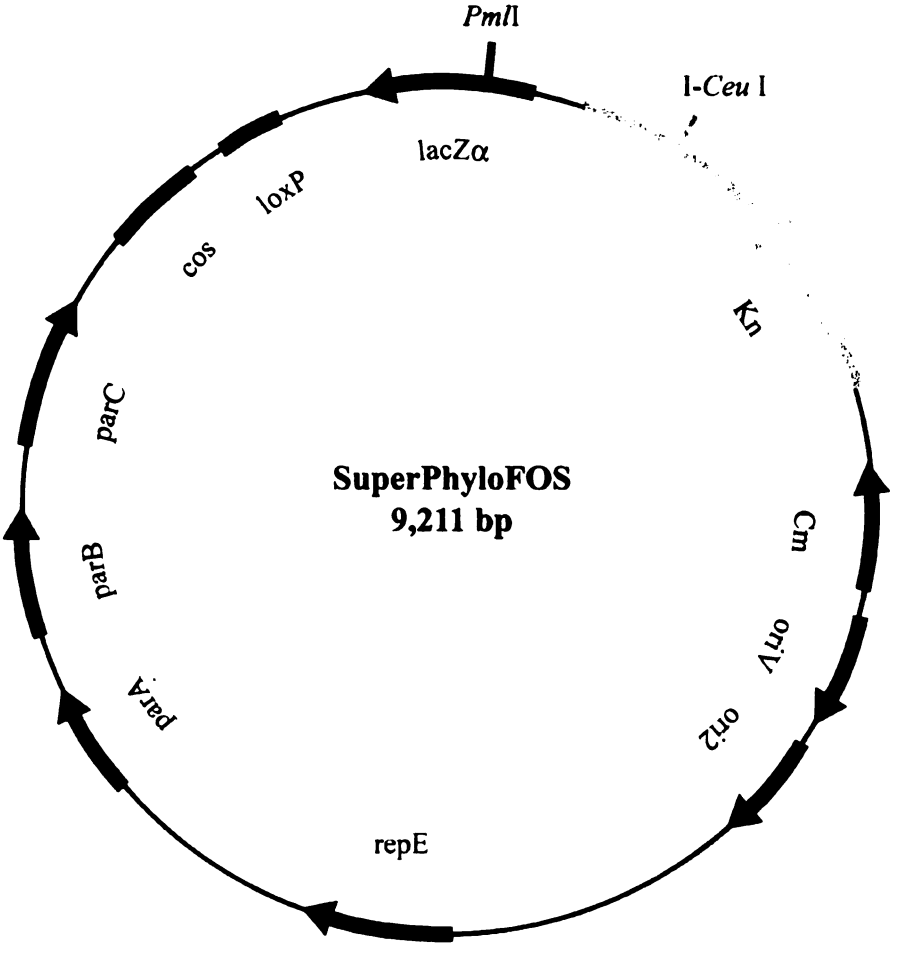

Figure 3: Map of the vector pSuperPhyloFOS (not drawn to scale). Section in light gray is the insert from pSCANS. Black section is the pCC1FOS backbone.

prepared by restricting 10.7 μg once with 40 U of *PmlI* (New England Biolabs) at 37°C overnight and inactivating the *PmlI* at 65°C for 20 min. *PmlI* produces blunt ends which were dephosphorylated by incubating at 37°C for 1 hr with 500 U of Shrimp Alkaline Phosphatase (SAP; Roche, Indianapolis, IN) in the provided buffer and inactivating the SAP at 65°C for 15 min. Next, the vector was cut once with 20 U of I-*CeuI* (New England Biolabs) in NEBuffer 4 for 5 hrs at 37°C and then the I-*CeuI* was inactivated at 65°C for 20 min. This resulted in the vector being cut into two pieces; the desired backbone into which DNA would be cloned and a small piece located between the *PmlI* and I-*CeuI* cut sites whose removal resulted in the loss of lacZ expression. Size selection was performed with a CHEF-DR™ II Pulsed Field Gel Electrophoresis (PFGE) Apparatus (BioRad, Hercules, CA) set to 12°C, 100 V, and 1 to 2 s pulses for 15 hrs to separate the two pieces of vector. The desired piece of vector was cut from the gel, electroeluted into dialysis tubing containing 1 mL of 0.5X TBE, and concentrated. Electroelution was performed using the PFGE apparatus set to 12°C, 50 V, and 1 to 2 s pulses for 3.5 hours. A Centricon-100 (Amicon, Inc., Beverly, MA) was used to concentrate the electroeluted DNA using the manufacturer's protocol. 20 μg of insert DNA (*X. campestris*) was prepared by first dephosphorylating with 60 U of SAP in NEBuffer 4 at 37°C for 1 hr and inactivating the SAP at 65°C for 15 min. The insert DNA was then restricted by adding 60 U I-*CeuI* to the inactivated SAP reaction and incubating for 5 hrs at 37°C followed by inactivation of I-*CeuI* at 65°C for 20 min. This treatment of both vector and insert DNA resulted in only the I-*CeuI* cut ends possessing phosphate groups; therefore these were the only ends capable of ligation. Since the I-*CeuI* site is non-palindromic, the vector was unable to ligate to copies of itself. Next,

ligation with 2000 U T4 DNA ligase (New England Biolabs) was performed with a mix of vector and insert DNA at 16°C overnight followed by inactivation of the ligase at 65°C for 20 min. Size selection with a horizontal gel was performed with a 0.7% agarose gel in sterile 0.5X TBE run at 4°C and 115 V for 1.5 hrs. This was done to eliminate small pieces of DNA. DNA greater than ca. 23 kb in size was cut out of the gel, electroeluted into 500 μL of 0.5X TBE at 115 V for 2 hrs, and the DNA ends blunted and phosphorylated with an End-It™ DNA-Repair Kit (Epicentre, Madison, WI) following the manufacturer's protocol. After this, another ligation reaction with the linear DNA was set up using 800 U of T4 DNA ligase and incubation at 16°C overnight. This caused the DNA to ligate into circular forms for transformation into *E. coli* by electroporation. Screening of the library was performed by cutting the cloned BACs once, running them on a horizontal gel and selecting those running at greater than the size of the BAC alone for sequencing. The forward sequencing primer used for end sequencing of the inserts was the pCC1FOS forward primer (FP) provided with Epicentre's CopyControl™ Fosmid Library Production Kit. A reverse sequencing primer (RP) was developed in the lab that was specific to SuperPhyloFOS (5' - GGT TGT AAC ACT GGC GAG - 3').

The cloning scheme outlined above was arrived at through many experiments and involved much trial and error. The three most challenging steps in creating a library using the I-*Ceu*I strategy were the creation of the vector, preparation of large pieces of insert DNA and vector, and the optimization of restriction, ligation and transformation. These steps are commented on in more detail in the following sections in order to provide guidance to others who may wish to use this or other large insert library strategies.

CONSTRUCTION OF THE BAC VECTOR:

A new vector, SuperPhyloFOS, was constructed which contains an I-*Ceu*I restriction site (Figure 3). The vector was constructed so that it could be used as a BAC or fosmid, although it was only employed as a BAC in the experiments described here. SuperPhyloFOS was created by inserting a portion of the pSCANS plasmid (a gift from Dr. John Dunn of Brookhaven National Laboratory) containing an I-*Ceu*I site and kanamycin resistance gene into a pCC1FOS (Epicentre) plasmid. When SuperPhyloFOS is transformed into *E. coli* strain JW366 (sold as One Shot™ Electrocompetent GeneHogs from Invitrogen; Carlsbad, CA or EPI300™-T1^R Phage T1-Resistant *E.coli* from Epicentre), it is inducible to high copy number upon addition of L-arabinose to the media. *E. coli* JW366 contains a defective transcription factor (TrfA) linked to an arabinose promoter that will act on the oriV of the vector. Replication begins and since the defective transcription factor cannot disengage from the oriV, replication continues indefinitely. For blue/white selection to occur, the vector needs to be cut with both I-*Ceu*I and a restriction enzyme with a site within the lacZα gene. Both kanamycin and chloramphenicol resistance genes are present for antibiotic selection.

The identity and properties of SuperPhyloFOS were confirmed experimentally. The sequences of both pSCANS and pCC1FOS are known, and using this information, a map of the theoretical sequence of SuperPhyloFOS was made. Restriction digests were used to confirm that the desired cloning sites were present and that the I-*Ceu*I site could be restricted properly. The lacZα gene was inducible with IPTG and cells were blue when in the presence of X-gal. It is important to note that induction with IPTG is required for colonies without inserts to turn blue on selective plates. Induction with L-

arabinose to high copy number was also functional as seen in comparisons of plasmid preparations from induced and non-induced cells. Once the identity and desired properties of SuperPhyloFOS were confirmed, attempts to clone DNA from a pure culture of *X. campestris* were made to test whether the cloning scheme was feasible.

PURE CULTURE AND ENVIRONMENTAL DNA EXTRACTION:

Pure culture DNA: Embedding cells within agarose plugs prior to cell lysis and DNA restriction is commonly used in construction of large insert libraries in order to reduce the amount of DNA shearing. However, since lysis and DNA restriction within plugs was found to be inconsistent between different batches of plugs the Marmur procedure [9, 10] was used to isolate genomic DNA. Genomic DNA from a pure culture of *X. campestris* isolated using the Marmur procedure yielded DNA ranging in size from 24 to 145.5 kb.

Environmental DNA: Initially, indirect DNA extraction methods were employed to obtain DNA from soil cells. Indirect methods were used first as they are less likely to result in the shearing of DNA because cells are extracted from the soil matrix before lysis. Blending, sonication, and combinations of the two were applied to soil in an effort to dislodge cells from sediment particles and isolate them from the soil matrix. The supernatant created from these techniques was then applied to an Optiprep density gradient (Axis-Shield, Oslo, Norway) with a density of 1.320 g mL^{-1}. Extracted cells were cast into plugs of 1% InCert agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME), stored until lysed, and after lysis the DNA was sized on a PFG. The indirect extraction method was inefficient, resulting in percent recoveries of cells ranging

from 0.09 to 1.9%. Most cells were lost during the extraction process when large particles were allowed to settle out of the supernatant, resulting in the settling out of any cells still attached to particulate. An additional issue was the presence of humic acids along with cells, which interfered with subsequent cell lysis and restriction within the agarose plugs.

Due to the above mentioned issues, a direct method of DNA extraction from soil based on that of Zhou and colleagues [11] was developed. This method has been shown to produce minimal shearing of DNA and employs a buffer containing sodium dodecyl sulfate (SDS), hexadecyltrimethylammonium bromide (CTAB), and proteinase K. A chloroform-isopropanol precipitation is then performed to recover the nucleic acids. As with the indirect extraction method, humic acid contamination is routine. To reduce the amount of humic acids present in the DNA, extracted samples were run on a horizontal gel. Large pieces of DNA moved slowly as one large band through the gel, while humics were drawn through more quickly. After the section of gel containing large pieces of DNA was excised, electroelution in a PFG apparatus was used to extract the purified DNA. DNA was then rinsed and concentrated using a Centricon-100 and sized by PFGE. DNA sizes ranged widely, from less than 9.4 kb to greater than 97 kb (Figure 4), which was considered acceptable for construction of BAC or fosmid libraries.

OPTIMIZATION OF RESTRICTION, LIGATION, AND TRANSFORMATION:

Restriction of vector and insert: Restriction of both vector and insert was found to work best under conditions where contamination from cellular or environmental components was minimized. For this reason, SuperPhyloFOS was isolated from the

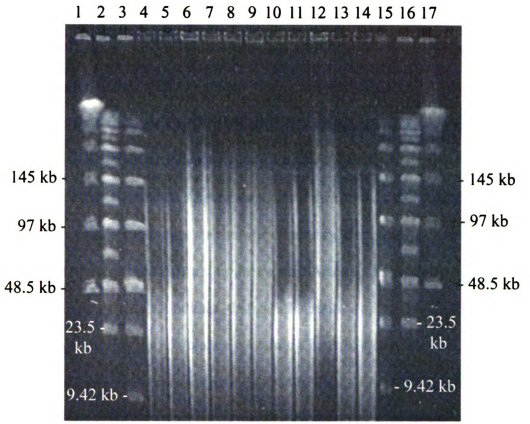

Figure 4: PFG of indirectly extracted soil DNA. Lanes 1 & 17 are Lambda Ladder PFG Markers; 2 & 16 are Mid-Range II PFG Markers, and 3 & 15 are Low-Range PFG Markers (all from New England Biolabs). Lanes 4 - 5 are from two extracts of deciduous forest soil; Lanes 6 - 7 are from two extracts of mid-successional, never-tilled soil. Lanes 8 - 11 are from four extracts of soil abandoned from agriculture; and Lanes 12 - 14 are from three extracts from conventional agricultural soil.

genomic DNA of its host cell with a CsCl gradient and concentrated by ethanol precipitation [12]. The CsCl gradient resulted in DNA with fewer contaminants and DNA yields were higher than with standard plasmid preparation kits. Genomic DNA from pure cultures was isolated with the Marmur procedure as described previously, resulting in minimal contamination from cellular components. As with pure cultures, the restriction of DNA from soil cells embedded in agarose plugs was problematic. There was a high amount of variability in the ability to lyse cells within the plugs and restriction required optimization for each batch of plugs. This was presumably due to the presence of humic acids and other contaminants co-extracted with the DNA.

Ligation: Ligations with *X. campestris* DNA were set up to maximize the amount of insert DNA present; therefore, molar ratios ranging from 1:10 to 1:50 of vector to insert were commonly used (assuming an average *X. campestris* DNA size of 20 kb), although preliminary results indicate that a 1:10 ratio worked best. The 1:10 ratio ligation resulted in 3 out of 10 clones containing inserts, while the 1:50 ratio ligation resulted in only 1 out of 10 clones containing inserts.

Transformation: A study was performed to determine what voltage would maximize the transformation efficiency of clones with inserts of approximately 100 kb, as well as whether desalting of the sample had an effect on transformation efficiency. Two clones from a common bean (*Phaseolus vulgaris* L.) BAC library constructed by Melotto and colleagues [13] were used in the study along with a 12 kb plasmid. Electroporation was carried out with 50 ng of DNA in cuvettes with 1 mm gaps on a GenePulser Xcell (BioRad) set to 100 Ω and 25 μF. Desalting was performed with agarose cones as recommended in Appendix B of the Epicentre CopyControl™ BAC

Cloning Kit. The best voltage for transformation of both BACs was 1300 V (Figure 5), while for the small plasmid it was 1700 V. Generally, as the size of the transformed DNA increased, its overall transformation efficiency decreased. Desalting resulted in increases in transformation efficiency of the 115 kb and 79 kb BACs of 37% and 142%, respectively. Therefore, a transformation voltage of 1300 V and desalting of the ligation reaction prior to transformation is recommended when creating BAC libraries.

SUMMARY:

The cloning scheme outlined in Figure 2 was successfully employed in cloning genomic DNA from *X. campestris*. Of the 20 clones screened by end sequencing of the insert, the largest insert size found was only 7.3 kb making the maximization of insert size the next logical step in this particular set of experiments. Once optimized, the procedure could be used to create BAC libraries of environmental DNA.

One option that was proven effective by Nesbø and colleagues [14] was very similar to that proposed here. An adaptor containing the I-*Ceu*I recognition sequence was ligated into the pCC1FOS (Epicentre) vector and the new construct used as a fosmid instead of a BAC. Fosmids are packaged as linear DNA into lambda phage particles and then inserted into a host cell through transduction; the DNA is then circularized and maintained in the cell. Because fosmid packaging requires a cos site (located on the vector) and a total of approximately 40 kb of linear DNA, all clones will contain large insert DNA ligated to the vector. SuperPhyloFOS also should be functional as a fosmid, and this may be the most efficient way to construct genomic libraries using the I-*Ceu*I strategy.

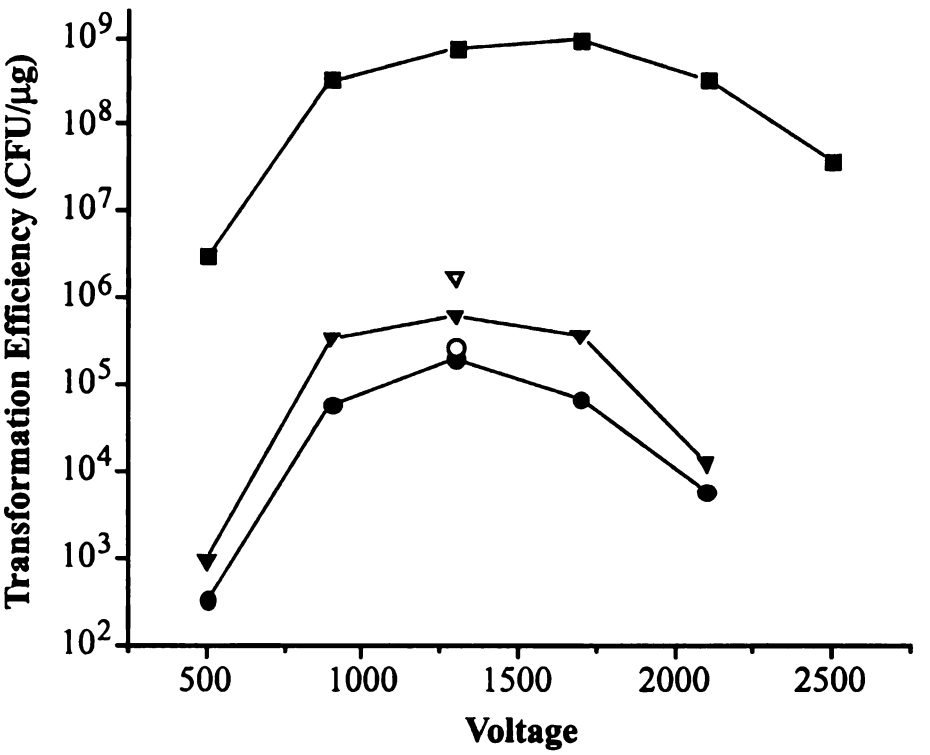

Figure 5: The dependence of transformation efficiency on voltage and desalting of sample prior to transformation. (■) 12 kb plasmid control, (▼) 79 kb BAC, (●) 115 kb BAC, (▽) desalted 79 kb BAC, and (○) desalted 115 kb BAC.

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