NOTE TO USERS

DISSERTATION

PINE WOOD SHAVINGS CONTRIBUTE NON-DENITRIFYING NITRATE REDUCERS AND MICROEUKARYOTES TO PERMEABLE BARRIER MICROCOSMS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPRARED UNDER OUR SUPERVISION BY GAIL BERARDINO-LANG ENTITLED PINE WOOD SHAVINGS CONTRIBUTE NON-DENITRIFYING NITRATE REDUCERS AND MICROEUKARYOTES TO PERMEABLE BARRIER MICROCOSMS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTORATE OF MICROBIOLOGY.

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ABSTRACT

PINE WOOD SHAVINGS CONTRIBUTE NON-DENITRIFYING NITRATE REDUCERS AND MICROEUKARYOTES TO PERMEABLE BARRIER MICROCOSMS

Wood products are used in permeable barriers as a reductant to enhance microbial denitrification to decrease the amount of nitrate from agricultural runoff which enters into tributaries in an effort to control the hypoxia that threatens fishing and recreational industries. Data with regards to the identities of the microbial populations in permeable barriers, especially denitrifiers and other nitrate reducers are required to design and manage permeable barriers for denitrification. Jar, syringe and flow-through column permeable barrier microcosms were analyzed. Diversity was assessed through EL-FAME ordination and fatty acid profiles by treatment while nitrate reduction activity was determined in isolates cultured anaerobically on high protein media with potassium nitrate. Isolates were subsequently tested for denitrification ability in durham tubes containing nitrate broth.

Addition of wood to soil in a permeable barrier affects the number of denitrifiers introduced as well as competing nitrate reducers and microeukaryotes. Abundant fatty acids in wood are produced by microeukaryotes while the abundant fatty acids in soil, exclusive of water samples, are produced by eubacteria. Microeukaryotes contribute to temporary fixation of nitrate in biomass as well as the release of ammonium through grazing of bacteria. There are significantly more culturable nitrate reducers in permeable

barrier microcosms than in soil controls. Wood controls have significantly more culturable nitrate reducers than soil; therefore, addition of wood increases nitrate reduction through the introduction of microbes as well as from the addition of carbon. Less than 1% of nitrate reducer isolates from wood denitrified which suggests that the majority of nitrate reducers in wood are non-denitrifying or that denitrification ability is inhibited by the wood. The denitrifiers most commonly isolated from permeable barrier microcosms are fluorescent pseudomonads and *Azospirillum* species which are potential candidates for denitrifier supplementation due to their ability to compete with non-denitrifiers, elude grazing, survive during periods of low nitrate input and scavenge micronutrients. Water samples from permeable barrier microcosms have less variance than solid samples, equal numbers of nitrate reducers and higher odds for isolation of denitrifiers which indicates that samples from wells and effluent from permeable barriers are important for analysis of denitrifiers in permeable barriers.

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DEDICATION

This dissertation is dedicated to my husband Jonathan E. Lang

for all his love and support.

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CHAPTER I

INTRODUCTION

1.0 Nitrate

Nitrate (NO_3^-) is an anionic nitrogen oxide compound composed of one nitrogen and three oxygen molecules, the most oxidized form of nitrogen with a valence of $^{+5}$. Nitrate is relatively inert in natural waters and concentrations in aquatic systems are low (0.5-0.8 ppm). Nitrogen concentrations in agricultural drainage water are mostly in the form of NO_3^- and may range from 1 – 100 ppm (43).

A five thousand square mile zone in the Gulf of Mexico becomes anoxic each summer due to eutrophication and hypoxia from excess nutrients such as nitrogen and phosphorous. As a result, the fishing and recreation industries along the gulf coast are adversely affected. The Mississippi River is the predominant source of excess nitrogen in the form of NO_3^- with agricultural non-point sources contributing 74% of this NO_3^-. The Mississippi River/Gulf of Mexico Task Force recommends that a 30% reduction in nitrogen load is required to reduce the hypoxic zone (56).

Biological action is the main factor in transformations of NO_3^- nitrogen (43). Riparian zones are natural permeable barriers for NO_3^- removal from surface and shallow ground water. Vegetation along stream banks and in the wetlands that adjoin streams supplies carbon from plant roots to bacteria in the rhizosphere for use in NO_3^- reduction reactions under oxygen-limited conditions. Nitrate and ammonium (NH_4^+) may also be assimilated by plants and become part of the vegetative biomass (10).

Engineered barriers are being explored because of eroded stream banks and insufficient vegetation in certain riparian areas or because the input of NO_3^- in other cases exceeds the ability of the natural riparian zone to successfully remove it. Permeable barriers are designed to supplement the subsurface soil with wood, leaves or straw to supply carbon and electron sources for the microbial reduction of NO_3^- and its removal from water as it passes through. An ideal barrier facilitates complete denitrification to nitrogen gas (N_2) and removal of excess nitrogen from the terrestrial and aquatic ecosystem. Incomplete denitrification to nitrous oxide (N_2O) is less desirable since N_2O is a greenhouse gas that contributes to the destruction of the ozone layer and global warming while N_2 is highly stable and unreactive.

Permeable barriers that are effective for the removal of NO_3^- from water are composed of soil, sand and sawdust (58), soil and sawdust (63, 64) or tree bark, wood chips, leaf compost and sand (7). The addition of easily decomposable carbon substances such as glucose, mannitol and sucrose have much greater effects than sawdust and lignin which are difficult to decompose but have limited application in the field due to economic

constraints (13). The recalcitrance of wood products to degradation is considered beneficial as it prolongs the ability of the barrier to reduce NO_3^- (64).

1.0.1 Wood in Permeable Barriers

1.0.1.1 Soil:Sawdust Denitrifying Barrier

Permeable barriers are buried in the soil to intercept surface (7) or groundwater (7,63,21,58) for the purpose of removing contaminants. Field experiments that examine the use of sawdust or wood shavings to stimulate the removal of NO_3^- from water are limited. A five-year investigation on carbon dynamics and denitrification with *Pinus radiata* sawdust in a permeable barrier with a ratio of 2:1 soil:sawdust (64) reports continuous removal of 95% of incoming NO_3^- ranging from 5-15 ppm. Microbial denitrification as measured by the DEA method (see section 1.4.1) is highly variable over the course of the trial and the specific microbial populations involved are not identified. The authors point out that estimates for the longevity of the effectiveness of a barrier to remove NO_3^- by denitrification do not take into account anaerobic degradation of complex organic matter by fermentation and don't consider the production of NH_4^+, which may be incorporated into biomass (17, 84) or adsorbed to clay particles (68) or organic matter (10).

1.0.1.2 Septic-System Denitrification

Initial leaching of dissolved organic carbon (DOC), as well as sulfate reduction and iron accumulation are the results of NO_3^- removal in a permeable barrier designed to denitrify septic-system effluent (58). The results suggest that reducing conditions are below the

optimum for denitrification. This investigation does not address the microbial community but the results suggest that lithotrophic denitrifiers that use sulfur may be involved.

1.0.1.3 Tile Drainage Bioreactors

The removal of agricultural NO_3^- from tile drainage with bioreactors using an inoculum from the black layer of sediment generates a decrease in sulfate as well as NO_3^-, plus increases reduced iron, manganese and carbonate. Increases in DOC in the effluent are also observed. Again, however, there is no report of the microbial community involved. (7) The black layer of sediment is usually associated with reducing conditions conducive to microbial sulfate and iron reducer populations.

1.1 Denitrification and Competing Nitrogen Oxide Reduction Reactions in the Soil

1.1.1 Nitrogen Oxide Reduction Pathways

The phenomenon of NO_3^- reduction by bacteria in the soil has been continuously investigated since Gayon & Dupetit first described it in 1886 (29, 13). Details of individual contributions to the field can be found in a number of reviews on the subject (73, 29, 13, 85). The biochemistry of denitrification distinguishes it from dissimilatory NO_3^- reduction, assimilatory NO_3^- reduction, fermentation of NO_3^-, codenitrification, chemodenitrification and the anammox reaction.

1.1.1.1 Denitrification

Denitrification is a microbial step-wise process that reduces the nitrogen oxide species of NO_3^- and NO_2^- to the gaseous nitrogen species nitric oxide (NO), N_2O and N_2 (13). As

there are other pathways that produce gaseous nitrogen oxides as intermediates, there is a second part to the definition of denitrification. Denitrification uses nitrogen oxides as terminal electron acceptors in the respiratory chain and results in the production of energy, in the form of ATP, plus an increase in microbial biomass (73). This theory was first proposed by Weissenberg in 1897 (13). Denitrification occurs under reducing conditions with eH of $^{+}$350 to $^{+}$100 mV (78). However, it has been reported that eH may not be a reliable indicator of denitrification *in situ* since it depends upon mineralization rates, availability of enzyme catalysts, proton and electron buffering capacity and the rate of oxygen diffusion. (48).

The chemoheterotrophic organisms involved in denitrification are facultative anaerobes. They use nitrogen oxides as terminal electron acceptors in the anaerobic respiration pathway. It is believed that they respire aerobically using oxygen as the terminal electron acceptor when it is available and switch to NO_3^- as the terminal electron acceptor when oxygen is not available. Since the same electron transport apparatus is used in both pathways, the switch from aerobic to anaerobic metabolism occurs quickly. Five electrons are used to reduce one molecule of NO_3^- (78) and the process is considered thermodynamically favorable when the reduction of NO_3^- is tied to the oxidation of organic carbon (43, 14). This is why organic carbon sources are used in permeable barriers.

Facultative anaerobic microorganisms capable of denitrification include phylogenetically diverse genera of eubacteria as well as fungi and archaea. Denitrifiers encompass

microbes with phototrophic and lithotrophic metabolisms as well as the general aerobic organotrophs. And there are species that are fermenters, halophilic, thermophilic, spore formers and nitrogen-fixers. Therefore, denitrifiers can survive in a wide array of environmental conditions. (85) Products of denitrification are a mix of NO, N_2O and N_2. The ratio of these products is dependent upon abiotic factors such as oxygen levels, saturation and pH, as well as microbial diversity.

Incomplete respiratory denitrification is the generation of N_2O as the final product of denitrification. Denitrifying strains isolated from the environment that lack the ability to reduce N_2O to N_2 are common and cultivation on lab media often causes new environmental isolates to lose the ability to reduce N_2O to N_2 . (73) Whether this ability to reduce N_2O to N_2 is routinely lost (and/or gained) in the environment has not been reported and is discussed further in section 1.1.8.

Since the first report of N_2O production by fungi (8), it has been shown that denitrification occurs in the mitochondria coupled to ATP synthesis (32). A cytochrome P-450nor is the NO reductase used to generate N_2O (76, 45). Further reduction to N_2 does not take place because there is no functional N_2O reductase (83). Fungal species, as well as some actinomycete species, that are currently known to denitrify either generate N_2O exclusively or only a very small percent of N_2 via codenitrification as described in section 1.1.1.7. *Penicillium, Aspergillus, Fusarium, Trichoderma, Chaetomium, Hansenula* and *Cylindrocarpon* have species that denitrify. *Fusarium solani* and

Cylindrocarpon tokinense have been reported to produce N_2; NO_2^- is reported to be a better substrate than NO_3^- for fungal denitrification. (85)

Nitric oxide and N_2O are soluble while N_2 is not. The microbial community in soil and sediment may act to inhibit the formation of N_2 and loss of precious nitrogen from the soil, perhaps dependent on the presence of nitrogen-fixers, reducing conditions and concentrations of NO_3^- and NH_4^+. Nitrous oxide may remain in the soil ecosystem dissolved in the soil solution which would facilitate fixation as the step to go from N_2 to N_2O is energetically costly.

1.1.1.2 Nitrogen-Fixation

Nitrogen-fixation may confound attempts to measure denitrification as it generates NH_4^+ from N_2 gas, which is then assimilated into microbial biomass. It is considered desirable in agricultural fields since it reduces the need to add nitrogen fertilizers. Added labile carbon has been reported to stimulate nitrogen-fixation in flooded soils (78). Bremner and Shaw (1958) point out that nitrogen-fixation may occur after the depletion of NO_3^- under anaerobic conditions where the carbon supply is in excess of that needed by denitrifiers to reduce the available NO_3^- (14).

Nitrogen-fixation is performed by many species of *Cyanobacteria* and other phylogenetically diverse eubacterial genera such as *Rhizobium, Azospirillum, Klebsiella, Erwinia, Citrobacter, Azotobacter, Rhodospirillum* and *Clostridium* either as free living organisms or in symbiosis with plants. Postgate (1998) postulates the free living

nitrogen-fixers are not of great importance in the nitrogen economy of the soil due to their need for large amounts of carbon. (54) However, as this study examines the effect of carbon amendments to subsurface soil, it is expected that free living nitrogen-fixing populations may be found and could be important in a wood amended subsurface ecosystem.

Nitrogenase is the enzyme that catalyzes nitrogen-fixation. It is sensitive to oxygen, suppressed by high ammonia (NH_3^+) and low phosphorous, and is inhibited by pH above 8 and below 5. The process requires energy, in the form of ATP, and molybdenum and sulfur are part of the enzyme complex. Other nitrogenases that use vanadium and iron in place of molybdenum have also been reported. (54)

1.1.1.3 Dissimilatory Nitrate Reduction to Ammonium (DNRA)

Dissimilatory NO_3^- reduction is a process found in organisms with fermentative rather than oxidative metabolism (73). This path results in the reduction of NO_3^- to NH_4^+, which is then excreted into the environment. Gaseous intermediates of NO and N_2O have been reported. The process requires eight electrons per mole of NO_3^- reduced and this path is generally used by fermentative bacteria that are not dependent on the presence of NO_3^- for growth under anaerobic conditions (78). Tiedje (1988) reports that this activity is greatest in carbon-rich, electron acceptor poor environments where the organism uses NO_3^- as an electron sink for energy generation (73). This type of metabolism is found in almost all the *Enterobacteriaceae* and *Vibrionaceae*, some *Pseudomonas* and *Bacillus* species as well as *Clostridium* species. A total of 73 genera have been identified (78).

The NH_4^+ generated by these eubacteria is not incorporated directly into biomass but is excreted into the environment where it may be taken up by other microbes or plants and incorporated into biomass. If conditions are alkaline, it may be converted to ammonia (NH_3^+) and volatilized. Soils that have high kaolinite content may fix NH_4^+ in the interlayers of the clay silicate minerals, organic matter can chelate it, or it may be oxidized to NO_3^- by nitrifying bacteria (10). It may also be converted to N_2 in the anammox reaction discussed in section 1.1.1.6.

1.1.1.4 Assimilatory Nitrate Reduction

Assimilatory NO_3^- reduction results in the reduction of NO_3^- to NH_4^+ and the incorporation of NH_4^+ into microbial biomass of the producer. This is a process that requires energy and occurs in numerous species of bacteria (28, 42) and yeast (67). It is inhibited by high concentrations of NH_4^+ (28) and eight electrons are used to reduce one mole of NO_3^- (67). Microbes prefer to transport NH_4^+ since more energy is needed to transport NO_3^- and then reduce it to NH_4^+ (52, 28).

1.1.1.5 Ammonia Fermentation

Ammonia fermentation is a eukaryotic NO_3^- metabolism that occurs under conditions more anoxic than suitable for denitrification. Fermentation of NO_3^- to NH_4^+ is coupled to acetogenic oxidation of ethanol, glycerol or glucose and also produces acetate (83). Cells cultured with glucose produce low amounts of NH_4^+ and more CO_2 than with other substrates. Zhou *et al.* (2002) suggest this ability may be widespread among soil fungi. This process is similar to acetogenic fermentation coupled to NO_3^- reduction seen in

some *Clostridium* species. There may be microsites within a barrier that would produce conditions favorable for this process.

1.1.1.6 The Anammox Reaction

The anammox reaction is part of an anaerobic NH_4^+ oxidation metabolism where NH_4^+ is used as an electron donor to reduce NO_2^- to N_2. This metabolism is found in organisms belonging to the order *Planctomycetales*, first isolated from waste water. (70) Anammox bacteria are irregularly shaped and grow slowly with up to two weeks required for cell division (29). They have membrane bound compartments called anammoxosomes where the anammox reaction occurs (77). The membrane of this structure contains unique ladderane membrane lipids (66). Anammox bacteria have also been found in the suboxic zone of the Black Sea (37). This reaction is expected under conditions of low carbon plus high NO_2^- and NH_4^+.

1.1.1.7 Codenitrification

Codenitrification is purported to occur in some *Streptomycete* species (36) and in denitrifying fungi (39). This path produces N_2O and small amounts of N_2, through the combination of a nitrogen atom from NO_2^- and a nitrogen atom from a source that is not NO_2^-. The mechanism is a nitrozation reaction involving the transfer of a nitroso group from NO_2^- to a nucleophilic nitrogen compound like an amino acid, catalyzed by NO_3^- reductase (39). Codenitrification would be expected in a permeable barrier if the species involved are present, however it is not known to what extent they would complete with other NO_3^- reducers for subtrates.

1.1.1.8 Chemodenitrification

Chemodenitrification does not involve microbes and results from the combination of hydroxylamine and NO_3^- under acidic conditions, in frozen soils that are thawed, and in dried soils that have been rewetted (8). Chemodenitrification may cause seasonal fluctuations in NO_3^- levels in the field. Samples for the present study were collected in June, were not frozen or dried prior to incubations, and had an alkaline pH.

1.1.2 Nitrate Reduction in Natural Consortia

Any of the nitrogen oxide species can be used as an initial substrate for reduction which makes for complicated interactions among soil microbes that possess a range of nitrogen oxide reductases. Some have reductases to allow for complete denitrification from NO_3^- to N_2 while others possess a limited palate of these enzymes. Since denitrification occurs in a stepwise manner, the amount of product produced in each successive step and its accumulation will affect the transcription of enzymes for the next step, as they are involved in feedback loops (85). Therefore not all denitrification reactions will result in complete denitrification through to the production of N_2, even if the genes for all the required reductases are present. This is dependent on microbial diversity as well as abiotic factors. The main abiotic factors are the type and amount of carbon and the degree of saturation of the soil which affect both denitrifiers and other nitrogen oxide reducers. (14) These abiotic factors are discussed further in sections 1.1.4 and 1.1.6 and 1.1.7.

The diversity of nitrogen oxide reducers will affect the amount of substrate produced at each step, dependent on the number of active organisms that possess the reductase

specific for that step, under the prevailing conditions. There are multiple forms of most of the reductases; they have different affinities for their substrate and variable tolerance to factors such as oxygen and pH (18, 19). Fungal denitrification may have ecological significance because the dominant gaseous end product is N_2O due to the lack of N_2O reductase. These fungi have the potential to generate N_2O in a wider range of soil aeration conditions than bacteria and they are believed to be widely distributed (39).

Tiedje (1988) suggests that it is the general ability to utilize carbon under aerobic conditions that influences prokaryote denitrifier populations (73). Under natural conditions the NO_3^- levels do not remain consistently high enough to support continuous denitrification. In addition, the subsurface soil may not be completely saturated dependent on seasonal water table levels. Since aerobic respiration is productive energetically, the facultative, heterotrophic denitrifier populations may grow quite large during these phases if there is no other limiting factor.

It is suggested that there exists a symbiotic relationship between denitrifiers and anaerobic cellulose fermentors, such as *Clostridium* (3). The denitrifiers use the breakdown products of cellulases and products of fermentation to prevent the repression of the enzymes. A carbohydrate fermentor producing acetic acid and H_2 generates twice the energy of fermentation to propionic or butyric acid. However, they may switch paths when H_2 builds up (3). Lithotrophic denitrifiers such as *Paracoccus*, *Alcaligenes*, *Bradyrhizobium* and *Pseudomonas* use H_2 and limit its accumulation (73).

Similar consortia with fermentors and heterotrophic nitrogen-fixers under anaerobic conditions with low nitrogen concentrations may exist. Some nitrogen-fixers are heterotrophs that can use a limited range of carbon substrates including acetate (78), which may be produced by carbohydrate fermentors (3). Some species of *Clostridium* are capable of nitrogen-fixation and degradation of wood products under anaerobic conditions and may thrive in the presence of wood when NO_3^- concentrations are low. Spirochetes have also been reported in anaerobic cellulolytic enrichments and may associate and interact with cellulose degrading bacteria (40).

Thus, the specific conditions in the soil that precede conditions that are favorable for denitrification would dictate the diversity of the denitrifiers as well as other nitrogen oxide reducers. The population sizes of the species present when denitrifying conditions are created, the ability of the species to tolerate the above mentioned factors that affect the rate of metabolism and the affinity of their particular reductases will be factors in the products of NO_3^- reduction.

Additionally, denitrification itself is only one part of the nitrogen cycle. Concurrent nitrification and denitrification intertwine the products and substrates of these reactions with that of dissimilatory and assimilatory NO_3^- reduction as well as ammonification and nitrogen-fixation. Other participants in the nitrogen cycle provide substrates or convert the products back into NO_3^- or NH_4^+. Assimilatory NO_3^- reduction utilizes the same substrates as denitrification. Therefore the potential exists for microbial biomass to be a temporary sink for NO_3^-.

1.1.3 Grazing by Microeukaryotes and Nitrogen Release

Turnover of microbial biomass due to microeukaryotic grazing releases nitrogen into the soil as NH_4^+. Protozoan grazers include raptoral feeders such as amoeba while chrysophyte flagellates are interceptor (suspension) feeders. Grazing activity is restricted to water films and water filled pores and it is believed that small pores will protect eubacteria from grazing. Clay soils generally have small pore sizes (10) while wood products have larger pores. Protozoan populations may increase for up to six weeks after the addition of dead plant material. Non-pigmented Enterobacteriaceae produce high yields of protozoa while pigmented eubacteria such as *Pseudomonas aeruginosa* and *Serratia marcesens* are not considered good food sources for protozoa. (60)

Selective grazing could impact populations of bacteria involved in NO_3^- reduction. Some microorganisms are resistant to amoebae grazing and have evolved to resist internalization or death after ingestion. There is evidence for intracellular survival of *Bradyrhizobium japonicam*, *Francisella tularensis*, *Legionella pneumonophilia, Flavobacterium*, *Mycobacterium leprae*, *M. avium* and *Helicobacter pylori*, among others and for extracellular survival by *Mesorhizobium, Burkholderia*, *Ralstonia picketti*, *Pseudomonas aeruginosa* and *Vibrio cholera*, among others. (24)

Cell size, motility and morphology can also affect grazing resistance in bacteria. Ronn *et al.* (2002) report clear separation of bacterial communities by DGGE profiles in treatments with suspension feeders versus amoebae. They report an increase in high G+C bacteria related to *Arthrobacter* while non pigmented gram negative eubacteria decrease.

They postulate that grazing affects bacterial communities via selective feeding, susceptibility to predation and subsequent effects on nutrient and substrate availability that decrease competition. (60)

1.1.4 Oxygen Levels and Water Saturation

The paradigm for much of the twentieth century states that denitrification is strictly an anaerobic process, inhibited by oxygen. However, evidence that denitrification can sometimes proceed in the presence of higher than expected concentrations of oxygen is mounting (71,36,39,48,51,82). Oxygen represses reductase enzyme synthesis or inhibits enzyme activity (73). Oxygen also inhibits NO_3^- utilization by preventing its uptake into the microbial cell (3, 44). This inhibition has been shown to be maximal at 0.2% oxygen saturation. The synthesis of NO_3^- transport proteins is induced by growth in the presence of NO_3^-. The transport systems for NO_3^- and NO_2^- are energy dependent and the rate of denitrification is affected by uptake of these substrates which is also influenced by carbon sources (3).

Water saturation plays a profound role in the soil with little loss of nitrogen when the moisture content is less than 60% of water holding capacity. Denitrification in soil with a thin layer of water, in ambient air, proceeds at the same rate as in a vacuum or in an atmosphere of N_2. (13) It could be that denitrification is a process that requires an aqueous component, with the water itself being an important factor aside from its ability to limit oxygen diffusion in the soil. Nitrate is soluble and relatively inert in water. It does not readily adsorb to or form complexes with organic matter (43). Therefore,

microbes that reduce it must be in the water to access it, or must be sessile with high volumes of water passing by. The intermediate products of NO_3^- reduction are also soluble and there is a significant correlation between water soluble carbon and denitrification (16). See section 1.1.6 for details.

1.1.5 Chemotaxis

Some denitrifiers exhibit chemotaxis for NO_3^- and it is purported as evidence that denitrifiers are not specialized for soil versus water habitats. *Pseudomonas sp.* represent 62% of the soil isolates that display this behavior. (73) There is also evidence of a chemotactic response by cellulolytic bacteria towards cellulose hydrolyses products (40) and of *Agrobacterium tumefaciens* towards NO_2^- as well as NO_3^- (41). Chemotaxis for substrates would give a competitive advantage to these microbes in aqueous environments.

1.1.6 Carbon Sources

An analysis by Hedin *et al.* (1998) finds that N_2O and NO_3^- persist and accumulate in subsurface water with dissolved organic carbon (DOC) levels of less than 2 ppm whereas subsurface waters with DOC above 4 ppm show no accumulation of N_2O-N above 0.6 ppb and no accumulation of NO_3^-N above 0.3 ppm. (27) Wheat and oat straw are less effective as carbon sources after being rinsed of water-soluble carbon because the insoluble carbon is recalcitrant (14). Soils tested for denitrification show significant correlation ($r=0.77$) between denitrification capacity and total organic carbon as well as very high correlation ($r=0.99$) with water-soluble organic carbon (16). Since the water

soluble fraction is highly correlated with denitrification it is expected that the permeable barrier aqueous solution will be enriched in denitrifiers.

1.1.7 Micronutrients, pH, eH, Temperature and Heavy Metals

The distribution of micronutrients such as Cu, Fe and Mo will affect NO_3^- reduction enzyme activity because they are co-factors in nitrogen oxide reductase complexes. The pH and eH of the soil, the temperature and the amount of substrate also are important factors. Nitrite is toxic to some denitrifiers in slightly acidic soils and low pH can favor the retention of NH_4^+ (13). At pH less than 6, Fe becomes more soluble and at pH below 5 Cu and Mo become less soluble which may influence the activity of the enzymes that require these metals. Above pH 7 Fe and Cu become less soluble while Mo does not (10). Low pH also favors the growth of fungi versus most eubacteria and so may alter the products of denitrification and nitrogen oxide reduction. Heavy metals (Cd, Cu, Zn) inhibit denitrification and increase ammonification in wetland sediment at concentrations of 500 or 100 mg Kg^{-1}, although lower concentrations (100 mg Kg^{-1}) of Cu or Zn significantly increase denitrification (61). The ability of microbes to scavenge for essential metals used in denitrification enzymes, which become less soluble due to pH changes, may give them a competitive advantage over those that cannot.

1.1.8 Genetic Regulation of Denitrification

There are over 80 genes that contribute to the process of denitrification. Detailed descriptions can be found in excellent reviews of the subject (9, 53, 69, 85). Denitrifiers encompass a wide range of phylogenetic classes and it is not possible to use 16S rRNA

genes to identify them as a singular group. Therefore, functional genes specific to denitrifiers have been explored as markers in natural and manmade systems.

Phillipot's 2002 review of denitrifying genes in prokaryotes reports that new studies reveal that the genetic basis of the denitrification is more complex than previously believed. Complete sequences for all of the genes involved are few and belong to well characterized and taxonomically related bacteria. A search for open reading frames in both complete and ongoing prokaryotic genome sequence databases adds to the list for analysis of the structure, phylogeny and organization of these genes. The gene clusters form operons which appear to be mosaic and were likely formed in part by horizontal gene transfer events, gene duplications and linkage. (53)

Hybridization to probes for some of these genes does not seem to correlate well with denitrification activity. This could be due to the presence of pseudogenes that result in false positives. Pseudogenes may have high sequence similarity to a probe but may not be conserved in residues at the active site of the protein. Molecular diversity and redundancy in the genome can result in false negatives. High diversity in the gene of interest may cause some organisms with functional genes not to hybridize to the probe or the probe may be for a gene with multiple functions and not critical for the generation of intermediates in the denitrification pathway. Regulatory genes may be required that are not probed for and may not be present or functional in the organisms tested. Finally, environmental conditions or mobile genetic elements that are not accounted for may be involved in regulation and function. The presence of a gene does not insure that it will be

expressed since they are regulated to different extents by the environmental conditions described above. Many of these enzymes require co-factors that may not be available in sufficient quantities to activate the enzyme; therefore the presence of a gene, or even its expression, does not always equate to function.

Nitrite reductase is the first enzyme in the path to catalyze a reaction that results in a gaseous product, nitric oxide (NO). There are currently two genes, *nirS* and *nirK*, that are known to code for NO_2^- reductase in denitrifiers. Probes for *nirS* and *nirK* have been used to quantify the presence of these genes in pulp and paper mill sludge and to correlate their presence with denitrification enzyme activity with variable results (46).

Nitric oxide is toxic to most microbes and it must be reduced quickly. There are multiple NO reductases that function under different optimal conditions and there is evidence that it is a signal molecule for transcription of a number of nitrogen oxide reductases. The *nir-nor* cluster is suggested to have remained highly conserved, despite shuffling of gene locations, because NO is so highly toxic to bacteria. (53,85)

Nitric oxide reductase is an enzyme found in all eubacterial denitrifiers tested to date. There are two genes that code for this protein, *cnorB* and *qnorB*. Most denitrifiers possess *cnorB,* however, *qnorB* is found in the genomes of some non-denitrifying organisms as well as some denitrifiers. The minimum level of amino acid identity among *cnorB* samples is 38% and it is 43.9% for *qnorB.* (12) Denitrifying fungi have a cytochrome P-450nor NO reductase (45, 76).

Currently, *nosZ* is the only gene described which codes for nitrous oxide reductase. This enzyme catalyzes the reduction of N_2O to N_2 (85). Experiments with *nirS*, *nirK* (74) and *nosZ* (20) molecular markers for denitrification do not produce significant correlation with activity in diverse communities. Disruption of *nirK* in *Nitrosomonas europea* does not inhibit NO or N_2O production (4). The *nirS, nirK* and *nosZ* genes are detected in sediment samples but only the NirS and NosZ mRNAs are detected (47). *Nir* probes and primer amplification do not correlate with RFLP patterns (80) and heterogeneity among genes may limit the ability of probes and primers to detect them (11, 26, 47, 55, 62, 80). Diversity analysis of *nirS* genes shows levels of nucleotide identity as low as 45.3% while the nucleotide identity among *nirK* genes is at 78.6% and higher (11).

1.2 Wood Amendments to Soil

Carbon source plays an important role in the function of denitrification and other microbial populations that compete for this substrate. Understanding the response of microbes to different sources is critical to the design and evaluation of NO_3^- removal systems. Economic considerations also play a role in design, and inexpensive, long lasting substrates are the most desirable. Sawdust has been recommended as an organic soil amendment for clay soils (59). Organic matter that is added to the soil is converted to humus, which imparts benefits for plant growth such as improved drainage and aeration and increased nutrient and water availability (10).

1.2.1 Carbon to Nitrogen (C/N) Ratio

The carbon/nitrogen (C/N) ratio must be taken into consideration when adding sawdust to soil supporting plant growth. Microbes require a C/N ratio of 20/1 in their nutrient source. Carbon source additions with greater than 20/1 ratios can result in a temporary deficiency of nitrogen for plants since the microbes will scavenge all the available nitrogen from the soil (2). When sawdust is used as an amendment in soil that supports plant growth, a supplemental addition of nitrogen is recommended for the first few seasons. It follows then that the addition of sawdust in a permeable barrier would lead to the use of excess NO_3^- in the water that passes through because the microbes that degrade the wood have a large supply of carbon but a limited supply of nitrogen. Fresh sawdust has a C/N ratio of 400 – 600 (10) which declines as it ages. Fresh sawdust is acidic with a pH of about 4.5 which increases with aging. The moisture content of fresh sawdust is approximately 30% and climbs to 60-70% after 5 years of aging. Composting prior to addition will affect the properties and is recommended when sawdust is used as an amendment for plant growth. (59)

Composting prior to incorporation into subsurface permeable barriers generated on a large scale may not be required if the sawdust does not encroach on the root zone of plants established above the barrier. However, the leaching of excess dissolved organic carbon into streams is not desirable and may be a problem if the C/N ratio is high and exceeds the ability of the microbial community to incorporate the carbon. (See Section 1.0.1.2)

1.2.2 Soil Microbes Involved in the Degradation of Wood

1.2.2.1 Wood Rot and Saprobic Fungi

Fungi secrete their degradation enzymes and are grouped according to the method of degradation. These are the soft rot, brown rot and white rot fungi. Soft-rot fungi exhibit limited degradation of lignin and degrade cellulose and hemicellulose components. Hardwoods are degraded more than softwoods. Fungi involved in this type of degradation include *Phialophora mutablis, Aspergillus niger, Fusarium oxysporum, Neurospora crassa, Trichoderma, Chaetomium sp., Daldinia concentrica* and *Penicillium*. (35)

Brown-rot fungi produce fast degradation of cellulose but many are unable to degrade and use pure cellulose especially in submerged environments (35). Lignin residues form a brown mass in the advanced stages of degradation. Solid state fermentation of pine sawdust by brown rot fungi results in higher yields with increased oxygen; a pH of less than four is required for degradation (1). The brown rot fungi generate a low pH during degradation due to organic acids (57) and attack mainly softwood (23). The brown-rot fungi include *Gleophyllum trabeum, Wolfiporia cocos, Lentinus lepideus* and others. (1)

White-rot fungi can attack all components of wood either simultaneously or selectively. Nitrogen in wood and other environmental factors may influence the type of attack. They produce an extracellular slime sheath. The biodegradation of lignin is oxidative. (35) Softwoods inhibit white rot fungi, so they mainly attack hardwoods (57). *Sporotrichum thermophile* and *Phanerochaete chrysosporium* are examples of white-rot fungi. (35)

In addition to wood rot fungi, some phycomycetes display saprophytic behavior as do member of the mucorales and numerous other fungal organisms. Phycomycetes may also be capable of parasitic behavior on other fungi, algae or each other. (5) Fungi are expected to be introduced into the permeable barrier with the addition of wood.

1.2.2.2 Bacteria that Degrade Wood

Bacteria slowly attack softwood and hardwood under high moisture conditions. They attack in conjunction with fungi to compensate for the lack of wood cell penetration ability. Bacteria use cell bound enzymes to degrade cellulose except for actinomycetes, which secrete enzymes into the environment. *Streptomyces* and other actinomycetes degrade lignin and cellulose (22) and cause partial solubilization (81). They can degrade and remove lignin from softwood and hardwood. However, these organisms are sensitive to low pH. Other lignin degraders are found in the genera *Erwinia, Aerobacter, Achromobacter, Pseudomonas, Xanthomonas* and *Acinetobacter* (35).

Both aerobic and anaerobic bacteria produce cellulases. These include *Clostridium, Cellulomonas, Bacillus* and *Pseudomonas*. Hemicellulase producers include *Streptomyces, Bacillus, Cellulomonas, Thermonospora* and *Chainia* as well as the anaerobic *Clostridium, Acetovibrio and Bacteriode*s (35). Cellulose decomposition by aerobic bacteria produces a mucilaginous mass of cells containing hemicellulose, various acids and pigments. The optimum pH is 6.5 to 8.2 with decomposition severely limited above pH 8.5 and below pH 6.0 (79).

1.2.3 Microbial Populations Associated with Sawdust Degradation

An investigation of the microbial community associated with spruce sawdust degradation included a comparison of ammonifying bacteria, fungi, actinomycetes and cellulose decomposers. Ammonifying bacterial populations peaked after 60 days and dropped sharply, fungal populations declined after 60 days and then leveled off, actinomycete populations were variable but more or less consistent for the 180 day trial and cellulose decomposers increased from 30 to 60 days, peaked and then dropped off. There was no assay for denitrifiers or discussion of specific microbial populations. (33)

Fewer microfungi and fewer fungal species were observed two years after the addition of pine sawdust to a fallow agricultural soil. There was a strong increase in the *Trichoderma harzianum* population which requires high carbon input for spore germination. Fungal populations that declined were unable to decompose cellulose and hemicellulose. (38)

1.3 Microcosm Studies

Microcosm studies are used to investigate microbial ecology because they homogenize the variance among samples and allow for controlled experimental variables. Problems with variance when measuring denitrification in field studies occur because of heterogeneity in the distribution of nutrients, substrates, gases and water, which create microbial hot spots that may or may not be captured during sampling (15, 50). These variables are difficult to control in the field and therefore there is a lack of correlation of function with treatment unless large samples are taken (49). However, the use of large

samples contradicts the need to investigate microbial processes at the resolution of the microenvironment. Therefore, this study utilizes homogenized microcosms to attempt to understand the relationship between treatment, microbial diversity and function in controlled experiments.

1.4 Functional Assays

Functional assays are used to determine the metabolic capabilities of microbial isolates. Nitrate reduction and denitrification ability are the metabolic pathways of interest for this investigation. Culture based methods limit the analysis to culturable organisms but allow for functional assays. Molecular approaches only give the potential for activity and are limited by prior knowledge of the genes involved. Since the molecular assays for nitrogen oxide reductases don't correlate well with function they are not applied here.

1.4.1 Denitrification Enzyme Activity (DEA)

Nitrogen is the major constituent of air; therefore it is difficult to quantify its generation as a product of denitrification. Measuring denitrification enzyme activity (DEA) is the most widely used method but it is flawed. The DEA assay is not tied to a specific organism or group and measures activity of all enzymes present in the sample. It artificially inhibits the formation of N_2 using acetylene and results in the accumulation of N_2O, which is measured. The rate of generation is used to indicate the activity of the denitrifiers. However the *in situ* rates don't seem to correspond to the potential expressed by DEA (72). DEA is unexpectedly high in dried soils and in perpetually anaerobic environments where denitrifiers are not expected to persist, but N_2O is produced by

organisms other than denitrifiers, and fungal denitrification will also impact the results. Due to these limitations this assay is not used.

1.4.2 Nitrate Reduction and Gas Production by Individual Isolates

Isolation of eubacterial colonies and subsequent testing for reduction of NO_3^- and NO_2^- and gas production in nitrate broth are used in this investigation to test for NO_3^- reduction and denitrification ability. Use of these methods allows for the identification of the organism performing the function and for the discrimination among several different NO_3^- reduction paths. Additionally, it generates cultures that may be used for further study and for potential inoculum of field installed permeable barriers. These assays are described in detail in the material and methods section of Chapter II.

1.4.3 Microbial Diversity

Molecular methods to assess diversity based on nucleic acids from complex microbial communities that include both prokaryotes and eukaryotes are somewhat expensive and require specialized equipment and reagents. Prior knowledge of the populations in the community under study can facilitate the use of this method but were not previously available. Ester-Linked Fatty Acid Methyl Ester (EL-FAME) extractions from environmental samples have also been applied to the comparison of microbial diversity between treatments and yield information regarding populations that are not easily cultured. This method will not reveal the presence of archaea but allows for comparisons of microeukaryote and eubacterial populations. Denitrifying archaea have halophilic and thermophilic metabolisms and are not expected to be found in the barrier. Archaea

belonging to the Crenarchaea line (75) and from a group most closely related to the planktonic archaea (6) have been identified in surface soils by 16S rRNA gene sequence analysis of uncultured prokaryotes but their activity in the soil remains uncertain. The EL-FAME method is also described in detail in the materials and methods section of Chapter II.

1.5 Significance, Goals and Hypotheses

The Mississippi River/Gulf of Mexico Task Force recommends that a 30% reduction in nitrogen load is required to reduce the annual hypoxic zone in the Gulf of Mexico that threatens the fishing and recreational industries in this region. The majority of the excess nitrogen, in the form of NO_3^- is from seasonal agricultural non-point sources. Permeable barriers using sawdust as a reductant to enhance microbial denitrification are proposed to reduce the NO_3^- entering into tributaries. Although initial investigations have shown that NO_3^- is removed from water passing through these barriers, there are no reports of specific denitrifier or NO_3^- reducing populations associated with wood amended subsurface soil. Data regarding the identities of the microbial populations found in permeable barriers, especially the denitrifiers and other NO_3^- reducers, are required in order to effectively design and manage permeable barriers for denitrification.

Tiedje (1988) reports that at low concentrations competition for the NO_3^- substrate is dependent upon the K_m of the enzyme. Denitrifiers are favored at low concentrations because they have a high affinity for NO_3^-. At high concentrations the competition between DNRAs and denitrifiers will be dependent on population size. Additionally, high C/N environments, such as those found in saturated permeable barriers without

consistently high NO_3^- inputs, are believed to favor DNRA populations. (73) In order to enhance denitrification in permeable barriers at high concentrations of NO_3^-, supplemental inoculation with denitrifiers may be necessary. Denitrifiers are found in a wide array of environmental conditions but little is known about which species are expected to thrive in permeable barriers.

The goals and hypotheses for this study are as follows:

1) Determine if the addition of pine wood increases NO_3^- reducing populations.

   H_0: Addition of pine wood shavings to a clay soil does not increase NO_3^- reducer populations over those found in the soil controls of jar microcosms.

   H_A: Addition of pine wood shavings to a clay soil will increase NO_3^- reducer populations found in the soil through the addition of new NO_3^- reducer populations associated with the wood. (Chapter II)

2) Assess the effects of pine wood on the overall diversity of the microbial community in a subsurface clay soil.

   H_0: Wood will not contribute microeukaryote populations to the mixture treatments in jar microcosms.

   H_A: Microeukaryotic populations will be introduced into the mixture with the wood. (Chapter II)

3) Determine if the denitrifier population is greater in water versus solid samples.

   H_0: The odds of isolating denitrifying nitrate reducers will be the same in the water versus the solid samples from saturated jar microcosms.

H_A: The odds of isolating NO_3^- reducers that are capable of denitrification will be greater in water samples than in solid samples due to the solubility of the substrates of the reaction. (Chapter III)

4) Determine if the addition of pine wood affects denitrifier populations.

H_0: The odds of isolating NO_3^- reducers that are capable of denitrification will be the same in the mixture and the soil treatments from jar microcosms.

H_A: The addition of wood to soil increases the odds of isolating denitrifiers. (Chapter III)

H_0: The odds of isolating a denitrifier will be the same in the wood as in the mixture or soil.

H_A: The odds of isolating NO_3^- reducers that are capable of denitrification from pure pine wood shavings will be lower than in the soil or mixture due to low pH or other inhibitory substances in the wood and the odds of isolating NO_3^- reducers that are capable of denitrification will be greater in the mixture (permeable barrier) and soil than in the wood due to favorable pH, nutrients and microbial inocula supplied by the soil. (Chapter III)

5) Isolate and identify denitrifier populations and other NO_3^- reducing eubacterial species in samples from soil:pine wood mixture (permeable barrier) microcosms. (Chapter III)

6) Identify microeukaryote populations affected by the addition of pine wood.

(Chapters II and IV)

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CHAPTER II

PINE WOOD CONTRIBUTES MICROEUKARYOTES AND NITRATE REDUCER POPULATIONS TO PERMEABLE BARRIER MICROCOSMS

2.0 Abstract

Excess NO_3^- in water bodies contributes to eutrophication and hypoxia which threatens fishing and recreational activities. Experiments to examine the use of wood products in permeable barriers for removal of NO_3^- from surface and groundwater have not generated data regarding the effects of wood on microbial communities required for efficient design and management. Short-term permeable barrier microcosm incubations in syringes and flow-through columns were used to test for the effects of sawdust on microbial cell counts in water versus solid samples and to test for a correlation between microbial cell counts and NO_3^- concentrations. Long-term incubations of a 2:1(v/v) clay soil:pine wood permeable barrier, plus soil and wood controls, in flint glass jars were used to test for the effects of wood shavings on NO_3^- reducer populations and EL-FAME diversity. The creation of a permeable barrier by mixing soil and wood created a community different from either by increasing available nutrients and merging inocula from two sources. The soil:wood mixture had a greater percentage of EL-FAMEs characteristic of microeukaryotes and significantly more eubacterial NO_3^- reducers over time versus soil alone and nitrate reducers were isolated in high numbers from pure pine wood. Abundant

fatty acids detected in the soil were characteristic of eubacteria while those from the wood and mixture microcosms were produced by microeukaryotes. Although eubacterial NO_3^- reducers are increased though the addition of wood, the removal of NO_3^- from wood based permeable barriers may be dominated by microeukaryotes.

2.1 Introduction

Riparian zones act as natural filters or barriers which inhibit the movement of agricultural non-point contaminants to surface waters. Enhancement of natural NO_3^- removal with engineered permeable barriers is being explored because of wetland and stream bank degradation due to erosion and loss of vegetation or because anthropogenic NO_3^- input exceeds the ecosystems ability to remove it. Fishing and recreational activities in natural waters are threatened due to the generation of seasonal anoxic zones as a result of the excess nitrogen or phosphorous (8). A permeable reactive barrier may consist of soil and wood products to provide a source of NO_3^- reducer inocula and a carbon substrate. The addition of easily decomposable carbon substances such as glucose, mannitol and sucrose have much greater but short-lived effects and have limited application in the field due to economic constraints (2). The recalcitrance of wood products to degradation is considered beneficial as it prolongs the ability of the barrier to reduce NO_3^-.

The ability of wood based barriers to support NO_3^- removal has been demonstrated. Shipper and Vodivic-Vukovic reported that a 2:1 soil:wood barrier consistently removed 95% of incoming NO_3^- ranging from 5 – 15 ppm, although denitrification enzyme assays taken from below the water table were highly variable over the course of their experiment. (10)

There is a paucity of published reports on how wood treatment affects or selects for NO_3^- reducing microorganisms in the soil, the relative contributions of NO_3^- reducing microbes associated with the wood and the percent of these organisms capable of denitrification. Experiments designed to answer these questions were conducted in long-term (six week and 24 weeks) microcosm incubations in flint glass jars. Supplemental experiments in plastic 60 cc syringes and plastic flow-though columns provided background data on short-term incubations.

The syringe microcosms were used to test four hypotheses with respect to short-term (3 day) permeable barrier incubations. First is that the addition of wood increases microbial cells. Second is that the addition of NO_3^- increases microbial cells. Third is that equal numbers of microbial cells will be found in the water and solid samples taken from saturated incubations. Fourth is that the number of microbial cells found in the water samples are correlated with the concentration, and therefore the removal, of NO_3^-. Flow-though column effluent, from the course of a 13 day experiment, was used to provide additional data for the fourth hypothesis.

The goals of the jar microcosm experiments were to determine how wood specifically affects the eubacterial NO_3^- reducer populations in a subsurface clay soil and the general overall diversity of the microbial community. The diversity of fatty acids was characterized because the culture selection method for NO_3^- reducers is biased towards fast growing eubacteria while fatty acid analysis can be used to compare populations across kingdoms. Microeukaryotic populations such as fungi, algae and protozoa are

affected by carbon inputs and are potential users of NO_3^- as well. The first two hypotheses presented in Chapter I were tested. The first null hypothesis states that the addition of pine wood shavings to a clay soil does not increase NO_3^- reducer populations over those found in the soil controls of jar microcosms. The alternate hypothesis states that the addition of pine wood shavings to a clay soil will increase NO_3^- reducer populations found in the soil through the addition of new NO_3 reducer populations associated with the wood. The second null hypothesis states that the wood will not contribute microeukaryote populations to the mixture treatments in jar microcosms. The alternate hypothesis states that microeukaryotic populations will be introduced into the mixture with the wood.

2.2 Materials and Methods

2.2.1 Syringe Microcosms

Incubations consisted of 20% wood:75% sand:5% sediment or 95% sand:5% sediment by volume. Wood was douglas fir sawdust and sand was washed filter sand. Sediment was taken from a shallow bank on the Poudre River just north of Picnic Rock in LaPorte, CO in November 2002 and used the same day. All incubations were saturated with an unbuffered trace mineral solution and sparged with nitrogen. (4) Samples were incubated in sterile, 60cc plastic syringes in an anaerobic chamber for up to three days at room temperature and analyzed by destructive sampling at 24 hours intervals. Nitrate treatments received 20 ppm NO_3-N in addition to the trace mineral solution. Water was removed with the plunger and fixed in 2% formaldehyde and 0.1% Triton-X 100. One gram samples of the saturated solids were diluted 1:10 in sterile water and fixed with 2%

formaldehyde and 0.1% Triton-X 100. Samples were stored for several days at 4°C prior to DAPI (4'6-diamindino-2-phenylindole) staining. Samples were stained with 1ug ml^{-1} DAPI solution for 15 minutes at room temperature in the dark, filtered onto 0.2 um black polycarbonate filters, mounted on slides and sealed under coverslips. Cells were viewed and counted with low fluorescent oil at 1000x magnification with an Olympus BH-2 Epifluorescent microscope with a Chroma UV filter set. The total number of cells ml^{-1} or g^{-1} was calculated according to the protocol of Bloem (1). Syringes were then double bagged in baggies and frozen at -80°C until EL-FAME analyses were performed.

2.2.2 Flow-Through Columns

Flow-through columns were designed and constructed by Lauren Glushik (4). Briefly, there were three columns of clear PVC tubing (1 x 8 inches). The bottom was sealed with cemented PVC cap and a threaded seal was attached to top. Sand and sawdust were washed and oven dried for 24 hours while the sediment was sieved to 2mm and allowed to stand at room temp (21°c) for 24 hours prior to use. Columns were filled with approximately 8.3 cc of media (75% filter sand; 20% unprocessed cherry sawdust (sieved to 2mm); 5% river sediment, by volume) with glass wool plugs at both ends. The top was attached with Teflon tape to assure a watertight seal.

Solution was pumped through the column at a rate of 0.136 ml/min with peristaltic pumps (Masterflex I/S). Two solutions consisted of 1) a buffered blank (pH 7.0 with sodium bicarbonate) trace mineral solution (Bergmann-Haas mixture without ammonium

nitrate 5% v/v) and 2) the same solution spiked with 30 ppm NO_3^-N . All columns were fed buffered blank solution for 24 hours to establish hydraulics.

Column A was a non-NO_3^- control with a wet/dry cycle. It was feed for 120 hours with the buffered blank solution then run dry by a combination of draining and gentle application of compressed air and allowed to stand for 70 hours. Flow was resumed with buffered blank solution for 120 hours. Columns B and C were fed for 120 hours with the 30 ppm NO_3^-N spiked solution and run dry as above. Column B was allowed to stand dry for 70 hours and flow was resumed with the NO_3^- spiked solution. Column C was filled with the buffered blank and allowed to stand for 70 hours at which time flow was resumed with the NO_3^- spiked solution.

Effluent was collected at 8 time points during 13 days of incubation (in the dark at room temperature). Effluent was collected and samples were prepared and analyzed as described in section 2.2.1. At the end of the 13 day trial solids were sampled and analyzed as described above. A temporally separated replication of the experiment was run. All variables were the same except for the river sediment which had a higher number of microbial cells in the second trial. Nitrate concentrations in the water samples from syringe and flow-through microcosms supplemented with NO_3^-N were supplied by Lauren Glushick (4) and were regressed on the total estimated microbial cells using SAS Version 8.0 (SAS Institute Inc., Cary, NC) and Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA).

2.2.3 Jar Microcosms

The soil was a composite from a stream bank excavation for installation of a permeable barrier for field testing (near Colorado State University's Environmental Research Center, Foothills Campus) of random samples dug with a hand trowel. The soil was from below the rhizosphere at a depth of from 3-5 feet. It was collected in mid June 2003. The vegetation along the stream was mostly native grasses and *Salix spp.* A few small aspens were within 50 yards of the site. The soil was classified in the field as clay and clay loam belonging to the Heldt series formed in alluvium from clay shale (7). Laboratory analyses of the subsurface soil samples (Soil, Water and Plant Testing Lab at Colorado State University) confirmed the clay texture. The pH of the soil was 7.8 and it had 1.9 % organic matter. Soil was stored at 4°C prior to incubation.

The untreated pine wood shavings (Rushmore Forest Products, Drywood Shavings, Hulett, Wyoming) and clay soil were sieved through a 4 mm stainless steel screen sterilized with 70% alcohol. The pH of the wood shavings was 4.5. Tap water was degassed for two hours to remove chlorine, and then filter sterilized with a 0.2 um filter. A 2:1 soil:wood shaving mix was created by volume, to mimic a NO_3^- removal barrier tested in the field. Table 2.1 lists the ingredients used for each treatment. Ten sterile 240 cc flint glass jars per treatment were assembled and covered with parafilm which allowed for air transfer but not water loss.

Jar microcosms were incubated at room temperature inside a cardboard shipping box which was placed inside a covered plastic container. An additional jar filled with water

was placed in the box and left open for humidity. The jars were weighed initially and at harvest to determine loss of water by weight. The changes in weights were consistently less than one percent. The air temperature during the six month incubation period ranged from 20 – 27°C. The moist treatments were approximately 70% moisture. The saturated experiments had a water column of approximately 1 cm. Because of the different water holding capacity of the clay and wood shavings, the ratio of water to substrate differed for the saturated treatments. The soil was 4.7:1 soil:water, the wood was 0.66:1 wood:water and the mixture was 0.96:1 mixture:water.

Three jars from each treatment were harvested after six weeks and 24 weeks of incubation. Sub-sample aliquots by weight for serial dilutions and plating, soil analyses and EL-FAME extractions were made with autoclaved scoops. Dilutions and plates were made the day of harvest as were pH measurements. Water was removed from the saturated treatments with sterile pipettes and transferred to sterile 15 ml polypropylene conical tubes. Dilutions and plates from the water were made the day of harvest; the remainder was stored at -20°C for EL-FAME analyses. The four remaining jars from the saturated wood treatment were harvested after 24 weeks for the biofilm analysis described in Chapter IV. The saturated soil did not form a visible biofilm after 24 weeks and the saturated mixture treatments had weak biofilms that were difficult to sample.

2.2.4 Nitrate Reducer Analyses

Soil dilutions were made with autoclaved water in sterile glass bottles, polypropylene tubes or eppendorf tubes. For isolations, duplicate plates at two dilutions were made for

each sample based on a trial run and ranged from 10^{-2} to 10^{-6} for NO_3^- reducing plates. Dilution water blanks and media blanks were included as controls. Aliquots of 100 ul of the dilution were pipetted onto the plates and spread with flame sterilized glass Dragalski spatulas. Nitrate reducing isolates were cultivated on nutrient agar (DIFCO, Becton-Dickenson, Franklin Lakes, NJ) supplemented with 5mM KNO_3 (Fisher Scientific, Fairlawn, NJ). Duplicate plates were incubated for five days in the dark at room temperature in gas pack chambers with BBL GasPak Plus Anaerobic Envelopes with Palladium Catalyst. BBL Dry Anaerobic Indicator Strips (Becton-Dickinson) were used to confirm anaerobic conditions. After incubation, colonies were counted for all plates. Nitrate and nitrite reduction, as well as denitrification ability of the isolates, were confirmed by incubation in nitrate broth in durham tubes and reaction to Nitrate A and Nitrite B reagents and zinc when necessary. Details of these tests and results are presented in Chapter III.

2.2.5 EL-FAME Analyses

Frozen samples for EL-FAME analysis were thawed and aliquots weighed and transferred to acid washed 35 ml glass centrifuge tubes with teflon-lined screw caps. Three grams of material were used for all solid samples except for wood shaving controls which used 1.5 g. Water samples had 20 mls centrifuged for 5 minutes at 3,000 x g. The supernatant was discarded and the pellet was extracted using 1/3 the reagents of the solid samples. FAME extractions were performed as described using a transesterification extraction (11). The extraction involved a mild alkaline methanolysis in 0.2 N KOH in methanol for one hour at 37°C, with each tube vortexed for 10 seconds every 10 minutes.

The pH was adjusted to neutral with acetic acid and followed by extraction with hexane. Tubes were centrifuged at 4°C at 440 x g (Sorvall HS-4 Swinging bucket rotor) for 20 minutes. The top two thirds of the supernatant was removed and transferred to acid washed 17 x 100 test tubes. The tubes were placed in a 40°C water bath and the hexane was evaporated under N_2. The precipitate was redissolved in 0.5 ml of hexane/methyl-tert butyl ether and transferred to gas chromatography vials.

An internal standard, 20 ug of c19:0, was added to each sample and samples were evaporated under N_2 prior to analysis by gas chromatography. An HP 5890 gas-liquid chromatograph (Hewlett Packard, Rolling Meadows, Illinois) with an HP Ultra 2 capillary column (5% diphenyl-95% dimethylpolysiloxane, 25 m x 0.2 mm) and a flame ionization detector were used for the analysis. EL-FAMEs were identified with the standard Eukary chromatographic program and peak naming table supplied by MIDI (Microbial ID, Inc., Newark, DE, USA). Mixed FAME standards were used as described by manufacturer's instructions to adjust and monitor the calibration of the system. Standard fatty acid nomenclature was used. The carbons were numbered from the aliphatic (ω) end of the fatty acid molecule. The number of double bonds was reported after the colon. The suffices 'c' and 't' referred to the cis and trans conformations, respectively. Hydroxy groups were noted with 'OH' and cyclopropane rings with "cy". The prefixes for iso- and anteiso-branched fatty acids were 'i' and 'a'.

2.2.6 Statistical Analyses

The jar microcosm experiment was a randomized complete block (RCB) design, with time as the blocking factor. ANOVA (SAS and Excel) was performed on the total estimated NO_3^- reducing colony forming units (NRCFUs) by treatment and on the total nmol EL-FAME by treatment at alpha=0.05. Natural log transformations were used to reduce heteroscedasticity in the NRCFU data and total nmol EL-FAME data.

Non-metric multidimensional scaling (NMS) ordination (PC-ORD Version 4.20) was applied to the EL-FAME fatty acid profiles using the manufacturer's recommended parameter settings, followed by one way ANOVA (SAS) on axis coordinates. NMS was used because of the non-normality of the data set. Arcsine square root transformations were applied to reduce heteroscedasticity, instead of natural log, because of the presence of zeros in the data. This transformation resulted in a slight improvement but the data were still not suitable for principle components analysis. NMS uses an iterative search to rank and place n entities on k dimensions (axes) while minimizing stress of the k-dimensional configuration. It is calculated from an n x n distance matrix and the n x p-dimensional main matrix, where n is the number of rows and p is the number of columns in the main matrix. (6)

Stress measures the departure from monotonicity in the relationship between the dissimilarity (distance) in the original p-dimensional space and distance in the reduced k-dimensional ordination space. Individual rows of the main matrix become points in the ordination space. NMS may generate axes that are weakly correlated so correlations are

calculated between all pairs of axes (orthogonality). Orthogonality is 100% when statistically independent, is calculated by the formula $1-r^2$ and is expressed as a percentage. NMS axes are not invariant with respect to adding axes, unlike PCA and other ordination techniques. (6)

Sorensen (Bray-Curtis) distance was used since it is considered most useful for ecological community data as it retains sensitivity in more heterogeneous data sets and gives less weight to outliers when compared to Euclidian distance. Sorensen distance or percent dissimilarity (PD) is a proportion coefficient measured in city-block space. The formula is 1-2W/(A+B) where W is the sum of shared abundances and A and B are the sums of abundances in individual sample units. This dissimilarity coefficient is converted to PD. An after-the-fact evaluation calculates the coefficient of determination (r^2) between distances in the ordination space and distances in the original space. Distances in the ordination space are measured with Euclidean distance, while the distance measure is the same as that used to construct the ordination for NMS, in this case Sorenson (Bray-Curtis). (6)

Monte Carlo tests and a Scree Plot confirmed the fit of the data to the statistical model by plotting stress against dimensionality for real and randomized data. Additionally, the coefficients of determination and orthogonality were computed for each axis. Kendall correlations for nonparametric data (tau) were calculated for sample units against ordination axes with the positions of species on the ordination axes compared to their abundances in a given sample unit for each sample unit. (6) It was calculated for each

species' abundance for each axes based on ranks. Results of these tests are presented in Appendix I.A. Ordination coordinates from PC-Ord were input into Sigma Plot 2001 for Windows (Aspine Software International, Leesburg, VA) to generate Figure 2.4.

2.3 Results

2.3.1 Short-Term Effects of Sawdust and Nitrate on Microbial Cell Counts in Syringe and Flow-Through Column Microcosms

The first set of experiments tested the short-term effect of sawdust and additional NO_3^- on cell numbers, as well as microbial diversity and biomass in syringe microcosms prepared with river sediment and sawdust. Four hypotheses were tested with the results for the first three presented in Figure 2.1. The number of cells in the syringe microcosms that contained sawdust (wood) were significantly higher than those that did not ($p<0.0001$) and this supports rejection of the null hypothesis that wood does not increase the number of microbial cells in permeable barrier microcosms, in favor of the alternative that the addition of wood does increase the number of microbial cells. Second, there was no significant difference in the number of cells in treatments with and without supplemental NO_3^- ($p=0.76$) which does not support rejection of the null hypothesis, that the presence of NO_3^- does not cause a short-term increase in the number of microbial cells. Third, there was no significant difference in the number of microbial cells in the solid versus water samples from saturated incubations ($p=0.51$) which also does not support rejection of the null hypothesis, that the number of cells will be the same in the water and solid samples of saturated microcosms.

The results of the tests for the fourth hypothesis are presented in Figure 2.2. Nitrate concentration was regressed on the number of microbial cells in water samples from the syringe and flow-through column microcosms. Nitrate concentrations significantly correlated with the number of microbial cells ($p<0.0001$) which supports rejection of the null hypothesis, that there is no correlation between cells and NO_3^- concentration, in favor of the alternative that the number of microbial cells found in the water samples will be correlated with the concentration, and therefore the removal, of NO_3^-. Although the syringe microcosms and flow-though columns had different types of sawdust (douglas fir and cherry respectively) and the inoculum was from temporally separated collection events of river sediment, the slope of the regression of NO_3^- on cell number in both the syringe and flow-though columns was the same at -0.002. This predicts two parts per billion decreases in NO_3^- concentration per microbial cell in the presence of sawdust, and a direct correlation between biomass and NO_3^- concentration. The r^2 for the regressions were 0.64 and 0.57, for syringe and flow-through respectively, suggesting that more than half of the variability in the data is due to microbial assimilation of NO_3^- during growth on organic carbon supplied from sawdust.

In order to understand the effect of wood on microbial cells in the long-term, as expected in a permeable reactive barrier, six and 24 week jar microcosm incubations were analyzed. One effect of sawdust is to increase the water holding capacity of soil, which in turn affects the microhabitat for both bacteria and microeukaryotes. Since the syringe microcosms were saturated, the effect of moisture could not be tested with this design. Therefore the long-term experiments included a variable for moisture.

2.3.2 Long-Term Effects of Wood and Moisture on Nitrate Reducers and EL-FAME Diversity in Jar Microcosms

Moist or saturated microcosms of soil, pine wood shavings or a 2:1 (v/v) soil:wood mixture (to approximate conditions in a NO_3^- removal barrier) were analyzed to determine the effect of addition of wood shavings on soil microbial populations, after six and 24 weeks of incubation, without additional input of NO_3^-. EL-FAME profiles were used to characterize the effects of wood on microbial eukaryote and bacterial population diversity. To examine a specific subset of organisms important for NO_3^- removal, NO_3^- reducers were selectively cultured to determine the effects of wood on cell numbers by inference from colony forming units. Two hypotheses were tested. First that the addition of pine wood shavings to subsurface clay soil increases the number of culturable NO_3^- reducers because pine wood shavings are an additional source of NO_3^- reducers and second that EL-FAMEs characteristic of microeukaryotic populations will be increased in the presence of the pine wood.

2.3.2.1 Nitrate Reducers from Jar Microcosms

After 5 days of incubation, colonies were counted on plates selective for NO_3^- reducers. Colony counts are in Appendix I.B. Figure 2.3a shows the mean CFU of NO_3^- reducers for each time point after natural log transformation. Untransformed CFUs ranged from 10^3-10^6 CFU g^{-1} in the moist and saturated solids and 10^2-10^5 CFU ml^{-1} in the water column above the saturated solids.

There were significantly more total NO_3^- reducing colony forming units (NRCFUs) g^{-1} or ml^{-1} in the mixture treatments than in the soil alone for all treatments and time points ($p=0.003$). The numbers of NRCFUs in the wood control and mixture treatments were not significantly different ($p=0.78$), suggesting that the wood was contributing NO_3^- reducers to the mixture. These results support rejection of the null hypothesis that the addition of pine wood shavings to a clay soil does not increase NO_3^- reducer populations over those found in the soil controls of jar microcosms in favor of the alternate hypothesis stating that the addition of pine wood shavings to a clay soil increases NO_3^- reducer populations found in the soil through the addition of new NO_3^- reducer populations associated with the wood.

There was no significant effect of time from six to 24 weeks for mixture ($p=0.13$) and soil ($p=0.78$) treatments. NRCFUs were similar at both time points, suggesting stable populations of NO_3^- reducer numbers. However there was a significant decline in NRCFU populations in the wood treatments over time ($p=0.02$). This suggests that competition from microbial populations introduced into a barrier with wood will decrease from six to 24 weeks. The mixture had similar numbers of NO_3^- reducers in the solid and water samples while the soil and wood treatments had significantly fewer NO_3^- reducers in the water samples ($p=0.04$ and $p=0.01$, respectively).

Nitrate reducers are phylogenetically diverse with multiple paths for NO_3^- reduction. The microbial community also has a variety of other bacteria and microeukaryotes. The culture method used here does not select for microeukaryotes, nor show changes in

population levels of non-NO_3^- reducers. However, populations grown oligotrophically in water agar (15% agar in sterile water) pour plates, run simultaneously with selection for NO_3^- reducers, showed the presence of 10^4 g^{-1} – 10^6 g^{-1} oligotrophic organisms. These were highest in the wood and mixture treatments and lowest in the soil. Many of these organisms produced spores characteristic of fungal colonies or actinomycetes. Fifteen oligotrophic isolates were tested for NO_3^- reduction and gas production in nitrate broth and slightly more than half (8/15) reduced NO_3^- to NO_2^-. See Appendix I.C for data.

2.3.2.2 EL-FAME Diversity in Jar Microcosms

2.3.2.2.1 Total nmols EL-FAME in Jar Microcosms

EL-FAME was applied to estimate differences in sample diversity by treatment.

The total nmol EL-FAME extracted from the samples were transformed using natural log and are shown in Figure 2.3b for comparison to natural log transformed NO_3^- reducers across samples. (See Appendices I.D and I.E) The nmols EL-FAME from the water samples from all treatments were one to three orders of magnitude lower than the respective moist or saturated solid treatments which were significant with $p<0.0001$. There was no significant effect of the block for time on mixture ($p=0.91$), soil ($p=0.69$) or wood ($p=0.62$). There was a significant difference between the mixture and soil samples ($p=0.003$) with approximately twice as many nmols EL-FAME in the mixture samples (Fig. 2.3b). The nmols EL-FAME in the wood and in the mixture samples were not significantly different ($p=0.14$).

2.3.2.2.2 Jar Microcosm EL-FAME Ordination

Figure 2.4 presents an ordination graph of the jar microcosm EL-FAMEs that shows distinct groupings by treatment with solids from the saturated and moist microcosms represented by a single symbol. These treatments were not significantly different from each other. The data was best described in two dimensions. Fatty acids with taus above 0.580 were chosen. These fatty acids contribute significantly to the separation of treatments with positive taus representing fatty acids enriched in samples in the positive portion of the axis and negative taus representing fatty acids enriched in the negative portion of the axis. Fatty acids characteristic of bacteria are associated with the positive values on Axis 2, while the negative values on Axis 2 are enriched in fatty acids characteristic of microeukaryotes. The positive values on Axis 1 were also enriched in fatty acids found in microeukaryotes.

Axis 2 (r^2= 0.594) of the ordination plot explains more of the variance in the data than Axis 1 (r^2=0.324), therefore the Axis 2 coordinates were used for statistical analysis using ANOVA. NMS statistics from PC-Ord and ANOVA statistics from SAS are presented in Appendix I.A. The mixture treatments had an EL-FAME community that was significantly different from the soil treatments ($p<0.0001$). All mixture treatments were also significantly different ($p<0.0001$) from wood treatments except for the saturated mixture treatment at 24 weeks. Fatty acids that contributed to the separation of wood and mixture treatments from the soil solid samples were characteristic of microeukaryotes and this separation was significant and suggested rejection of the second null hypothesis that states wood will not contribute microeukaryote populations to the mixture

treatments in jar microcosms in favor of the alternate hypothesis stating that microeukaryotic populations will be introduced into the mixture with the wood. There was no significant difference in EL-FAME diversity between saturated or moist solids for each treatment. Comparison of the closed (6wk) and open (24 wk) symbols shows a trend over time for the soil and mixture solids samples toward fatty acids representative of microeukaryotes but it is not significant. The saturated soil water samples were not significantly different from the wood with the exception of the moist wood treatment from 24 weeks. This is due to dominant microeukaryote fatty acids in the soil saturated water. The specific microeukaryote populations involved are investigated further in Chapter IV and possible reasons for microeukaryote dominance in soil water and not soil solid samples are discussed in Chapters III and IV.

2.4 Discussion

2.4.1 Short-Term Effects of Added Wood and Nitrate in Syringe and Flow-Through Column Microcosms

Short-term experiments demonstrated that the addition of wood significantly increased cell numbers in syringe microcosms and that cell numbers were significantly correlated with NO_3^- removal in both syringe and flow-through microcosms. The addition of NO_3^- did not significantly increase microbial cell counts in syringe microcosms; however these microcosms were sparged with nitrogen to induce anaerobic conditions and nitrogen-fixation may have played a role in microbial growth confounding the effects of the added NO_3^-. The long-term effects of wood on microbial communities in the absence of supplemental nitrogen or NO_3^- were investigated because NO_3^- levels fluctuate in nature.

It has been postulated that nitrate reducing microbes have evolved to use NO_3^- opportunistically as an energy source and that the ability to survive heterotrophically is an important factor in microbial community dynamics that involve these organisms (13).

2.4.2 Long-Term Effects of Wood and Moisture on Nitrate Reducers and EL-FAME Diversity in Jar Microcosms

Long-term microcosms without added NO_3^- were created to simulate substrates in a permeable reactive barrier for NO_3^- removal. The number of NO_3^- reducing colony forming units (NRCFUs) cultured from treatments did not change significantly from six to 24 weeks in the soil or mixture; therefore NO_3^- removal rates attributed to these populations would be expected to remain consistent, assuming there are no other limiting factors. NRCFUs were cultured from pure pine wood which implies that the wood contributes NO_3^- reducers to the mixture microcosms, serving as both a source of microbial inoculum and carbon. These populatons significantly declined over time.

Moist and saturated microcosms were examined to identify the effects of moisture levels on solid samples and to generate a small water column to investigate population differences in solid versus water samples. The amount of water for the moist microcosms was determined by saturation of a sample and subsequent drainage by gravity to simulate field moist conditions. Determination of the percent moisture in these samples was approximately 70%. It has been reported that there is little loss of nitrogen when soil is below 60% of water holding capacity (2). The NRCFUs in moist and saturated treatment solids were not significantly different for the mixture, soil or wood.

Greater nmol of EL-FAME in the wood treatments, compared to the soil or mixture treatments, may have been due to carbon availability and/or the greater water holding capacity of the wood; however a significant difference between moist and saturated wood was not observed. Therefore under these conditions it is more likely the added nutrients and microbial inoculum from the wood contributed to the increase. The soil was a source for macro and micronutrients. (See Appendix I.F for soil analyses results.) The creation of a permeable barrier by mixing soil and wood created a community different from either by increasing available nutrients and merging inocula from two sources.

Distinct microbial communities by treatment were supported by the predominant fatty acids identified by EL-FAME. The association of fatty acids with specific populations is based on fatty acids extracted from pure cultures as reported in Ratledge and Wilkinson (1988). Although there is substantial overlap in fatty acid profiles among microbes, some generalizations can be made which can be applied to a broad view of the community. Gamma linolenic acid (18:3ω6c) was reported to be characteristic of some protozoan and fungal phycomycetes and was also reported to be produced by species of basidiomycetes and zygomycetes as well as algae and Cyanobacteria. (9) It was the abundant fatty acid associated with the negative values of axis two in Figure 2.4 and contributed significantly to the separation of samples by treatment. It was found to be enriched in many of the wood treatments and some of the mixture treatments and the soil water. This fatty acid is not found in higher plants (9) and indicates that the separation by treatment is not an artifact of fatty acids extracted from pine wood resins. This potential is discussed further in Chapter IV.

The soil solids were enriched in fatty acids commonly found in gram positive and gram negative bacteria. These bacterial fatty acids were also components of the mixture treatments. Although the wood solids contained high numbers of bacterial NO_3^- reducers, they represented a small proportion of the overall population in these treatments in comparison to microeukaryote specific fatty acids.

The taxa that are known to produce the fatty acids enriched in the wood treatment samples are microeukaryotic saprophytes and parasites. This does not exclude the possibility that fungal denitrifiers or fermentors may be present in the microcosms because some fungi do not produce either alpha or gamma linolenic acid (9). A shift towards microeukaryotic EL-FAMEs from six to 24 weeks suggests increased grazing although the changes were not significant. This possibility is also discussed further in Chapter IV.

2.4.3 Detection Limits

Actinomycete populations were detected on water agar control plates run simultaneously with the NO_3^- selective plates. Actinomycetes are cellulose and lignin degraders with some strains reported to denitrify. They might be expected to be selected for in the presence of wood. There was no evidence of 10Me18:0, a biosignature fatty acid that is usually associated with actinomycetes (9) and these populations may have been below the limit for detection by EL-FAME. Water from the saturated soil treatments did not yield fatty acids associated with eubacteria, although eubacterial NO_3^- reducers were isolated

from this water at 10^2 - 10^3 ml^{-1} (Figure 2.3a). This may represent a detection limit for eubacterial characteristic fatty acids under these conditions.

Archaea would not be detected using the EL-FAME method because their membranes do not contain fatty acid methyl esters. The three genera of archaea known to contain species that denitrify, *Haloarcula, Halobacterium* and the hyperthermophilic *Pyrobaculum* are organisms associated with high salt or high temperature environments (16). They were not expected under the experimental conditions of the microcosm incubations.

The media for NO_3^- reducers used in these experiments was selective for fast growing eubacteria. The anammox reaction is an alternate path to N_2 production by members of the Planctomycetales that use NH_4^+ and NO_2^- under anaerobic conditions. This group has been reported to be important in removing NH_4^+ from waste water in aquifers (3) and in seawater (5). These organisms are slow growing, require NH_4^+ and NO_2^- and would not likely be cultured with the method used for this investigation. They have recently been cultured from soil using media supplemented with antibiotics (14). The fatty acid iso-, n-, 9- and 10-methyl-hexadecanoic acid is characteristic for planctomycetes (12) and *i*17:1 at 10 was detected at less than 1% in some of the wood and mixture samples. This suggests that a small population of planctomycetes may be present but does not indicate whether they are capable of the anammox reaction. It has been reported that planctomycetales populations increase after blooms of microeukaryotes in the soil which could be related to chitinolytic activity on fungal cell walls. (15)

2.5 Conclusions

Short-term syringe and flow-through column microcosm experiments generated data to support several hypotheses tested. The addition of wood significantly increased microbial cell counts with significant increases in the numbers of cells in both the water and solid fractions of saturated syringe microcosms. Also, the number of microbial cells was significantly correlated with NO_3^- concentrations in both the syringe and flow-through column experiments. The presence of NO_3^- did not lead to a significant increase in microbial cell growth during short-term syringe microcosm incubations but results may have been confounded by the use of nitrogen to create anaerobic conditions.

Hypotheses with regard to the long-term effects of pine wood on the microbial community were supported by the data generated from jar microcosm experiments. The addition of pine wood shavings to subsurface clay soil caused a significant increase in the number of culturable NO_3^- reducers. Nitrate reducers were isolated from incubations of pure pine wood shavings and EL-FAMEs characteristic of microeukaryotic populations were increased in the presence of the pine wood. EL-FAME analysis was a useful tool for the estimation of microeukaryote and eubacterial populations but may be subject to detection limits based on population size. The number of NO_3^- reducers isolated from the pine wood controls significantly decreased from six to 24 weeks and may have implications for long-term microbial community dynamics in a permeable barrier.

Water samples had less variance then solid samples for both NRCFUs and nmol EL-FAME from jar microcosms. The number of NRCFUs in the jar microcosm saturated

mixture water and solid samples were not significantly different, nor were the number of cells in the water versus solid samples of syringe microcosms. These results indicate that water samples may be used for analysis of barriers and may be preferred to solid samples because there is less variability in the data, from both jar and syringe microcosms and the number of cells in the water is significantly correlated with NO_3^- concentration in the syringe and flow-through microcosms.

The abundant fatty acids found in the soil microcosms are produced by eubacteria although soil water samples were enriched in microeukaryotic fatty acids. The abundant fatty acids found in the wood and mixture microcosms are produced by fungal saprophytes and parasites such as chytrids, oomycetes and rusts as well as protozoa, algae and some Cyanobacteria. This suggests that although eubacterial NO_3^- reducers are increased though the addition of wood, the removal of NO_3^- from wood based permeable barriers may be dominated by microeukaryotes through assimilation and turnover of nutrients via parasitism, saprophytic activity and grazing.

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Treatment	Soil (cc)	Wood (cc)	Water (cc)
2:1 Mixture moist	50	25	25
2:1 Mixture saturated	50	25	75
Soil moist	50	0	10
Soil saturated	50	0	50
Wood moist	0	75	45
Wood saturated	0	75	85

Table 2.1 Jar microcosm components

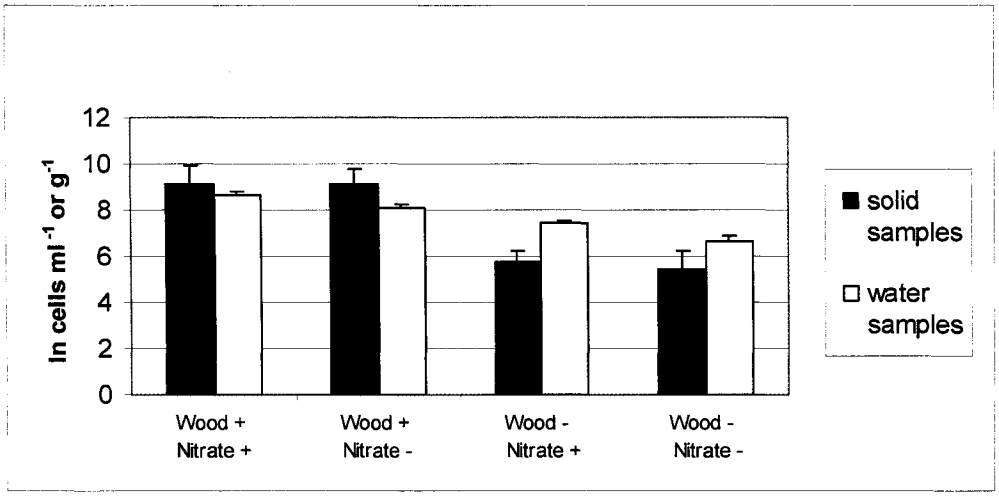
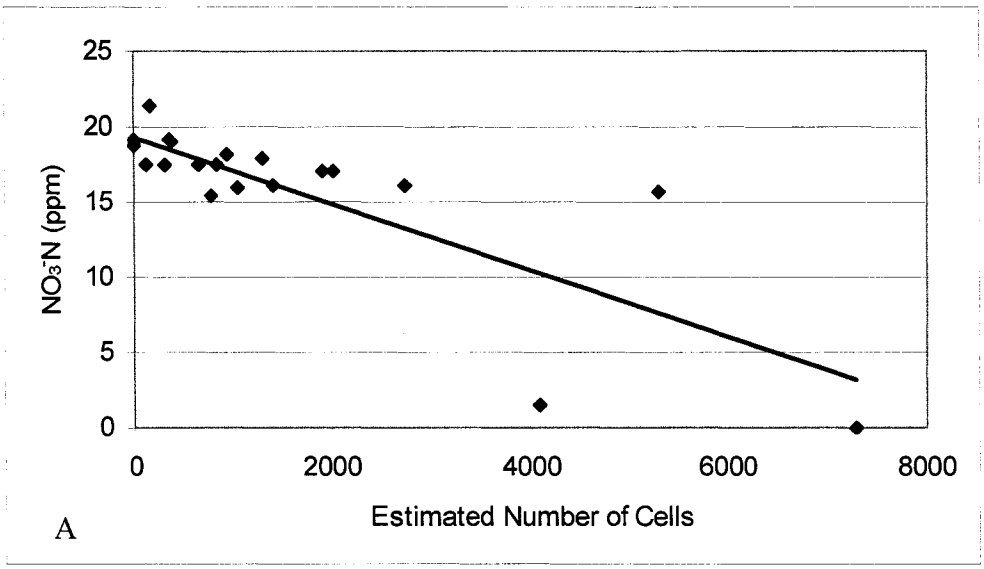


Figure 2.1 Effects of wood and 20 ppm NO_3^-N on the total number of estimated cells in syringe microcosms after three days incubation. Error bars reflect the standard deviation of three replicates. There is no significant difference between water and solid samples or in treatments with and without nitrate. Wood containing treatments have significantly more cells than those that do not.



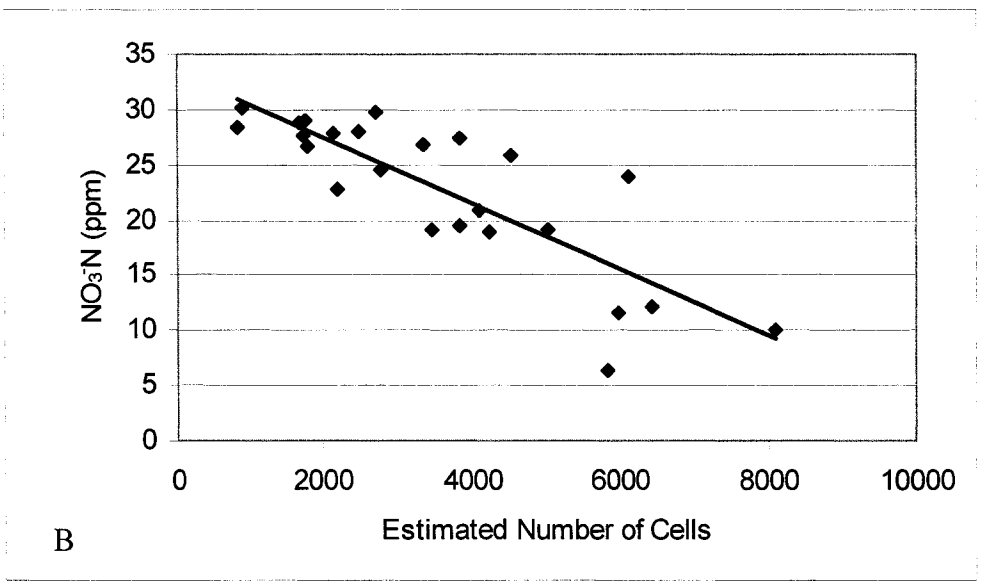


Figure 2.2 Regression of nitrate concentration (ppm) on the number of microbial cells illustrates that the concentration of nitrate decreases with an increase in the number of microbial cells. (a) Regression of NO_3^-N on estimated number of microbial cells in syringe microcosm solution where n = 22, adj $r^2 = 0.64$, SEM = 0.0004, $p < 0.0001$, $y = -0.002x + 19.4$ (b) Regression of NO_3^-N on estimated number of microbial cells in flow-through column effluent from a 13 day trial with 30 ppm NO_3^-N where n = 32, adj $r^2 = 0.568$, SEM = 0.004, $p<0.0001$, $y = -0.002x + 32$. See Appendix I.G. for regression statistics.

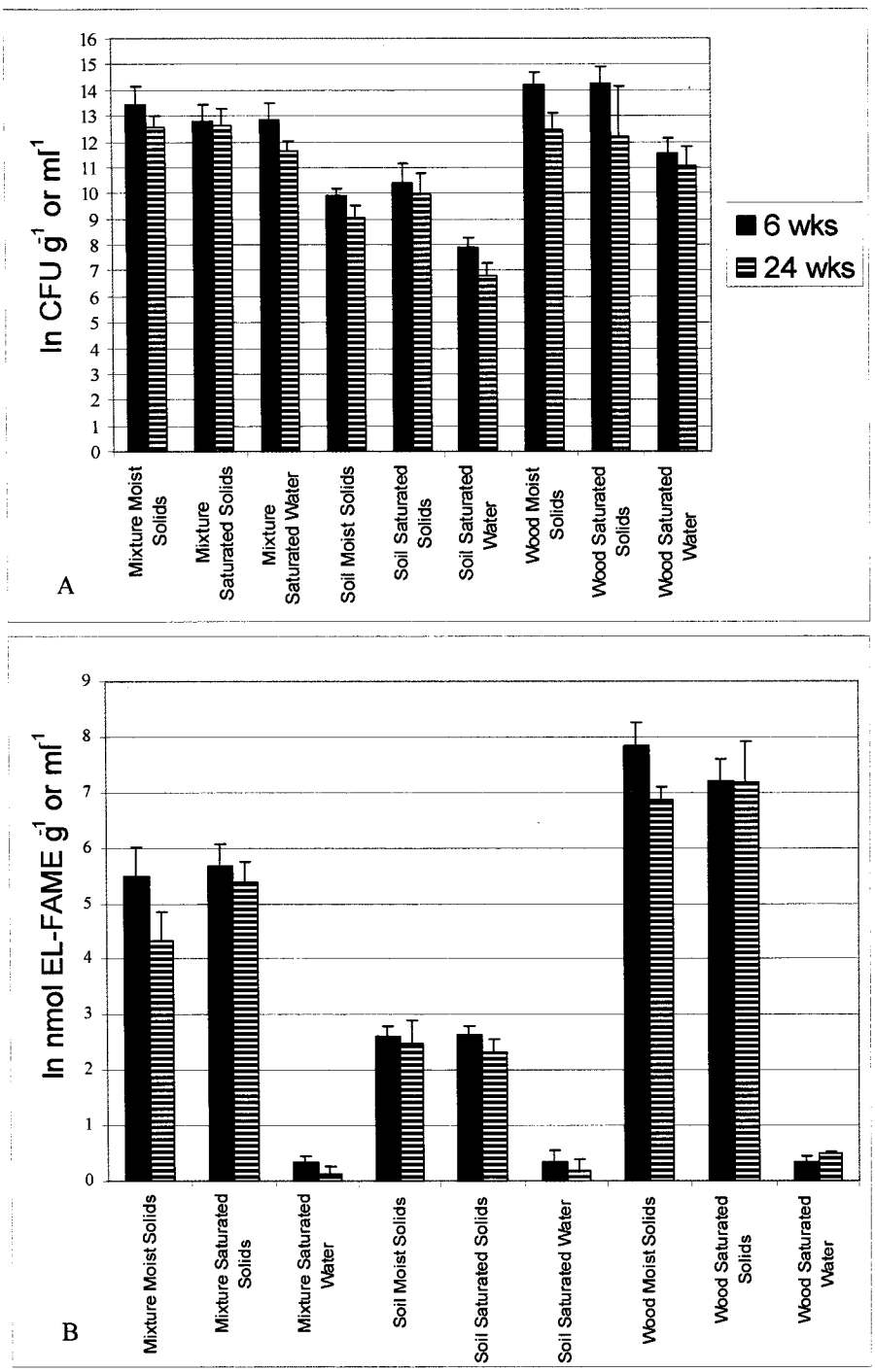
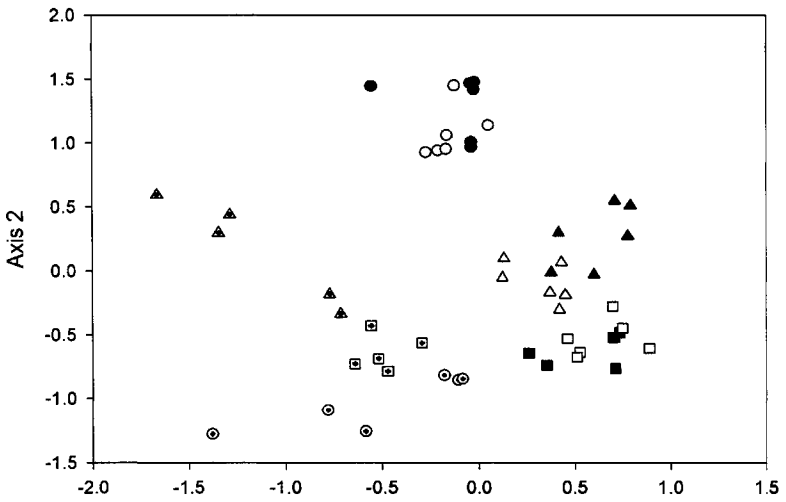


Figure 2.3 Nitrate reducer colony forming units (NRCFUs) and total nmol ester linked fatty acid methyl esters (EL-FAME) from jar microcosms. Total estimated NO_3^- reducing colony forming units (a) and total nmol EL-FAME (b) g^{-1} of moist and saturated jar microcosms and ml^{-1} water from saturated microcosms after six and 24 weeks incubation. Data was transformed using natural log with a constant of one used for EL-FAME to avoid negative log. Error bars represent the variance of three reps.



Axis 2	
tau	fatty acid
0.692	i15:0
0.696	a15:0
0.962	16:1w6c
0.709	16:1w7c
0.648	i17:1g
0.655	18:1w9t/7c
-0.657	18:3w6c

Axis 1	
tau	fatty acid
0.603	20:2w6c
0.589	20:1w9c

Figure 2.4 Non-metric multidimensional scaling (NMS) ordination of EL-FAMEs extracted from solids and water samples from jar microcosms. Saturated and moist treatment solids are represented by the same symbol (no significant effect of moisture). The coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space were 0.324 for axis one and 0.594 for axis two with a cumulative r^2 of 0.918. The axes were orthogonal (99.7% orthogonality). Kendall's Correlation values refer to fatty acids that are enriched in the samples. Positive and negative numbers correspond to the relative contribution of the fatty acid to the groupings in the positive or negative ends of the axis. The *i* and *a* fatty acids are characteristic of gram positive eubacteria while 18:1ω9t/7c is enriched in gram negative eubacteria and 18:3ω6c is found in microeukaryotes.

CHAPTER III

PINE WOOD SHAVINGS CONTRIBUTE NON-DENITRIFYING NITRATE REDUCERS TO PERMEABLE BARRIER MICROCOSMS

3.0 Abstract

Wood products are proposed for use in permeable barriers as a reductant to enhance microbial denitrification to reduce the amount of nitrate from agricultural runoff entering into tributaries in an effort to reduce hypoxia that threatens fishing and recreational industries. Data regarding the identities of the microbial populations found in permeable barriers, especially the denitrifiers and other nitrate reducers are required in order to design and manage permeable barriers for denitrification. A combination of 16S rRNA gene sequence similarity and Restriction Fragment Length Polymorphisms (RFLP) of 16S rRNA genes was used to identify culturable denitrifiers from jar microcosm incubations. Only 1 of 128 nitrate reducer isolates from wood denitrified indicating that the majority of nitrate reducers in wood are non-denitrifying or that denitrification ability is inhibited by the wood. The denitrifiers most commonly isolated from permeable barrier microcosms are fluorescent pseudomonads and *Azospirillum* species which are potential candidates for denitrifier supplementation due to their ability to compete with non-denitrifiers, elude grazing, survive during periods of low nitrate input and scavenge micronutrients. The odds of isolating dentrifiers were higher for water than for solid

samples supporting the use of water samples to monitor denitrifiers in permeable barriers. The odds for isolation of denitrifiers from the mixture increased with time and were significantly greater than those from the soil or wood controls. It was reported in Chapter II that the total number of nitrate reducers in wood controls significantly decreased with time. It is possible that the increase in the odds of selection for denitrifiers in the mixture over time was due to reduced competition from non-denitrifying nitrate reducers introduced from the wood.

3.1 Introduction

Riparian vegetation provides organic carbon to soil dwelling denitrifiers for use in the reduction of NO_3^- to nitrogen gas (N_2), which, ideally, removes excessive nitrate (NO_3^-) from water prior to entry into streams and rivers (6). Permeable soil barriers supplemented with carbon sources such as wood, straw, leaves or bark have been explored for use as carbon and electron sources for the microbial reduction of NO_3^- to N_2 and its subsequent removal from the terrestrial ecosystem in locations where excessive NO_3^- presents a risk for eutrophication of waterways and subsequent hypoxia that threatens fishing and recreational activities (5,15,28,29,30).

The effects of pine wood shavings on selection for denitrifying isolates from jar microcosms of a 2:1 (v/v) soil:wood mixture plus soil and wood controls were tested here. Pine wood was used because it is more recalcitrant than other materials and therefore may provide carbon and electron sources for a longer period of time plus it is widely and readily available (29). There were no previous reports of data on the identities

of specific denitrifiers and NO_3^- reducing populations found in wood amended subsurface soil. The isolation and identification of denitrifying organisms suited for survival in a barrier in the absence of high concentrations of NO_3^- can be used for analyses of suitability as inocula for denitrifying permeable barriers. Nitrate reducing isolates were tested for gas production and the reduction of NO_3^- and NO_2^- in nitrate broth with durham tubes to characterize denitrification ability. Isolates were identified by sequence similarity to16S rRNA genes and by comparison of RFLP patterns.

There was a paucity of data regarding the partitioning of denitrifiers between water and solid portions of denitrifying permeable barriers. The use of water samples to monitor the effectiveness of a barrier with regards to the denitrifier populations has advantages over solid samples due to less variability in the data and fewer potential inhibitory substances for molecular analysis. It is also a mobile component of the barrier. Odds ratios were applied to determine if water samples from saturated jar microcosms significantly increased the chances of isolating denitrifiers versus from the solid samples and whether there was an increase in the odds of selection of denitrifiers in the mixture over the soil and wood controls.

The experiments described below test three of the hypotheses outlined in Chapter I. The first of these null hypotheses states that the odds of isolation of denitrifying NO_3^- reducers will be the same in the water versus the solid samples from saturated jar microcosms with the alternative hypothesis that the odds of isolation of NO_3^- reducers capable of denitrification will be greater in water samples than in solid samples due to the

solubility of the substrates of the reaction. The second null hypothesis is that the odds of isolation of NO_3^- reducers that are capable of denitrification will be the same in the mixture and the soil treatments from jar microcosms. The alternative hypothesis is that the addition of wood to soil increases the odds of isolation of denitrifiers from the mixture. The third null hypothesis states that the odds of isolation of NO_3^- reducers that are capable of denitrification are the same for the wood contols of jar microcosms as for the soil and mixture. The alternate hypothesis is that the odds of isolation of NO_3^- reducers that are capable of denitrification will be greater in the soil and mixture than in the wood due to the favorable pH, nutrients and microbial inocula supplied by the soil and the low pH of the wood. An additional goal is to isolate and identify denitrifier populations and other NO_3^- reducing eubacterial species in samples from soil:pine wood mixture (permeable barrier). The isolates can be used to establish NO_3^- reducer community dynamics in the microcosms and to culture organisms that have the potential to be used as supplemental sources of denitrifier inoculum.

3.2 Materials and Methods

3.2.1 Jar Microcosms

Incubations were assembled as described in Chapter II. Soil was from a stream bank excavation (Colorado State University, Fort Collins, CO) from a depth of up to two meters. It was classified as belonging to the Heldt series formed in alluvium from clay shale (24). Carbon and nitrogen analyses were performed by the Soil, Water and Plant Testing Laboratory at Colorado State University and data are presented in Table 3.1.

Drywood Shavings were from Rushmore Forest Products (Hulett, WY). Soil and wood were sieved through an ethanol sterilized 4 mm stainless steel screen. Incubations were brought to 70% moisture or saturated with degassed, filter sterilized (0.2um) tap water in flint glass jars and covered with parafilm. Triplicate incubations were harvested after six and 24 weeks in the dark at room temperature.

3.2.2 Selection of Nitrate Reducers and Test for Denitrification

Soil dilutions were made with autoclaved water in sterile glass bottles, polypropylene tubes or eppendorf tubes. For isolations, duplicate plates at two dilutions were made for each sample based on a trial run and ranged from 10^{-2} to 10^{-6}. Dilution water blanks and media blanks were included as controls. Aliquots of 100ul of the dilution were pipetted onto the plates and spread with flame sterilized Dragalski spatulas.

Nitrate reducing isolates were cultivated on nutrient agar (DIFCO, Becton-Dickenson, Franklin Lakes, NJ) with 5mM KNO_3 (Fisher Scientific, Fairlawn, NJ), duplicate plates were incubated for five days in the dark at room temperature in gas pack chambers with BBL GasPak Plus Anaerobic Envelopes with Palladium Catalyst. BBL Dry Anaerobic Indicator Strips (Becton-Dickinson) were used to confirm anaerobic conditions. Nitrate reducing isolates from each treatment were tested for reduction of NO_3^- and NO_2^- and for gas production in nitrate broth (DIFCO, Becton-Dickenson) in durham tubes using DIFCO nitrateA and nitrateB reagents, or REMEL (Lenexa, KS) Bactidrop nitrateA and nitrateB reagents after up to one week of incubation at room temperature in the dark. One gram of zinc dust was used to confirm negative reactions. Duplicate assays

were performed on 33% of the isolates and over 90% of the duplicates gave the same results. Isolates were chosen to represent all unique colony types, however some were not readily separated and therefore not all unique colony types could be tested.

3.2.3 Six Week Jar Microcosm Isolates

Half of each tested colony from six week plates was streaked on nutrient agar and the other half was used to inoculate the nitrate broth to test for denitrification. Several loopfuls of culture from the nutrient agar plates were then used to extract DNA with the FastDNA Kit (BIO101, Carlsbad, CA). Extracted DNA was sent to MidiLabs (Newark, DE) where PCR and sequencing reactions were performed. 16S rRNA gene sequences of approximately 500 bp length were received from MidiLabs corresponding to *E. coli* positions 005 and 531 (see Appendix II for sequence data). Sequences were identified by similarity to sequences in GenBank using Blastn. (1)

3.2.4 Twenty Four Week Jar Microcosm Isolates

Duplicate samples of 150ul were removed from nitrate broth cultures, prior to addition of reagents, and mixed with glycerol to make frozen stock which was stored at -80°C. Isolates from frozen stock samples were cultured in nitrate broth and centrifuged at 10,000 x g for 20 minutes. DNA was extracted from cell pellets using the FastDNA Kit and a 1300 bp fragment of the 16S rRNA gene was amplified using 63f and 1387r primers (21). DNA was digested with the restriction enzyme Msp1 (Fisher Scientific) according to the manufacturers protocol using *E. coli* as a positive control.

RFLP samples were run on native polyacrylamide gels for approximately three hours at 10°C. A 50 bp ladder (Invitrogen, Carlsbad, CA) was run for comparison. Bands were visualized by silver-staining (4). Band sizes were calculated by regression of distance migrated on the number of base pairs per band of the ladder described above and digestion was confirmed based on estimated band size calculated from known sequences. Patterns were compared and similar patterns were rerun next to each other for verification. (See Appendix II for RFLP gels)

16S rRNA gene amplicons to be sequenced were purified with the Wizard DNA Purification Kit (Promega, Madison, WI). Amplicons were sequenced at UC Davis (Sacramento, CA). Sequences of approximately 1300 bp length for forward and reverse sequences were received from UC Davis. Sequences were compiled using Seqman II (DNASTAR, Madison, WI) and identified by similarity using Blastn at NCBI (1).

3.2.5 Flow-Through Column Microcosm Isolates

Flow-through columns were designed and constructed by Lauren Glushik as described in Chapter II (15). After 13 days of incubation at room temperature in the dark, effluent was collected for dilution plating and columns were disassembled for solids sampling and dilution plating. Isolates were cultured on nutrient agar with 5mM KNO_3. Isolates were not tested for denitrification ability. DNA extractions were performed as described above. 16S rRNA genes were amplified using primers 517f and 1406r (17). DNA was purified with the Qiagen QIAquick PCR Purification Kit (Valencia, CA). Forward and reverse sequences of approximately 860 bp were obtained from MacroMolecular Labs

(Colorado State University, Ft. Collins, CO). Sequences were compiled and identified as described above.

3.2.6 Statistical Analyses

Small sample sizes from some of the six week incubations were due to low numbers of isolates on high dilution plates and overlapping colonies that could not be readily isolated. Colonies were selected based on unique colony morphology and similar colonies with high counts were tested more often. However diversity analyses were not possible because not all colonies on all plates were tested. The assays for NO_3^- reducer isolation and gas production used a high protein media which discouraged fermentation and the generation of CO_2 gas which can yield false positives (33). In order to make a statement about the effect of treatment on the selection for denitrifiers, odds ratio were determined by the method of Ott and Longnecker (25) for (α=0.05). Twenty four week sample denitrifiers had a constant of one added because the wood samples had zero denitrifiers.

3.3 Results

3.3.1 Jar Microcosm Carbon and Nitrogen Analyses

Table 3.1 lists the results of the nutrient analyses on composite samples of microcosms from each treatment for each time point. No results are available for water from saturated microcosms as there was not enough volume for carbon to nitrogen (C/N) ratio determinations after FAME analysis and culturing. Initial C/N ratios were highest in wood and lowest in the soil. The C/N of the soil and mixture increased from zero to six

weeks due to the decrease of nitrogen. The C/N ratio of the wood treatments dropped substantially after six weeks of incubation due to an increase in nitrogen which may have been the result of nitrogen-fixing by free living eubacteria and were consistent with the soil and mixture treatments. The C/N ratios after six and 24 weeks were highest in the mixture and lowest in the wood. There was an increase in the C/N ratio of the moist solids from the mixture and soil treatments from six to 24 weeks but a decrease in the C/N ratio of the wood. A decrease in the C/N ratio in the saturated mixture and soil incubations from six to 24 weeks and an increase in the saturated wood ratio was observed. Levels of NH_4^+ increased in all treatments between six and 24 weeks.

3.3.2 Jar Microcosm Denitrifiers

Nitrate reducers from all treatments and time points were grown on nutrient agar plates supplemented with 5 mM KNO_3 incubated anaerobically at room temperature in the dark. This culture method was biased toward fast growing eubacteria (33); however, we make the assumption that these organisms are also fast growing *in situ* and would be more competitive for NO_3^- under reducing conditions *in situ* than slower growing organisms and therefore react quickly to sporadic and seasonal NO_3^- inputs. Representatives of all colony types were tested for gas production in nitrate broth, which indicated denitrification activity. A total of 426 isolates were tested, 107 from 6 weeks and 319 from 24 week samples (Table 3.2.a). The odds ratios for isolation of denitrifiers from total nitrate reducers were calculated for soil, wood and mixture treatments.

At 6 weeks, it was 14.8 times as likely that denitrifiers would be isolated in the soil microcosms versus the wood. Nutrient level determinations showed high levels of NO_3^- in the soil and low levels in the wood. At 24 weeks, it was 79.4 times as likely that denitrifiers would be selected in the soil versus the wood. Both of these are significant. Denitrifiers were very rare in the wood microcosms; only one out of 128 of the NO_3^- reducer isolates tested was a denitrifier. Therefore, the NO_3^- reducing isolates from wood treatments reported in Chapter II are predominantly non-denitrifiers. This supports rejection of the null hypothesis that the odds of isolation of NO_3^- reducers that are capable of denitrification are the same in the soil control as in the wood controls of jar microcosms in favor of the alternative hypothesis that the odds of isolation of NO_3^- reducers that are capable of denitrification will be greater in the soil than in the wood due to favorable pH, nutrients and microbial inocula supplied by the soil and lower in the wood due to acidic pH or other inhibitory substances.

Odds ratio analyses determined that after 24 weeks, it was 2.27 times as likely that denitrifiers would be cultured in the mixture versus the soil treatments and 180 times as likely that they will be cultured in the mixture versus the wood. These are both considered significant at $\alpha=0.05$. Odds ratios were not significant for six week samples. Between six and 24 weeks, there was an increase in percent denitrifiers of total NO_3^- reducers tested from the mixture saturated solids and saturation water. The null hypothesis that the odds of isolating NO_3^- reducers that are capable of denitrification will be the same in the mixture and the soil treatments from jar microcosms is not rejected at

six weeks, but is rejected at 24 weeks in favor of the alternative hypothesis that the addition of wood to soil increases the odds of isolating denitrifiers from the mixture.

The null hypothesis that the odds of isolating NO_3^- reducers that are capable of denitrification is the same in the mixture (permeable barrier) as in the wood controls of jar microcosms is also not rejected at six weeks, but rejected at 24 weeks in favor of the alternate hypothesis that states that the odds of isolating NO_3^- reducers that are capable of denitrification will be greater in the mixture (permeable barrier) than in the wood due to favorable pH, nutrients and microbial inocula supplied by the soil. It was reported in Chapter II that the total number of NO_3^- reducers in wood controls significantly decreased with time. It is possible that the increase in the odds of selection for denitrifiers in the mixture over time was due to reduced competition from non-denitrifying NO_3^- reducers introduced from the wood or due to adaptation by denitrifiers previously inhibited by the conditions in the wood.

Odds ratios for moist solids, saturated solids, and saturated water (Table 3.2) were also calculated to look specifically at the difference between water and solids because substrates for denitrification are soluble, therefore it was expected that a high percent of denitrifiers would be isolated from water samples. At 6 weeks the odds ratio was greater, but not significant for water versus solids. At 24 weeks the odds were 2.84 times greater that denitrifiers would be cultured from the water column versus the saturated solids samples which was significant at $\alpha=0.05$; therefore at six weeks the null hypothesis that the odds of isolating denitrifying nitrate reducers will be the same in the water versus the

solid samples from saturated jar microcosms was not rejected but at 24 weeks it was rejected in favor of the alternative hypothesis stating that the odds of isolating NO_3^- reducers that are capable of denitrification will be greater in water samples than in solid samples due to the solubility of the substrates of the reaction.

3.3.3 Identification of Jar Microcosm Nitrate Reducers

Long-term microcosms without added NO_3^- were constructed to simulate the substrate in a 2:1 soil:wood permeable reactive barrier for NO_3^- removal. The number of NO_3^- reducing colony forming units (NRCFUs) cultured from the microcosms did not change significantly from six to 24 weeks in the soil or mixture; however, the percent of denitrifiers increased in the mixture from 23% at six weeks to 62% at 24 weeks. Although there were a large number of NO_3^- reducers in the wood treatments, there were virtually no denitrifiers detected. Therefore it was important to identify NO_3^- reducers in the six week soil and mixture treatments and then examine the mixture denitrifiers in 24 week samples to determine whether there was an increase in diversity, an explosion in a specific population or adaptation of a population that previously tested negative for denitrification.

Sixty-five colonies from the KNO_3 supplemented nutrient agar plates from six week samples were chosen for 16S rRNA gene sequencing. Colonies were chosen from each treatment that were unique and represented both denitrifiers (gas production in nitrate broth) and other NO_3^- reducers because what appeared to be strains within the same species had variable denitrification ability. Table 3.3 lists 16S rRNA gene sequence

similarity, plate dilution and results of nitrate broth assays for these isolates. In the soil treatments, a greater diversity of denitrifying organisms was represented by unique colonies, with isolates from alpha, beta, and gamma proteobacteria. In the mixture treatments alpha and gamma proteobacterial isolates were *Azospirillum* species and fluorescent pseudomonads. The single denitrifier from the wood treatments had high sequence similarity to an alpha proteobacterium, *Pseudomonas sp. G-179* (reclassified as a Rhizobiaceae), used to characterize a Cu-containing NO_3^- reductase for comparison with other denitrifiers (35).

To determine if the same species were responsible for denitrification in the mixture treatments at six and 24 weeks, RFLP patterns were generated for fifteen of the six week denitrifying species from the mixture and soil treatments that had already been sequenced (Table 3.3) and compared those with RFLP patterns of 45 denitrifying isolates from the 24 week mixture incubations (Table 3.4). Twenty-three (51%) of the isolates from 24 weeks had one of four patterns found in six week samples. The balance of samples fell into three new patterns. Representatives from each new pattern were identified by 16S rRNA gene sequencing (Table 3.4). The isolates from the unique patterns had highest percent similarity to *Azospirillum sp. B506* and *A. sp.* DA6-2-2, *Pseudomonas fluorescens* and *Ralstonia sp.* LMG 19089.

The predominant species of denitrifiers in the mixture water samples were *Azospirillum* species (alpha proteobacteria) while the predominant species in the mixture moist solids were members of the fluorescent pseudomonads (gamma proteobacteria). In the

saturated solids, the species were evenly distributed between the two groups. Patterns 10 and 14 each had a single isolate but the sequences were too noisy to identify. *A. sp.* DA6-2-2 was found in the mixture at six weeks but tested negative for denitrification and may be an example of adaptation.

3.3.4 Flow-Though Column Isolates

Since NO_3^- is soluble, dissolved carbon is highly correlated with denitrification and chemotaxis has been reported for NO_3^- reducing organisms, it was expected that non-attached NO_3^- reducers will be present in effluent. Nitrate reducers were isolated from both the effluent and solid portions of flow-through columns of filter sand and sawdust inoculated with river sediment after 13 days of incubation. These were identified by 16S rRNA gene sequence similarity, are presented in Table 3.5 and include fluorescent pseudomonads and beta proteobacteria.

3.4 Discussion

3.4.1 Effects of Addition of Wood to Soil for Barrier Construction

Permeable barriers created with the intent of restoring riparian vegetation may require supplemental nitrogen in the rooting zone because carbon and nitrogen ratios above 20/1 will result in microbial scavenging of nitrogen to the detriment of plant life (6). A second option is to install barriers below the rhizosphere and amend the rooting zone with nutrient rich top soil, then plant vegetation to restore riparian area. The C/N results for jar microcosm mixture treatments suggest that a permeable barrier would be unsuitable for plant growth in cases where runoff contained less than approximately 2% total

nitrogen for an extended period. The mixture and soil treatment samples both showed a decrease in total percent nitrogen from zero to six weeks, consistent with denitrification activity which would release nitrogen in gaseous form. High C/N ratios have the potential to cause leaching of (dissolved organic carbon) DOC which is in excess of that assimilated by the microbial community.

The wood treatments had an increase in total nitrogen which may have been the result of nitrogen-fixation activity. Genera that have species of nitrogen-fixers such as *Clostridium* and *Azospirillum* were isolated from the wood. Bremner and Shaw (1958) reported that in their investigations of denitrification, nitrogen-fixation may have occurred after the depletion of NO_3^- under anaerobic conditions where the carbon supply was in excess of that needed by denitrifiers to reduce the available NO_3^- (7). After 24 weeks the mixture saturated samples also had decreased carbon and increased nitrogen, possibly from nitrogen-fixation. *Azospirillum* were among the most common NO_3^- reducer genera isolated from the 24 week mixture water samples from saturated microcosms. Overall, the highest C/N ratios were found in the mixture and the lowest were in the wood. Wood microcosms may have supported more nitrogen-fixation which increased total nitrogen and decreased the ratio. The mixture had nutrients supplied from the soil and NO_3^- from this source likely supported NO_3^- reduction and an increase in the C/N ratio.

There was an increase in NH_4^+ in all treatments over time. Increased NH_4^+ over time may be due to protozoan and/or fungal grazing on bacteria and subsequent nutrient turnover

since bacteria contain more nitrogen than can be assimilated by the microeukaryotes who release the excess as NH_4^+. Alternately, it can be the result of dissimilatory NO_3^- reduction to ammonium (DNRA). DNRA is postulated to act as an electron sink, since it uses eight electrons per NO_3^- reduction versus five for denitrification, and is expected in a high C/N environment under anaerobic conditions (32). Candidates for this type of reduction are in Table 3.3 and were positive for NO_3^- and NO_2^- reduction but negative for gas production in nitrate broth. The majority are gamma proteobacteria from the *Pseudomonas* genus with a single alpha proteobacterium.

The increase in carbon observed in all of the 24 week samples could be due to abiotic factors. Small amounts of carbon dioxide can react in soil and form carbonic acid, carbonates and bicarbonates with base forming cations (6). Increased NO_3^- in the moist wood could be due to grazing or the activity of eubacterial nitrifiers; however, nitrification is inhibited by high C/N ratios and by high moisture. Therefore the experimental conditions were more suitable for nitrogen-fixation and denitrification (6).

3.4.2 Denitrifiers from Jar Microcosms

Permeable reactive barriers for NO_3^- removal are usually constructed by mixing local soils with wood products, so the population of NO_3^- removers in the barrier is obtained from those materials. Previously published numbers of NO_3^- reducers from soils cite denitrifier populations from $1 - 5 \times 10^6$ g^{-1} (14). NO_3^- reducing populations in this study ranged from 10^3 - 10^6 CFU g^{-1} in the moist and saturated solids and 10^2 - 10^5 CFU ml^{-1} in the water column above the saturated solids. Denitrifiers from soil were reported to

represent 25-33% of NO_3^- reducers (14). This study determined the percent dentrifiers of NO_3^- reducers in the incubated subsurface clay soil to be 42% at both six and 24 weeks. The mixture had 23% denitrifiers at six weeks and 62% denitrifiers at 24 weeks, suggesting an initial effect of competition from other NO_3^- reducers, possibly introduced from the wood, with subsequent selection for denitrifiers over time.

At six weeks the only significant difference was in the odds ratio for denitrifiers in the soil versus wood. The decreased NO_3^- at six weeks in the soil treatments may have been due in part to the denitrifiers which were isolated from these treatments. The soil had ideal initial conditions for denitrification; there was a loss of nitrogen from the microcosms and denitrifiers were isolated (Table 3.4). However, due to the loss of NO_3^- via denitrification the C/N ratio increased and conditions did not remain favorable for denitrifiers.

In contrast to the soil, only one of 128 isolates tested from wood microcosms was able to denitrify. This may be a result of the acidic pH of the wood which ranged from 4.8 to 5.1. Nitrite is toxic to some denitrifiers in slightly acidic soil and low pH can favor the retention of NH_4^+ (33). The single denitrifier from the wood treatments had high sequence similarity to an alpha Proteobacterium, *Pseudomonas sp. G-179* (reclassified as a Rhizobiaceae) but a second isolate with similar sequence similarity to *P. sp. G-179* did not denitrify. It has been reported that denitrification ability can spontaneously be lost or gained with sub-culturing, is related to nitrous oxide reductase activity and postulated to be either a result of NO_2^- toxicity or due to unstable genetic material (14).

The *nap* genes in organisms such as *Ralstonia eutropha* and *Pseudomonas sp.* G-179 are on endogenous plasmids. The transfer of a Cu NO_3^- reductase gene (*nir*K) to a *P. stutzeri* mutant for cytochrome cd1 nitrite reductase (*nir*S) restored mutationally interrupted denitrification. The *nir*S mutation was due to a transposon Tn5 insertion. Mutagenesis of *nos*X has been reported to stop reduction of N_2O by *Sinorhizobium meliloti*. This protein has been suggested to function in transport of Cu or its insertion in the apoenzyme binding site. Environmental control of *nos*Z involved the presence of nitrogenous oxides and anaerobiosis which were required for significant expression in *P. stutzeri* and *P. fluorescens*. (26)

What appear to be closely related isolates by 16S rRNA gene sequence comparison, exhibited differing ability to produce gas in nitrate broth. The most common denitrifier isolate from the six week soil samples was *P. borealis* which was reported to be a dominant ribotype in subsurface soils from California grassland (19). Isolates with a high percent similarity to *P. borealis* found in the soil moist solids were denitrifiers; those sampled from the soil saturated solids reduced NO_3^- and NO_2^-, but did not produce nitrogenous gases or only reduced NO_3^- to NO_2^-. *Azospirillum sp.* DA6-2-2 was isolated from the mixture at six weeks and tested negative for denitrification but isolates of what appear to be the same species from 24 week mixture incubations were able to denitrify.

There was one case of multiple isolates with the identical sequence that had different NO_3^- reduction paths. The morphology and denitrification ability differed when cultured in the wood versus the soil and mixture. The sequence has 100% similarity to

Pseudomonas sp. LCY11. A single isolate each in the mixture and in the soil denitrified while the balance of those in the mixture and those in the wood did not. These may have been different strains and the 500 bp sequence may not have been long enough to resolve them.

At six weeks there was no significant difference in the odds ratios for mixture treatments versus wood because denitrifiers in the mixture may have been out competed by other NO_3^- reducing organisms introduced from wood. There was a significant decrease in the number of NO_3^- reducers in the wood controls from six to 24 weeks. At 24 weeks there was an increase in denitrifiers as a percent of the total NO_3^- reducers tested from mixture saturated solids and saturated water samples. The odds of selection for denitrifiers at 24 weeks in the mixture were significantly greater than in the wood or soil. Isolates related to *Azospirillum* and *Pseudomonas* were found at both six and 24 weeks in the mixture and the increase in the percent denitrifiers in the mixture over time is attributable to an increase in the numbers of these populations.

Some species of *Azospirillum* are diazotrophs (27), as well as denitrifiers and the ability to fix nitrogen may have been a selective advantage due to the high C/N in the mixture incubations. RFLP pattern 2 was the most common among 24 week denitrifiers from mixture samples. Two of these isolates were identified as having high sequence similarity to *Azospirillum sp. B506* which is reported to be a nitrogen-fixer (12). Other genera with N_2 fixing species, *Erwinia, Clostridium, Citrobacter* and *Rhizobium* (27), were isolated from the mixture and/or wood. The addition of wood may be selecting for

nitrogen-fixers which would aid the in survival of dentrifiers during seasonal fluctuations of nitrogen in a high carbon environment. This activity would definitely confound attempts to measure NO_3^- removal, since we have shown NO_3^- accumulated in the microcosms without an outside source of additional NO_3^-.

The majority of the *Pseudomonas* isolates from the jar microcosms had high sequence similarity to fluorescent pseudomonads. Many fluorescent *Pseudomonas* species are saprophytic and phytopathogenic with some human or animal pathogens (11). They are ubiquitous and are the most common denitrifying isolates from soils worldwide (14). Saprophytic species would have a selective advantage in mixture microcosms due to the high carbon content. Those that respire with NO_3^- as well would be expected to grow under both aerobic and anaerobic conditions, in the presence and absence of excess NO_3^-.

3.4.3 Denitrifiers in Water Samples from Jar Microcosms

Assays of microbial communities in the runoff that enters riparian zones are needed because runoff may transport endogenous microbes, biofertilizers, biopesticides and fecal contamination from livestock. Antibiotics, heavy metals and organic chemicals such as pesticides will impact the microbial community in the field. Analyses of the microbial community in the water that leaves the barrier and enters into streams are also required since it is possible that pathogens and potentially toxic intermediate products of bioremediation as well as NO_3^- reducers will move with the water. Fluorescent pseudomonads related to *P. veronii* and *P. gessardii*, along with *P. alcaligenes*, were isolated from the effluent of flow-though columns of cherry sawdust, filter sand and river

sediment with and without supplemental NO_3^- (Table 3.3). This demonstrated that NO_3^- reducers could move with the water as it passes through the permeable barrier.

In the static jar microcosms, the odds of selecting for denitrifiers were significantly higher in the water versus the solid portion of saturated microcosms. This was not unexpected as a strong correlation has been shown between dissolved organic carbon and denitrification activity. Soils tested for denitrification showed significant correlation ($r=0.77$) between denitrification capacity and total organic carbon as well as very high correlation ($r=0.99$) with water-soluble organic carbon (8). Experiments discussed by Tiedje *et al.* (1994) suggest that some denitrifiers are motile and show tropism for dissolved NO_3^- (33). Lee *et al.* (2002) reported that *Agrobacterium tumefaciens* demonstrated chemotaxis towards NO_3^- and NO_2^- (20). Transport of microbes and nutrients with water in the field may homogenize the soil solution. Alternately, the community in the solution may change as it passes through pure soil, pure wood or the mixture.

3.4.4 Nitrate Reducers in Jar Microcosms

NO_3^- reducers that also degraded wood or used products of carbohydrate fermentation such as H_2 and acetate were expected (3). Strains of *Pantoea agglomerans (Erwinia herbicola)* have been shown to couple the oxidation of acetate or H_2 to the dissimilatory reduction of Fe, Mn and Cr (13) and to be important in the regulation of water content in the wheat rhizosphere through the improvement of soil aggregation due to the production of exopolysaccharides (2). *Pantoea agglomerans* was isolated from the mixture.

Eubacterial cellulase producers have been reported in the genera of *Clostridium*, *Bacillus*, and *Actinomycetes* and lignin degraders are found in the genera *Actinomycetes, Erwinia, Achromobacter* and *Pseudomonas* (10, 18). Some *Actinomycetes* were isolated on water agar that were capable of NO_3^- reduction to NO_2^-, but not denitrification (data not shown). *Clostridium* species were isolated from the wood and the mixture and *Pseudomonas* from the wood, soil and mixture. *Achromobacter* was isolated from the soil and *Erwinia herbicola (P. agglomerans)* from the mixture.

Coates *et al.* (2002) recently demonstrated that *Pseudomonas sp.* NMX used reduced humic substances as electron donors in NO_3^- reduction, as did *Agrobacterium tumefaciens* and *Dechloromonas* species. Humic substances were also shown to stimulate mineralization of complex carbon compounds in anaerobic incubations (9). *Pseudomonas*. sp. NMX and *Dechloromonas* were isolated from the soil and *Agrobacterium tumefaciens* from the mixture incubations.

Some of the NO_3^- reducing isolates from the mixture have been reported to be capable of degrading other substances such as aliphatic polyesters by *Ralstonia sp.* LMG 19089 (31). Others are potential commensals of earthworms and termites (Table 3.3). Matthies *et al.* (1999) reported evidence that denitrifying bacteria in the gut of earthworms were stimulated to produce N_2O in the presence of NO_3^- but not NH_4^+ (22). The selection for these types of organisms can have unforeseen consequences on the transformations of nitrogen and other substances in a permeable barrier.

Some of the isolates are potential pathogens such as *Erwinia herbicola* and *A. tumefaciens*. *Pseudomonas* sp. NZ099 was reported to be a causative agent of blotch disease in *Agaricus bisporus* and is from the *P. putida* lineage (16). An isolate from the wood treatments with high sequence similarity to this organism was abundant on anaerobically incubated nutrient agar plates supplemented with KNO_3, but had slow growth in nitrate broth and did not reduce NO_3^- to NO_2^- after one week.

3.4.5 Microeukaryotes in Jar Microcosms

Previous analysis of the jar microcosm microbial communities by EL-FAME (Chapter II) revealed the presence of microeukaryotes, which may affect the community dynamics of NO_3^- reducers. Weekers *et al.* (1995) showed that *A. tumefaciens* was not affected by *Acanthoamoeba* grazing (34). Matz *et al.* (2004) reported that the pigment violacein produced by some *Janthinobacterium* species caused acute toxicity in nanoflagellates that ingested the bacterium (23). *Janthinobacterium* species were isolated from the moist mixture and produced a purple/black pigment on nutrient agar supplemented with 5 mM KNO_3. The role of protozoan grazing is discussed further in Chapter IV.

3.5 Conclusions

The pine wood incubations selected for non-denitrifying nitrate reducers that belonged to genera with known cellulase producers and nitrogen-fixers. The addition of pine wood to soil resulted in a significant increase in the odds of selection for denitrifiers as a percent of NO_3^- reducers in the mixture versus the wood or soil alone after 24 weeks of incubation. The increase was found to be due in part to the presence of fluorescent

pseudomonads and *Azospirillum.* Most fluorescent pseudomonads are saprophytes and many *Azospirillum* species are nitrogen-fixers. This consortium could between them degrade wood, fix-nitrogen and denitrify when conditions prevailed. These organisms may be useful as microbial supplements to a permeable barrier.

Tiedje reports that at low concentrations of NO_3^-, the competition for the substrate will be dependent on the K_m of the enzyme. Since denitrifiers have a high affinity for NO_3^- they will be favored at low concentrations. At high concentrations the competition between DNRAs and denitrifiers will be dependent on population size. Additionally, high C/N environments, such as those found in saturated permeable barriers without consistently high NO_3^- inputs, are believed to favor DNRA populations. (33) Therefore, in order to enhance denitrification in permeable barriers at high concentrations of NO_3^-, supplemental inoculation with denitrifiers may be necessary.

The odds of selection of denitrifiers as a percent of NO_3^- reducers from water versus solid samples of saturated static microcosms were significant after 24 weeks and culturable NO_3^- reducing organisms in the *Pseudomonas* genus were transported with flow-through column effluent. This supports the conclusion presented in Chapter III which states that water samples are appropriate for the assessment of denitrifiers in permeable barriers and additionally, that inoculum of barriers with denitrifiers may be facilitated though a liquid medium.

3.6 Future Studies

The introduction of microbial denitrifiers into permeable barriers may be necessary to increase the population size in order to successfully compete with DNRAs for NO_3^- at high concentrations. Further studies with denitrifier isolates enriched from native soil should be conducted to determine whether they could be successfully introduced into a permeable barrier. Transport of microbes and nutrients with water in the field may homogenize the soil solution. Alternately, the community in the solution may change as it passes through pure soil, pure wood or the mixture. Analysis of water samples from permeable barriers may be used to test this hypothesis.

The introduction of foreign genes has potential for dissemination through horizontal gene transfer to unforeseen and possibly detrimental consequences. Permeable barriers could become a breeding ground for the emergence of new pathogens due to the influx of antibiotics, biopesticides, genetically modified organisms and fecal contaminants in the agricultural runoff. Isolates should be tested for their sensitivity and antagonism to antibiotics and chemicals in agricultural runoff prior to introduction into a barrier. Additionally, effluent from permeable barriers should be monitored for pathogens as well as dissolved organic carbon and nitrogen species.

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Treatment	# of Weeks	Total % Carbon	Total % Nitrogen	ppm NH_4^+N	ppm NO_3^-N	ppm NO_2^-N	C/N
Mixture*	0	47.90	7.51	NA	NA	NA	6
Mixture Moist Solids	6	42.98	0.54	1.98	7.11	0.042	80
Mixture Moist Solids	24	44.31	0.44	2.99	4.77	0.021	100
Mixture Saturated Solids	6	44.33	0.44	1.25	5.27	0.015	101
Mixture Saturated Solids	24	43.49	0.51	3.11	8.73	0.033	85
Soil	0	47.30	11.30	1.11	15.7	<0.01	4
Soil Moist Solids	6	41.25	0.63	1.04	4.73	0.035	66
Soil Moist Solids	24	45.39	0.57	1.34	4.38	0.022	79
Soil Saturated Solids	6	41.34	0.53	2.07	5.12	0.028	78
Soil Saturated Solids	24	46.42	0.64	2.77	6.99	0.033	72
Wood	0	50.54	0.16	NA	NA	NA	310
Wood Moist Solids	6	42.58	0.74	1.12	4.78	0.021	58
Wood Moist Solids	24	47.52	0.79	4.55	7.29	0.028	60
Wood Saturated Solids	6	44.32	0.84	2.43	6.25	0.035	53
Wood Saturated Solids	24	46.21	0.62	3.44	5.34	0.025	74

Table 3.1 Carbon and nitrogen levels from jar microcosms by treatment
* Calculated from soil and wood data

A	6 wk Nitrate Reducing Isolates			24 wk Nitrate Reducing Isolates		
	Denitrifier	Non-Denitrifier	Total	Denitrifier	Non-Denitrifier	Total
Soil	20	27	47	35	49	84
Wood	1	20	21	0	108	108
Mixture	9	30	39	79	48	127
Total	30	77	107	114	205	319

Odds of Isolating Denitrifiers	6 wk Nitrate Reducing Isolates				24 wk Nitrate Reducing Isolates			
	Odds	s.e.	95% CI	95% CI	Odds	s.e.	95% CI	95% CI
Mixture versus Soil	0.41	0.48	1.04	0.16	2.27	0.29	**3.97**	**1.30**
Mixture versus Wood	6.00	1.09	51.10	0.70	180.00	1.02	**1331.77**	**24.33**
Soil versus Wood	14.81	1.07	**119.77**	**1.83**	79.35	1.03	**595.46**	**10.57**

B	6 wk Nitrate Reducing Isolates			24 wk Nitrate Reducing Isolates		
	Denitrifier	Non-Denitrifier	Total	Denitrifier	Non-Denitrifier	Total
Moist Solids	13	25	38	41	59	100
Saturated Solids	5	27	32	32	78	110
Water	12	25	37	59	50	109
Total	30	77	107	132	187	319

Odds of Isolating Denitrifiers	6 wk Nitrate Reducing Isolates				24 wk Nitrate Reducing Isolates			
	Odds	s.e.	95% CI	95% CI	Odds	s.e.	95% CI	95% CI
Saturated versus Moist	0.36	0.59	1.14	0.11	0.59	0.29	1.05	0.34
Water versus Moist	0.92	0.49	2.41	0.35	1.69	0.28	2.91	0.98
Water versus Saturated	2.59	0.60	8.41	0.80	2.84	0.28	**4.93**	**1.63**

Table 3.2 Odds ratios for isolation of denitrifiers by substrate treatment (a) and by moisture treatment (b) for jar microcosms. A total of 426 nitrate reducing isolates were tested for the ability to produce gas and for the reduction of nitrate and nitrite in nitrate broth which indicates denitrification. The odds for isolation of a denitrifier from among the nitrate reducing isolates by treatment were calculated by the formula odds = (denitrifiers/total nitrate reducers)/(non-denitrifiers/total nitrate reducers). The odds ratios were determined by dividing the odds for situation one by the odds for situation two. The 95% Confidence Interval was calculated by adding or subtracting 1.96*S.E. to the odds ratio. S.E. was determined by taking the square root of the sum of 1/frequency for all four counts used in the odds ratio. If the 95% CI contains 1 then odds are not significantly different at α=0.05. The 95% CI's in bold typeface are significant at alpha=0.05. For example, the odds of isolating a denitrifier from the nitrate reducing isolates in the mixture at six weeks (A) were 0.41 or less than half that of isolating a denitrifier from the soil; however, this was not significant at alpha=0.05. The odds of isolating a denitrifier from the nitrate reducing isolates in the mixture at 24 weeks (A) was 2.27 times greater than that of isolating a denitrifier from the soil and this was significant.

Treatment	NRCFUs g^{-1} or ml^{-1}	Lab ID	RFLP Pattern	Identification by 16s rDNA Sequence Similarity	Blastn Identities	%	Div	Reduced NO_3^-	Reduced NO_2^-	Gas
Mixture Moist Solids	10^5	106-23		AF348508 *Pseudomonas corrugata from* sugar beet rhizosphere soil	518/522	99	G	+	+	+
Mixture Moist Solids	10^5	106-02		AJ492826 *P. tremae* type strain: CFBP 6111	520/521	99	G	+	+	+
Mixture Moist Solids	10^5	106-07	3	AF105385 *Psuedomonas sp.* PsG from Pinyon-Juniper Soil	519/521	99	G	+	+	+
Mixture Moist Solids	10^5	106-11		AJ292426 *Pseudomonas kilonenesis from agricultural soil/ P. brassicacearum*	516/516	100	G	+	+	-
Mixture Moist Solids	10^5	106-03		AF105385 *Psuedomonas sp.* PsG from Pinyon-Juniper Soil	519/521	99	G	+	-	-
Mixture Moist Solids	10^5	106-28		AY1182232 *Janthinobacterium sp*. An8 from deep sea sediment	516/519	99	B	+	-	-
Mixture Moist Solids	10^5	106-26		AY1182232 *Janthinobacterium sp*. An8	516/519	99	B	+	-	-
Mixture Moist Solids	10^5	106-24		AJ551147 *Citrobacter murliniae* genomospecies 11strain CDC 2970-59	523/525	99	G	+	-	-
Mixture Moist Solids	10^5	106-19		AY691545 *Pantoea agglomerans* ChDC YP3	521/523	99	G	+	-	-
Mixture Moist Solids	10^5	106-13		AY691545 *Pantoea agglomerans* ChDC YP3	521/523	99	G	+	-	-
Mixture Moist Solids	10^5	106-12		gi142272 *Agrobacterium tumefaciens*	465/467	99	A	+	-	-
Mixture Moist Solids	10^5	106-10		AY653222 *Pseudomonas sp.* KBOS	512/520	98	G	+	-	-
Mixture Moist Solids	10^5	106-05		AF005656 Gamma proteobacterium MS-1	513/522	98	G	+	-	-
Mixture Saturated Solids	10^5	128-05	4	AY510013 *Pseudomonas sp*. LCY11, Antarctic Soil PAH degrader	519/519	100	G	+	+	+
Mixture Saturated Solids	10^5	128-34		AY510013 *Pseudomonas sp*. LCY11	519/519	100	G	+	+	-
Mixture Saturated Solids	10^5	128-32		AY510013 *Pseudomonas sp*. LCY11	519/519	100	G	+	+	-
Mixture Saturated Solids	10^5	128-31		AY510013 *Pseudomonas sp*. LCY11	519/519	100	G	+	+	-
Mixture Saturated Solids	10^5	108-11		AY510013 *Pseudomonas sp*. LCY11	519/519	100	G	+	+	-
Mixture Saturated Solids	10^5	128-06		AJ292426 *Pseudomonas kilonenesis* from agricultural soil	516/516	100	G	+	+	-
Mixture Saturated Solids	10^5	128-21		AY228334 *Clostridium chromoreductans* Anaerobic Cr(IV) reducer from soil	491/497	98	F	+	-	-
Mixture Saturated Solids	10^5	128-18		AY691545 *Pantoea agglomerans* ChDC YP3	521/523	99	G	+	-	-
Mixture Saturated Solids	10^5	128-16		AJ551147 *Citrobacter murliniae* genomospecies 11strain CDC 2970-59	523/525	99	G	+	-	-
Mixture Saturated Solids	10^5	128-14		AJ551147 *Citrobacter murliniae* genomospecies 11strain CDC 2970-59	523/525	99	G	+	-	-
Mixture Saturated Water	10^5	128-19	3	AJ012712 *Pseudomonas borealis* direct submission	521/521	100	G	+	+	+
Mixture Saturated Water	10^5	128-11	1	AY061963 *Azospirillum sp*. NS01 Oxalotrophic	468/469	99	A	+	+	+
Mixture Saturated Water	10^5	128-04		AY661991 Uncultured clone 300A-A08 Nitric acid contaminated groundwater	518/519	99	A	+	+	-
Mixture Saturated Water	10^5	128-03		AJ292426 *Pseudomonas kilonenesis* from agricultural soil	516/516	100	G	+	+	-
Mixture Saturated Water	10^5	128-25		gi505516 *Azospirillum brasilense* NCIMB 11860*	466/481	96	A	+	-	-
Mixture Saturated Water	10^5	128-22		AF408940 *Pseudomonas sp.* C16C from soil on shipping container	517/519	99	G	+	-	-
Mixture Saturated Water	10^5	128-12		AY118223 *Azospirillum sp.* DA6-2-2 from Red River Delta, Vietnam	459/479	95	A	+	-	-
Soil Moist Solids	10^4	126-11		AY510013 *Pseudomonas sp*. LCY11, Antarctic Soil PAH degrader	519/519	100	G	+	+	+
Soil Moist Solids	10^4	126-19	3	AJ012712 *Pseudomonas borealis* direct submission	521/521	100	G	+	+	+
Soil Moist Solids	10^4	126-07		AJ012712 *Pseudomonas borealis*	518/521	99	G	+	+	+
Soil Moist Solids	10^4	126-05		AJ012712 *Pseudomonas borealis*	519/521	99	G	+	+	-
Soil Moist Solids	10^4	126-03		AY039404 Earthworm Cast Bacterium C20D1	485/488	99	F	+	-	-
Soil Moist Solids	10^4	104-02		AY336548 *Paenibacillus sp.* pfB19 endophytic communities	530/535	99	F	+	-	-
Soil Moist Solids	10^4	104-01		AY504448 *Bacillaceae bacterium* C22 hindgut of *Tipula abdominalis*	537/537	100	F	+	-	-
Soil Saturated Solids	10^4	127-1A	7	AF237784 *Achromobacter piechaudii ATCC 43552*	467/468	99	B	+	+	+
Soil Saturated Solids	10^4	127-10		AF105387 *Pseudomonas sp*. Psl from pinyon-juniper soil	519/519	100	G	+	+	+
Soil Saturated Solids	10^4	127-03	3	AJ012712 *Pseudomonas borealis*	520/521	99	G	+	+	+
Soil Saturated Solids	10^4	127-07A		AJ012712 *Pseudomonas borealis*	518/521	99	G	+	+	-
Soil Saturated Solids	10^4	127-09A		AJ012712 *Pseudomonas borealis*	514/521	98	G	+	-	-
Soil Saturated Solids	10^4	127-12		gi2073371 *Paenibacillus lautus NRRL NRS-666T*	529/536	98	F	+	-	-
Soil Saturated Water	10^3	104-21	7	AF237784 *Achromobacter piechaudii ATCC 43552*	467/468	99	B	+	+	+
Soil Saturated Water	10^3	104-06	6	AF422668 Uncultured bacterium clone d091 Superfund in-situ Dechlorination	517/517	100	B	+	+	+
Soil Saturated Water	10^3	104-27	5	AB021388 *Pseudomonas mephitica reclassified as Janthinobacterium lividium*	514/514	100	B	+	+	+
Soil Saturated Water	10^3	104-19	8	AF170356 *Dechloromonas sp*. SIUL dissimilatory (per)chlorate reducers	516/516	100	B	+	+	+
Soil Saturated Water	10^3	104-18	12	AF191739 *Ensifer adhaerens* ATCC 33212	462/466	99	A	+	+	+
Soil Saturated Water	10^3	104-16	3	AY035996 *Pseudomonas lini* from bulk soils and rhizosphere	518/519	99	G	+	+	+
Soil Saturated Water	10^3	104-13	3	AJ012712 *Pseudomonas borealis*	518/521	99	G	+	+	+
Soil Saturated Water	10^3	104-10		AF105387 *Pseudomonas sp*. Psl from pinyon-juniper soil	519/519	100	G	+	+	+
Soil Saturated Water	10^3	104-03	13	AY336564 *Pseudomonas sp.* pfB35 from field grown potatoes	492/519	94	G	+	+	+
Soil Saturated Water	10^3	104-17		AY661989 Clone 010B-H12 from groundwater with nitric acid uranium waste	491/495	94	G	+	-	-
Soil Saturated Water	10^3	104-24		AF482685 *Pseudomonas sp.* NMX	507/517	98	G	+	-	-
Soil Saturated Water	10^3	104-22		AF482685 *Pseudomonas sp.* NMX	507/517	98	G	+	-	-
Soil Saturated Water	10^3	104-12		AF482685 *Pseudomonas sp.* NMX	508/517	98	G	+	-	-
Soil Saturated Water	10^3	104-11		AF482685 *Pseudomonas sp.* NMX	507/517	98	G	+	-	-
Soil Saturated Water	10^3	104-07		AJ012071 *Acidovorax sp* isolate G8B1 denitrify on dimethylmalonate	517/517	100	B	+	-	-
Soil Saturated Water	10^3	104-05		AJ012071 *Acidovorax sp* isolate G8B1	516/517	99	B	+	-	-
Soil Saturated Water	10^3	104-04		AY336541 *Pseudomonas sp* pfB12 from endophytic communities	517/519	99	G	+	-	-
Wood Moist Solids	10^5	108-08		AY228334 *Clostridium chromoreductans*	491/497	98	F	+	-	-
Wood Moist Solids	10^5	108-06		AF092549 *Clostridium algidixylanolyticum*	511/514	99	F	+	-	-
Wood Saturated Solids	10^6	108-17		AF109171 *Pseudomonas sp.* G-179 (classified as a rhizobium)	462/470	98	A	+	+	+
Wood Saturated Solids	10^6	108-19		AF109171 *Pseudomonas sp.* G-179	464/470	98	A	+	+	-
Wood Saturated Solids	10^6	108-20		AY510013 *Pseudomonas sp*. LCY11, P.E. 140, AY838534 Clone P020	519/519	100	G	+	+	-
Wood Saturated Solids	10^6	108-14		AY510013 *Pseudomonas sp*. LCY11	519/519	100	G	+	+	-
Wood Saturated Solids	10^6	108-13		AY510013 *Pseudomonas sp*. LCY11	519/519	100	G	+	+	-
Wood Saturated Solids	10^6	108-27		AY527741 Uncultured ph5Lac302-37	531/535	99	F	+	-	-
Wood Saturated Solids	10^4	108-26		AF395027 *Paenibacillus sp.* TB9	513/527	97	F	+	-	-
Wood Saturated Solids	10^6	108-16		AY118222 *Azospirillum sp.* Mat2-1a	460/466	98	A	+	-	-
Wood Saturated Water	10^4	110-02		AY510013 *Pseudomonas sp*. LCY11	519/519	100	G	+	+	-
Wood Saturated Water	10^4	110-01		AY510013 *Pseudomonas sp*. LCY11	519/519	100	G	+	+	-
Wood Saturated Water	10^5	108-22		AF388207 *Pseudomonas sp*. NZ099	518/518	100	G	-	-	-

Table 3.3 Nitrate reducer isolates from six week incubations of soil, wood and soil:wood mixture jar microcosms. Isolates were tested for NO_3^- and NO_2^- reduction and gas production by incubation in durham tubes of nitrate broth for up to one week at room temperature. Div=division; G=gamma proteobacteria, A=alpha protoeobacteria, B=beta proteobacteria, F=firmicute.

RFLP Pattern	Lab ID	Identification by Sequence Identity	Blastn Identities	%	D	# of Similar RFLP Patterns Saturated Water	Saturated Solids	Moist Solids	Total
1	Table 3.3	*Azospirillum sp. NS01*			A	1	4	0	5
2	23-7	*Azospirillum sp. B506*	1034/1051	97.3	A	16	0	3	19
	23-2	*Azospirillum sp. B506*	1054/1070	97.1	A				
	24-56	*Azosprillum sp. DA6-2-2*	1018/1047	97.0	A				
	24-36	*Azosprillum sp. DA6-2-2*	1173/1206	97.0	A				
3	Table 3.3	*Pseudomonas borealis, P. lini, P. sp. PsG*				1	3	10	14
4	Table 3.3	*P. sp. LCY11*				1	2	2	5
5	Table 3.3	*Janthinobacterium lividium*				0	0	0	0
6	Table 3.3	*Uncultured clone from Superfund Site*				0	0	0	0
7	Table 3.3	*Achromobacter piechaudii*				0	0	0	0
8	Table 3.3	*Dechloromonas sp. SIUL*				0	0	0	0
9	16-1	*AJ270259 Ralstonia sp. LMG 19089 (soil, Japan)*	1220/1221	99.9	B	0	1	0	1
11	24-4	*P. fluorescens*	1267/1272	99.6	G	0	0	1	1
12	Table 3.3	*Ensifier adhaerens*				0	0	0	0
13	Table 3.3	*P. sp. pfB35*				0	0	0	0

Table 3.4 Denitrifiers from jar microcosm mixture incubations at 24 weeks. DNA samples from 45 nitrate reducing isolates that tested positive for denitrification were digested with the restriction enzyme MSP1 and the restriction fragment length polymorphisms compared to those from six week isolates. DNA was sequenced from isolates representing RFLP patterns not detected at six weeks (patterns 2, 9, 11). The balance of the RFLP pattern isolates are listed in Table 3.3. NRCFUs for all treatments were 10^5 g^{-1} or ml^{-1}. See Appendix II for sequence data, Blastn summaries and RFLP gel patterns. Isolates were tested for NO_3^- and NO_2^- reduction and gas production by incubation in durham tubes of nitrate broth for up to one week at room temperature. D=division; G=gamma proteobacteria, A=alpha protoeobacteria, B=beta proteobacteria.

Treatment	Lab ID	Identification by 16s rDNA Sequence Similarity	Blastn Identities	%	D
Effluent A	136-6	AB056120.1 *Pseudomonas veronii*	522/522	100	G
	136-5	AF074384 *Pseudomonas gessardii*	783/834	93	G
Effluent B	136-2	AY297790.1 *Pseudomonas alcaligenes*	522/522	100	G
	136-3	AF390747.1 *Pseudomonas alcaligenes LB19*	827/860	96	G
Saturated Solid A	136-10	AB056120.1 *Pseudomonas veronii*	522/522	100	G
	136-11	AB056120.1 *Pseudomonas veronii*	522/522	100	G
Saturated Solid B	136-19	AJ551147.1 *Janthinobacterium sp. An8*	514/519	99	B
	136-18	AY360350.1 *Acidovorax sp.* 040703/8067L	798/847	94	B
Saturated Solid C	136-23	AB056120.1 *Pseudomonas veronii*	522/522	100	G
	136-27	AF105387 *Pseudomonas sp. Psl*	519/519	100	G
	136-20	AJ233429.1 *Serratia fonticola* (strain DSM 4576)	799/844	94	G
	136-26	AJ233429.1 *Serratia fonticola* (strain DSM 4576)	821/849	96	G

Table 3.5 Flow-through column nitrate reducing isolates. Nitrate reducing isolates were obtained from Effluent C but were not identified due to noisy sequence data. The NRCFUs for all treatments were estimated to be 10^4 g^{-1} or ml^{-1}. Cherry sawdust and river sediment were used in the construction of the columns. D=division; G=gamma proteobacteria, B=beta proteobacteria.

CHAPTER IV

PHENOTYPIC AND GENETIC PHYLOGENY OF MICROEUKARYOTES AND IDENTIFICATION OF DOMINANT MICROBIAL POPULATIONS IN PERMEABLE BARRIER MICROCOSMS BY FATTY ACID ANALYSIS

4.0 Abstract

Fatty acid profiles of pure cultures from the literature and experimental microcosm extracts were used to generate cluster dendrograms in order to more fully explore fatty acid distribution relationships in the complex microbial communities of permeable barrier microcosms. The classification of dominant populations in the microcosm samples by fatty acid dendrogram was supported by results from cultural and direct observational methods. The results confirmed that microeukaryotes may play an important role in the dynamics of nitrate transformations in permeable barriers constructed with pine wood. The fatty acid profile cluster dendrogram was also compared to an 18S rRNA gene sequence phylogram for microeukaryotes. Taxa that linked with strong support in the phylogram and were also linked in the dendrogram included the Crysophyceae and Haptophyceae algae as well as the Zygomycete fungi which formed two distinct groups with both methods. The Cryptophyceae algal branch had weaker support in the phylogram but was linked in the dendrogram. Other taxa that linked with weaker support in the phylogram were non-monophyletic in the dendrogram and included

Hyphochytriomycetes, Mastigomycetes (Oomycetes), Bacillariophyceae and Chytridiomycetes. A difference in diet or growth conditions such as temperature and light, as well as morphological differences in fruiting structures may contribute to different fatty acid profiles in organisms with a common lineage while convergent evolution or horizontal gene transfer of genes involved in lipid synthesis could lead to the clustering of seemingly unrelated taxa.

4.1 Introduction

Chapter II presented results of jar microcosm incubations of subsurface clay soil, pine wood shavings and a 2:1 mixture with significantly different EL-FAME microbial diversity by treatment and the dominant fatty acids present in the microcosm samples suggested the presence of eubacteria as well as diverse microeukaryotes. The method that was applied to more specifically identify the dominant microbial populations associated with the microcosm samples was the creation of a dendrogram from an ordination analyses generated from a database of the percent fatty acids of total fatty acids (fatty acid profiles) reported in the literature for pure cultures.

Biomarker fatty acids used to determine the presence or absence of specific populations may overlook organisms with relatively simple profiles or no unique fatty acids. It was hypothesized that fungal or protozoan grazing was a factor in nutrient turnover in the jar microcosm incubations since there was an increase in NH_4^+ levels which is characteristic of nutrient turnover by grazing (Chapter III). The fatty acid biomarker 20:4 used to identify protozoan populations was not detected in our samples; however, this fatty acid

has been reported in high percentage in *Paramecium* but to be absent in other protozoans such as *Tetrahymena* and *Acanthamoeba* (7,13).

Additionally, environmental conditions may affect the level of production of some biomarker fatty acids. For example, there was a lack of polyunsaturated biomarkers for algal populations in our samples. However, heterotrophically grown algae were reported to be lower in polyunsaturated fatty acids (17). The experimental conditions of the present investigation may have reduced the presence of these biomarkers to below detection since the microcosms were incubated in the dark. Cryptophyceae, Dinophyceae, Bacillariophyceae, Chrysophyeae, Chlorophyceae, Euglenophyceae and Tathophyceae algae have all been reported to use acetate for growth in darkness (23) which may have been supplied as degradation products of wood.

Gamma linolenic acid (18:3ω6c) was present in many of the bulk extraction samples and was significant in the grouping of samples by non-metric multidimensional scaling (NMS) ordination (see Chapter II). Gamma linolenic acid is characteristic of some protozoa such as *Crithidia* and *Tetrahymena* (7,13), fungal phycomycetes (Chytrids, Oomycetes) and also some species of Basidiomycetes and Zygomycetes (Mucorales) as well as some algae and Cyanobacteria (13). Other microeukaryotes produce alpha linolenic acid (18:3ω3c) which is characteristic of higher plants and was not detected in the microcosm samples. Yet other microeukaryotic organisms have been reported to produce neither omega-3 nor omega-6 linolenic acids (13). Alternately, these fatty acids may be produced in amounts too low to be detected.

Temperature differences have been reported to lead to changes in the degree of saturation but fatty acids were still qualitatively similar (13). Fungi grown in pure cultures that ranged from 20-25°C had no differences that affected classification (16). Most of the data from fungal pure cultures were from incubations at 25°C. The microcosms used for this study were incubated at room temperature.

The assumption was made that if the cultural microbial community is dominant in the environmental community; then the fatty acid profile will be similar to those of the dominant pure cultures grown under like conditions. These data are important because they allow the researcher to determine the relative contributions of the cultural community, whether they represent the dominant communities and can also be used to identify dominant populations that were not cultured by the selection methods used.

Culturable populations are important because they can be tested for functional abilities such as NO_3^- reduction. However, how they interact with other dominant populations may affect their ability to function under different treatments. Once functional ability has been established through cultured organisms, the fatty acid analyses can be used to monitor and track changes in the structure of the microbial community and to infer contributions to NO_3^- transformations when interpreted together with chemical analyses.

4.2 Materials and Methods

4.2.1 Fatty Acid Extractions from Permeable Reactive Barrier Materials

The fatty acid profiles were from the previously reported jar and syringe microcosm incubations (Chapter II). Fatty acids found in biofilms were also analyzed. They were obtained from the air-water interface of four additional 24 week saturated wood jar microcosms and compared with the water and saturated solid matrix fractions from the same jars described in Chapter II. Biofilms were removed by aseptic technique from the water surface with loops and transferred to glass centrifuge tubes. Water and wood samples and the EL-FAME extraction procedure were also previously described in Chapter II.

4.2.2 Fatty Acid Profiles from Pure Cultures

Fatty acid profiles were compiled from the literature for microeukaryote and eubacterial organisms that produce fatty acids similar to those found in the jar and syringe microcosm environmental communities (Table 4.1). When possible, data from organisms that were cultured under conditions similar to the microcosm incubations (room temperature in the dark) were used. Classification of microeukaryotes from the percent total fatty acid cluster dendrogram was compared to tree topology from an 18S rRNA gene sequence alignment of similar or closely related species. The fatty acid profile cluster dendrogram was used to identify dominant microbial populations found in experimental jar and syringe microcosm incubations.

4.2.3 Fatty Acid Statistical Analyses

Fatty acids profiles for pure cultures of microeukaryotes and eubacteria were retrieved from the literature and these, along with extractions from the experiments described above, were entered into an Excel (Microsoft) spreadsheet database. The database is presented in Appendix III. The database was used to create cluster dendrograms following NMS ordination in PC-ORD for Windows which was described in Chapter II (11). Transformations to the data such as arsine square root reduced the heteroscedasticity of the distribution but did not result in improvement in the non-metric ordination or cluster analyses and therefore the data was analyzed without transformation. The distance measure used was Sorensen (Bray-Curtis) distance which was also described in detail in Chapter II. The Sorensen coefficient retains sensitivity in heterogeneous data sets and is less sensitive to outliers than Euclidean distance and therefore more useful for ecological community data. (11) There is a potential problem with systematic error in cluster analysis if the data are not ultrametric and distance measures for sister taxa are missing (18). The linkage method used was farthest neighbor (maximum distance) as opposed to UPGMA or nearest neighbor because it provided the lowest percent of chaining. However, since the linkage methods did not yield identical results, the data was not ultrametric and therefore, some systematic error was probably introduced.

4.2.4 18S rRNA Gene Sequence Analyses

18S rRNA gene sequences for the same or closely related species to those in Table 4.1 were retrieved from GenBank (NCBI). Sequences were aligned using ClustalX (20) and

exported to PAUP (19) for tree building and topology analyses. Ambiguous sites were excluded from the analysis. The GTR model was chosen because it had the highest (maximum) log likelihood score (-lnL) when compared to JC69, F81 and HKY85. The GTR model was then used to estimate pinvar (the percent of invariable sites) and discrete gamma to account for among site rate variation. The tree presented in Figure 4.2 was created with the GTR parameters, estimating gamma and with pinvar=0 because this topology had the maximum -lnL. Trees were viewed with TreeView (12).

4.3 Results and Discussion

4.3.1 Fatty Acid Cluster Dendrogram

Historically, microeukaryotes have been classified based on morphological measurements. In more recent years, nucleic acid data has been used to confirm these relationships. However, there are instances where morphological relationships are not the same as those suggested by nucleic acids. Fatty acid profiles were applied as a method for classification of microeukaryotes as compared with 18S rRNA gene analysis to see if relationships that were not resolvable by 18S rRNA or morphology alone may be elucidated.

Fatty acid profiles for pure cultures as reported in the literature for microeukaryotes were subject to a cluster analysis along with fatty acid profiles from environmental extracts of jar and syringe microcosms to identify dominant populations of microeukaryotes based on fatty acid profiles. The cumulative r^2 after NMS ordination was 0.81 and the orthogonality was 95.5%. The dendrogram separated into two main branches, designated

A and B (Figure 4.1). Branch A contained the soil solid samples, bacteria, algae, Glomeromycota and mixture water samples. Branch B contained Cyanobacteria, algae, fungi, protozoans, slime molds, mixture solids and water, soil water, wood solids and water and syringe solids samples. These are further divided into sub-branches, ten of which are numbered to facilitate discussion in section 4.3.2.

4.3.2 Comparison of Microeukaryote Classification by Fatty Acid Profiles to 18S rRNA Gene Sequence Phylogeny

Some of the associations seen in the 18S tree (Figure 4.2) were similar in the fatty acid dendrogram. These included the grouping of Crysophyceae (heterokont) algae and Oomycetes (Mastigomycetes) together, and the grouping of one of several clusters of Zygomycete fungi associated with Chytrids. Cryptophyceae also clustered together in both analyses but are closer to yeasts and protozoa in the 18S tree and closer to bacteria and algae in the fatty acid dendrogram, suggesting a lineage from the fungi and protozoa but possible convergent evolution with bacteria and algae. The Haptophyceae algae also clustered together in both analyses and are related to the Oomycetes and Crysophyceae (branches 5 and 6).

4.3.2.1 Oomycetes, Hyphochytriomycetes, Heterokont Algae and Protozoans

Van der Auwera *et al.* (1995) reported that the Oomycetes formed a monophyletic group, most closely related to the Hyphochytriomycetes and heterokont algae, with *Hyphochytrium catenoides* as the lone Hyphochytriomycete representative (21). The 18S rRNA (Figure 4.2) gene sequence analysis supported this relationship.

The phylogram had 54% support for a branch which contained the Oomycetes and Hyphochytriomycetes. Heterokont algae (Bacillariophyceae, Xanthophyceae and Crysophyceae) formed monophyletic branches that were closest to the Hyphochytriomycetes and Oomycetes.

The *Hyphochytrium, Rhizidomyces* and *Saprolegnia* species used for fatty acid profile are the same as those in 18S phylogram. However, the association of these organisms in the fatty acid dendrogram is not in agreement with the phylogram. Oomycetes are not linked with the Hyphochytriomycetes in the dendrogram. In this case, the Hyphochytriomycete, *H. catenoides,* is found in branch 8 grouped with Chytrids and amoeba and was most similar to the Glomeromycota, arbuscular mychorrhizal (AM) fungi *Gigaspora gigantica* and *G. rosea*. The Hyphochytriomycete *Rhizidiomyces apophysatus* was found on branch 5 with Haptophyceae and Chlorophyceae algae and Cyanobacteria. The hyphochytriomycetes are both saprophytic and parasitic, whereas the AM fungi are obligate plant symbionts. *Rhizidomycetes* are parasitic on oomycetes while *Hyphochytrium* are parasitic on algae and higher fungi (15).

The Cryptophyceae algae *Cryptomonas ovata* and *Chroomonas* sp. grouped with the Dinophyceae alga *Procentrum* (Figure 4.1 - branch three). Members of the Cryptophyceae and Dinophyceae produce trichocysts which are flagella related structures (17) and this is an example of similar fatty acid profiles due to similar morphology. The phylogram (Figure 4.2) placed the Cryptophyceae and Dinophyceae in separate branches.

The slime molds *Physarium* and *Dictyostelium* grouped together on a sub-branch of Branch 9 linked to *Trypanosoma*, Chytrids and *Candida* whereas the 18S phylogram placed the Dictosteliida on a branch with the Acanthamoebidiae. The Hyphomycete fungi (*Neurospora crassa, Penicillium crysogenum, Aspergillus, Sporobolus, Fusarium)* clustered together on a branch linked most closely to the ascomycete *Chaetomium.*

4.3.2.2 Zygomycete Fungi

Zygomycetes formed a non-monophyletic group in the phylogram with the *Mortierella* species on a branch, along with the AM fungi and Chytrids, that had 66% support. This concurred with the phylogeny reported by Voigt and Wostemeyer (1999) (22). Berbee *et al.* (1992) suggested that some Zygomycetes may group with Chytrid water molds due to unequal rates of substitution among the Zygomycetes lineages (3). The balance of the Zygomycete species was on a separate branch with 79% support.

The fatty acid dendrogram showed the Zygomycete fungi in branch 8 (Figure 4.1). The Zygomycetes *Thaminidium elegans*, *Mucor miehei*, *Mortierella sp.*, *Cuninghamella sp.* and *Rhizopus sp.* formed a group, similar to the 18S phylogeny, but the Zygomycete *Phycomyces blakesleeansus* was more closely related to the Hyphomyctes and Chytrids in branch 8. The Chytrid *Monoblepharis*, associated with Zygomycetes in the 18S phylogram, was found linked to the Oomycete *Phytophotora* and the protozoan animal pathogen *Leishmania* (branch 9) but not to Zygomycete fungi in the fatty acid dendrogram.

4.3.2.3 Glomeromycota

Branch 8 contained a group of Glomeromycota and Chytrids. The AM fungi *Gigaspora gigantica* and *G. rosea* are Zygomycetes that were recently transferred to a new phylum, Glomeromycota, and they formed a branch with the Hyphochytriomycete *H. catenoides*, the cellulolytic Hyphomycete *Cryptococcus albidis var. albidis*, Chytrids (*Rhizophlyctis roseae*, *Blastocladiella emersonii)* and *Acanthameoba*. *Blastocladiella emersonii* formed a group with *Acanthameoba* and has been reported to display pronounced amoeboid movement in zoospores (15). A second group of Glomeromycota, the AM Fungi *Scuttelospora* which are obligate plant symbionts, clustered with eubacterial nitrifiers, the bacterium *Erwinia herbicola,* and heterokont algae (Bacillariophyceae, Xanthophyceae). *E. herbicola* is a plant pathogen. The Glomeromycota were linked in the 18S phylogram with both genera related to Chytrids.

4.3.2.4 Eubacteria (Figure 4.1)

The eubacteria are polyphyletic and are found primarily in branches 1, 2, 4, 5, and 6. Fatty acid distributions for *Streptomycetes* were difficult to find. The species used may not be representative of the genus and linked most closely with fungi in branch 6. The link with fungi could be due to the hyphal morphology displayed by *Streptomycetes*.

Branch one had alpha, beta, delta, epsilon and gamma proteobacteria along with a Firmicute. Branch two was a mix of fungi, Cyanobacteria, algae and beta proteobacteria and linked the NH_3^+ oxidizing beta proteobacteria *Nitrosolobus sp.* and *Nitrosomonas europea* with the nitrogen-fixing plant symbiont *Erwinia herbicola* and two AM fungi

from the genera *Scuttelospora*. *Thiobacillus denitrificans* was in this branch as well and was most similar to a few Cyanobacteria and algae. *Anabaena* was found in branch 5 linked with Haptophyceae and Chlorophyceae algae and the Hyphochytriomycete *R. apophysatus*. The rest of the Cyanobacteria were in branch 7 linked to Zygomycetes and the protozoans *Tetrahymena* and *Crithidia*. Specialized membranes related to nitrogen biogeochemistry present in these eubacteria may contribute to their clustering with microeukaryotes.

The alpha proteobacteria *Prostecomicrobium, Caulobacter*, *Azospirillum brasilense,* and *Nitrobacter winogradsky,* as well as the beta proteobacterium *Thiobacillis ferroxidans* were in branch 4 while the neighboring branch was a cluster of opportunistic pathogens *Listeria denitrificans, Staphylococcus aureus, Legionella pneumonophilia,* and *Sporocytophaga myxococcoides*.

4.3.3 Identification of Dominant Populations in Permeable Barrier Materials

4.3.3.1 Pores and Protozoans

Ronn *et al.* reported that dead plant material increased protozoan populations in soil for up to six weeks and that inorganic nitrogen increased in the soil in the presence of protozoan populations (14). They also reported that protozoan populations were found in the water filled pores and water films. Both the moist and saturated treatments had water filled pores. The wood had higher water holding capacity than the soil and mixture as reported in Chapter II.

4.3.3.2 Jar Microcosm Mixture Samples

Four fatty acid profiles for six week mixture solid samples (moist and saturated) clustered in branch 8 and were most similar to parasitic fungi and *Acanthamoeba* fatty acid distributions. Since the dominant fatty acids found in the microcosm samples reflect grazing protozoans and parasitic fungi it is feasible that predation and nutrient turnover were factors in NH_4^+ accumulation in these microcosms (Chapter II).

Eubacterial NO_3^- reducers isolated from the mixture moist and saturated solids at six weeks were *Janthinobacterium, Citrobacter murlinae, Pantoea agglomerans (Erwinia herbicola), Agrobacterium tumefaciens*, *Clostridium* and fluorescent pseudomonads. *Agrobacterium tumefaciens* and *Ralstonia picketti* have been reported to be resistant to grazing by amoeba (14) and *Janthinobacterium* produced a violet pigment (violacein) that has been reported to be toxic to nanoflagellates (9). The 24 week mixture incubations and the balance of the six week incubations, exclusive of water samples, were found in branch 10. The closest branches are clusters of protozoans, fungi and slime molds.

The mixture water samples were split in two groups. Variability in microbial community dynamics in the well-homogenized replicates may have been due to hot spots in the inoculum (wood shavings or soil). One 24 week sample grouped with the alpha and beta proteobacteria. Among these was *Azospirillum,* the most commonly isolated NO_3^- reducer genus from the 24 week mixture water samples (Chapter III), the denitrifier *Caulobacter* and the NO_2^- oxidizer *Nitrobacter*. The remaining mixture water samples

were most similar to a diverse group of fungi, algae and Cyanobacteria. Nitrate reducers from the six week mixture water samples also included species of fluorescent pseudomonads which are reported to lyse fungal hypha (8). The Chryosphyceae algae from the *Ochromonas* genus were found in a cluster adjacent to the branch containing the mixture water samples and are reported to graze upon eubacteria.

4.3.3.3 Jar Microcosm Soil Samples

It is believed that small pores like those found in clay soils protect bacteria from grazing. (14). This seemed to be the case with our clay soil microcosms as fatty acid profiles from either the moist or saturated solid samples did not group with microeukaryotes. The moist and saturated soil solid samples showed a strong similarity to *Desulfovibrio vulgaris* in branch one. This was not surprising since there was a high concentration of sulfate in the original soil (See Appendix I). The soil water samples, however, grouped with protozoan populations and are discussed below. Nitrate reducing isolates from these treatments included eubacteria from the genera of *Paenibacillus, Bacillus*, *Achromobacter* and fluorescent pseudomonads (Chapter III). These were among the bacteria that had fatty acid profiles similar to that found in the moist and saturated soil solid samples (branch 1).

The saturated soil and wood water profiles were found in branch 9 and grouped with slime molds, protozoans and fungi. An unidentified slime mold was isolated on plates selective for NO_3^- reducers from the saturated wood treatment in pilot dilutions. The water in the saturated soil solid samples was very cloudy and protozoans may have been

able to feed on eubacteria attached to suspended particles. The fungi may or may not be active since spores are reported to be transported in water (4). The saturated soil water NO_3^- reducing eubacterial isolates were from the genera of *Pseudomonas, Dechloromonas, Ensifer* and *Acidovorax* but the nitrate reducer colony forming units (NRCFUs) for these NO_3^- reducers were low (10^2-10^3 ml^{-1}) and the populations were too small to be detected by the EL-FAME extractions (Chapter III).

4.3.3.4 Jar Microcosms Wood Samples

Fatty acid profiles from the wood jar microcosm treatments and the syringe microcosms were found in branch 10 with the 24 week moist and saturated mixture solid samples from jar microcosms. Eubacterial NO_3^- reducing isolates from the wood jar microcosm treatments were in the genera *Clostridium, Pseudomonas* and *Paenibacillus* (Chapter III). Protozoan grazers have been reported to favor the survival of gram positive organisms and to have a preference for non-pigmented Enterobacteraceae (14).

NRCFUs were highest in the wood treatments (Chapter II); however, the predominant fatty acids suggest that this population was eclipsed by the microeukaryotes. The dominant fatty acids have patterns that were more closely aligned with saprophytic and cellulolytic fungi and protozoans. This supported the hypothesis of microeukaryote contribution to the transformation of NO_3^- through assimilation and the release of NH_4^+ as a result of grazing as reported in Chapter III. Slime molds, fungi and protozoans have fatty acid distributions that are most similar to the wood solid treatment samples.

Gutierrez *et al.* (2002) reported that basidiomycetes produced novel unsaturated lipids during the decay of eucalyptus wood that may be a factor in lignin decay. It is possible that some of the unidentified fatty acids unique to the pine wood containing microcosms were produced by fungi involved in degradation. These were identified by the authors as being unsaturated dicarboxylic acids (6); however, dicarboxylic acids represented less than 1% of fatty acids, when detected, in the microcosm samples.

4.3.3.5 Biofilms from Jar Microcosms and Syringe Solids

The biofilm and wood fractions of the saturated wood jar microcosms (branch 10) were similar while the water samples grouped with saturated soil water in branch 9 discussed above. The three day incubation of the syringe microcosms with and without 20 ppm NO_3-N did not yield detectable differences in the microbial community which seemed to be dominated by microeukaryotes. The three day incubations were most similar to the 24 week mixture solids, both saturated and moist. The total nmols retrieved in the syringe microcosms were low because only 5% of the volume of the microcosm was from the sediment inoculum and the wood and filter sand were washed and oven dried prior to use. Micrographs of the jar microcosm saturated wood biofilms (Figures 4.3 and 4.4) revealed phycomyete and other fungal morphology. Micrographs from syringe microcosms showed alga and phycomycetes and supported the presence of these populations as well.

4.3.3.6 Community Dynamics

Differences in microbial communities grazed by amoeba that feed on attached bacteria versus the Chryptophyte flagellate suspension filter feeder *Spumella* have been reported (14) while Malajczak (1983) discussed interactions among fungi and eubacteria. *Penicillium* and *Rhizoctonia* were reported to lyse *Mucor* while *Rhizobium, Bacillus*, and *Streptomyces* inhibited fungi through the production of antibiotics and fluorescent pseudomonads caused hyphal lysis (8).

Matz and Kjelleburg (2005) argued that bacterial defense mechanisms against predation and transitions in evolution that involved multicellularity and pathogenesis were driven by selective predation (10). Convergence of organisms due to specialized membranes and diet may have contributed to similarities in fatty acid distributions of organisms that are not related by vertical inheritance. Some organisms such as the spirochetes and obligate intracellular pathogens derive fatty acids from their host and therefore it is difficult to obtain fatty acid profiles that indicate their presence as the fatty acids they produce are dependent on their host or on the media (13).

A problem that limits this analysis is that researchers do not always publish the complete fatty acid analysis on their isolates, as they are looking for specific biomarkers. In addition, databases for commercial diagnosis of strains are proprietary and the percent distributions are not provided in reports of pure cultures identified using these programs. Additional taxa may result in better clarification of relationships among total fatty acids.

4.4 Conclusions

This method identified dominant populations of phycomycetes and algae in the samples as supported by microscopic observation and of *Azospirillum* by 16S rRNA gene similarity of cultured isolates. The fatty acid distributions in moist and saturated soil solid samples were most similar to *Desulfovibrio vulgaris*. The soil inoculum used for the microcosms had high (>200 ppm) levels of sulfate.

Phycomycetes are saprophytic or parasitic on each other or algae and were shown to be present in the jar microcosm wood and mixture samples, with the exception of the saturated mixture water samples, and may have been a factor in nutrient turnover. These organisms were also found in the syringe microcosms as supported by direct observation of the samples. Protozoan grazers are also suspected due to similarities in fatty acid profiles to the microcosm extracts. Protozoa prefer non-pigmented Enterobacteraceae, many of which are DNRAs; therefore protozoan grazing may play a role in limiting these populations subsequently reducing competition with denitrifiers for the NO_3^- substrate.

Taxa linked with strong support in the phylogram that were also linked in the dendrogram included the Crysophyceae and Haptophyceae algae as well as the Zygomycete fungi which formed two distinct groups with both methods. The Cryptophyceae algal branch had weaker support in the phylogram but was also linked in the dendrogram. Other taxa had weak support for monophyletic branches in the phylogram and were non-monophyletic in the dendrogram and included Hyphochytriomycetes, Mastigomycetes (Oomycetes), Bacillariophyceae and Chytridiomycetes.

Fatty acid profiles linked Cryptophyceae algae with the Dinophyceae alga, which produce trichocysts (flagella related structures) but they are on separate branches in the 18S phylogram. The Chytrid *Blastocladiella emersonii* formed a group with *Acanthameoba* in the dendrogram and has been reported to display pronounced amoeboid movement in zoospores. A difference in diet or growth conditions such as temperature and light, as well as morphological differences in fruiting structures may contribute to different fatty acid profiles in organisms with a common lineage. Convergent evolution or horizontal gene transfer of genes involved in lipid synthesis for specialize membranes could lead to the clustering of seemingly unrelated taxa.

This was an efficient and cost effective method for the assessment of community dynamics across microbial kingdoms in microcosms and can be applied to characterize dominant microbial populations in samples from permeable barriers installed in the field as well as any other environmental or clinical mixed microbial community samples. The addition of more fatty acid profiles to the database can help with non-ultrametric issues through the introduction of sister taxa.

4.5 Future Studies

Due to time and economic constraints fatty acid analyses on isolates from the microcosms were not performed. They were identified through 16S rRNA gene sequence similarity and RFLP analyses. However, future studies with these organisms as potential inoculum for denitrifying permeable barriers should include the determination of fatty acid profiles.

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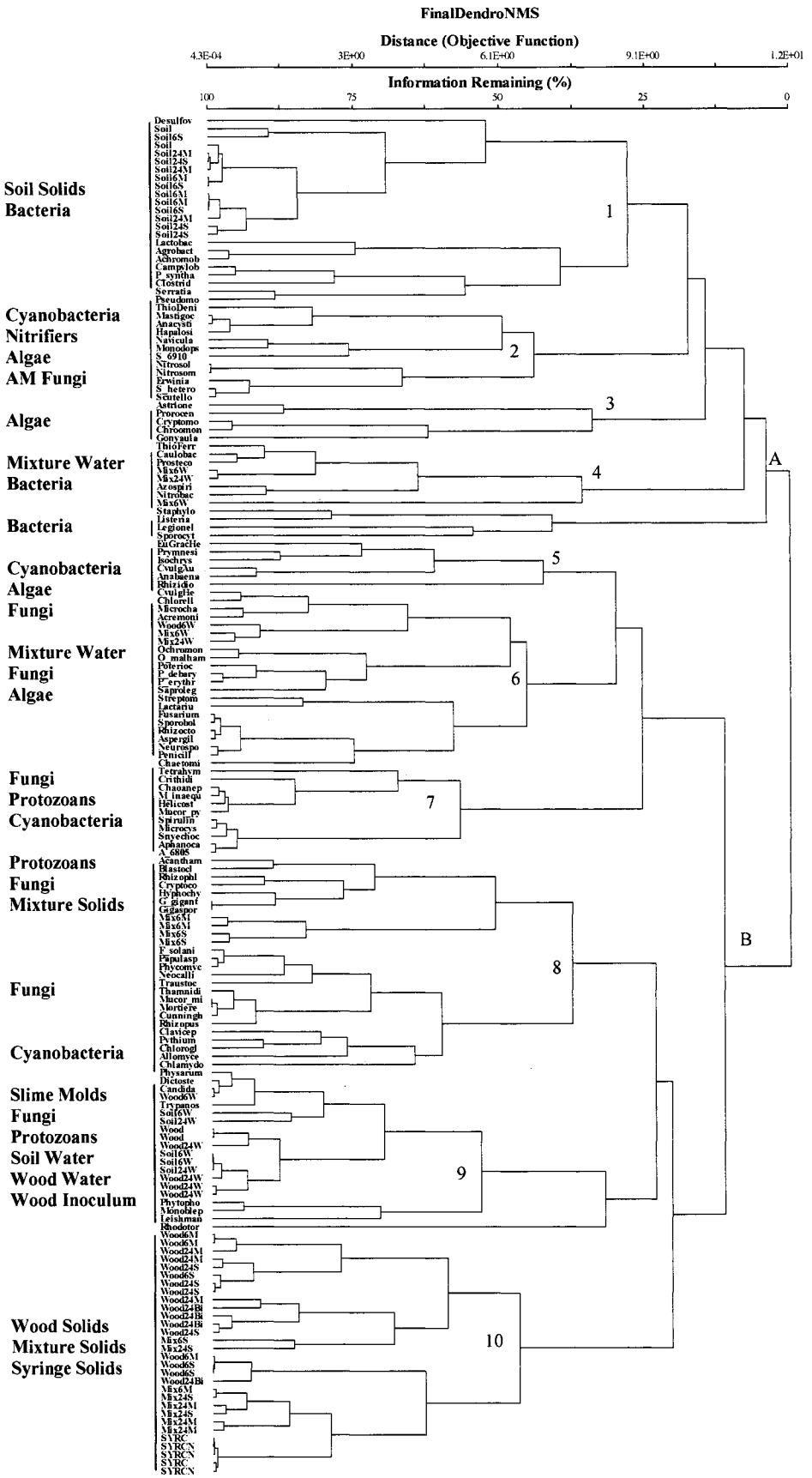


Ref	Organism	Fig4.1	Division
3	*Acanthamoeba*	Acantham	Acanthamoebidae
2	*Streptomyces hygroscopicuis 111-81NL*	Streptom	Actinobacteria
1	*Azospirillum brasilense*	Azospiri	Alpha proteobacteria
1	*Caulobacter cresentens*	Caulobac	Alpha proteobacteria
1	*Prostecomicrobium pneumaticum*	Prosteco	Alpha proteobacteria
1	*Nitrobacter winogradsky*	Nitrobac	Alpha proteobacteria
1	*Agrobacteria tumefaciens*	Agrobact	Alpha proteobacteria
1	*Neurospora crassa*	Neurospo	Ascomycete
1	*Claviceps purpurea*	Clavicep	Ascomycete
5	*Chaetomium sp.*	Chaetomi	Ascomycete
1	*Navicula pelliculos*	Navicula	Bacillariophyceae
1	*Astrionella japonica*	Astrione	Bacillariophyceae
1	*Lactarius rufus*	Lactariu	Basidiomycete
7	*Sporocytophaga myxococcoides*	Sporocyt	Bacteriodetes
1	*Achromobacter biotypes 1 & 2*	Achromob	Beta proteobacteria
1	*Nitrosolubus sp.*	Nitrosol	beta proteobacteria
1	*Nitrosomonas europea*	Nitrosom	beta proteobacteria
1	*Thiobacillus dentirificans*	ThioDeni	beta proteobacteria
1	*Thiobacillus ferridoxins*	ThioFerr	beta proteobacteria
4	*Chlorella vulgaris (Hetero/Dark)*	Chlorell	Chlorophyceae
4	*Chlorella vulgaris (Hetero)*	CvulgHe	Chlorophyceae
4	*Chlorella vulgaris (Auto)*	CvulgAu	Chlorophyceae
1	*Chlamydomonas reinhardtii*	Chlamydo	Chlorophyceae
1	*Ochromonas danica*	Ochromon	Chrysophyceae
1	*Poteriochromonas stipata*	Poterioc	Chrysophyceae
1	*Ochromonas malhamensis*	O_malham	Chrysophyceae
1	*Allomyces javanicus*	Allomyce	Chytridiomycete
1	*Blastocladiella emersonni myc*	Blastocl	Chytridiomycete
1	*Rhizophlyctis rosea*	Rhizophl	Chytridiomycete
1	*Traustochytrium roseum*	Traustoc	Chytridiomycete
1	*Neocallimastix frontalis*	Neocalli	Chytridiomycete
1	*Monoblepharis sp.*	Monoblep	Chytridiomycete
1	*Cryptomonas ovata*	Cryptomo	Cryptophyceae
1	*Chroomonas sp.*	Chroomon	Cryptophyceae
4	*Spirulina platensis*	Spirulin	Cyanobacteria
4	*Anabaena cylindrica*	Anabaena	Cyanobacteria
4	*Snyechoccus 6801*	Snyechoc	Cyanobacteria
4	*Snyechoccus 6910*	S_6910	Cyanobacteria
4	*Aphanocapsa 6702*	Aphanoca	Cyanobacteria
4	*Aphanocapsa 6805*	A_6805	Cyanobacteria
4	*Microcystis 700*	Microcys	Cyanobacteria
4	*Microchaete 6305*	Microcha	Cyanobacteria
4	*Clorogloea fritschii*	Chlorogl	Cyanobacteria
4	*Mastigoclades laminosus*	Mastigoc	Cyanobacteria
4	*Hapalospiphon laminosus*	Hapalosi	Cyanobacteria
4	*Anacystes nidulans*	Anacysti	Cyanobacteria
1	*Desulfovibrio vulgaris*	Desulfov	Delta proteobacteria
5	*Acremonium sp.*	Acremoni	Deuteromycotina
1	*Dictyostelium sp.*	Dictoste	Dictosteliida
1	*Gonyaulax polyedra*	Gonyaula	Dinophyceae
1	*Prorocentrum micans*	Prorocen	Dinophyceae
1	*Campylobacter jejuni*	Camphylob	Epsilon protobacteria
4	*Euglena gracilis (hetero)*	EuGracHe	Euglenophyceae
1	*Clostridium beijerinkii*	Clostrid	Firmicute
1	*Serratia marcesens*	Serratia	Gamma proteobacteria
1	*Erwinia herbicola*	Erwinia	Gamma proteobacteria
1	*Pseudomonas flourescens*	Pseudomo	Gamma proteobacteria
1	*Pseudomonas synthanxa*	P_syntha	Gamma proteobacteria
1	*Legionella pneumonophila*	Legionel	Gamma proteobacteria
6	*Scutellospora heterogama*	S_hetero	Glomeromycota
6	*Scutellospora pellucida*	Scutello	Glomeromycota
6	*Gigaspora gigantica*	G_gigant	Glomeromycota
6	*Gigaspora rosea*	Gigaspor	Glomeromycota
1	*Prymnesium parvum*	Prymensi	Haptophyceae
1	*Isochrysis galbana*	Isochrys	Haptophyceae
1	*Hyphochytrium catenoides*	Hyphochy	Hyphochytriomycete
1	*Rhizidiomyces apophysatus*	Rhizidio	Hyphochytriomycete
1	*Rhizoctonia solai mycelium*	Rhizocto	Hyphomycete
1	*Fusarium moniliform*	Fusarium	Hyphomycete
1	*Fusarium solani*	F_solani	Hyphomycete
1	*Penicillium crysogenum*	Penicill	Hyphomycete
1	*Aspergillus niger*	Aspergil	Hyphomycete
1	*Sporobolomyces roseus*	Sporobol	Hyphomycete
1	*Papulaspora sp.*	Papulasp	Hyphomycete
1	*Cryptococcus albidus var. albidus*	Cryptoco	Hyphomycete
1	*Candida humicola*	Candida	Hyphomycete
1	*Rhodotorula rubra*	Rhodotor	Hyphomycete
1	*Staphylococcus aureus*	Staphylo	Low g+c gram positive
1	*Lactobacillus buchneri*	Lactobac	Low g+c gram positive
1	*Listeria denitrificans*	Listeria	Low g+c gram positive
1	*Saprolegnia parasitica 24 hr*	Saproleg	Mastigomycete
1	*Pythium deebaryanum*	P_debary	Mastigomycete
1	*Pythium ultimum 14 days 20C*	Pythium	Mastigomycete
1	*Phytophora cactorum*	Phytopho	Mastigomycete
1	*Phytophora erythroseptica*	P_erythr	Mastigomycete
3	*Physarum polycephalum*	Physarum	Myxogastromycetidae
3	*Tetrahymena pyriformis W-1*	Tetrahym	Tetrahymeidae
3	*Trypanosoma lewisi - blood agar*	Trypanos	Trypanosomatidae
3	*Crithidia ap.- blood agar*	Crithidi	Trypanosomatidae
3	*Leishmania tarentolae (trager's C)*	Leishman	Trypanosomatidae
1	*Monodopsis subterraneous*	Monodops	Xanthophyceae
1	*Phycomyces blakesleeanus*	Phycomyc	Zygomycete
1	*Thamnidium elegans spores 8 days 25C*	Thamnidi	Zygomycete
1	*Rhizopus sp. 14 days 28C*	Rhizopus	Zygomycete
1	*Chaoanephora cucurbittarum -1*	Chaoanep	Zygomycete
1	*Helicostylum elegans*	Helicost	Zygomycete
1	*Mucor inaequisporus*	M_inaequ	Zygomycete
1	*Mucor miehei 14 days 28C*	Mucor_mi	Zygomycete
1	*Mucor pyriforme*	Mucor_py	Zygomycete
5	*Cunninghamella sp.*	Cunningh	Zygomycete
5	*Mortierella sp.*	Mortiere	Zygomycete

Table 4.1 Sources for pure culture fatty acid profiles

1. Ratledge & Wilson (1988)
2. Gesheva & Rachev (2000)
3. Korn *et al.* (1965)
4. W.D.P. Stewart, Ed. (1974)
5. Stahl & Klug (1996)
6. Bentivenga & Morton (1996)
7. Weyant *et al.* (1995)

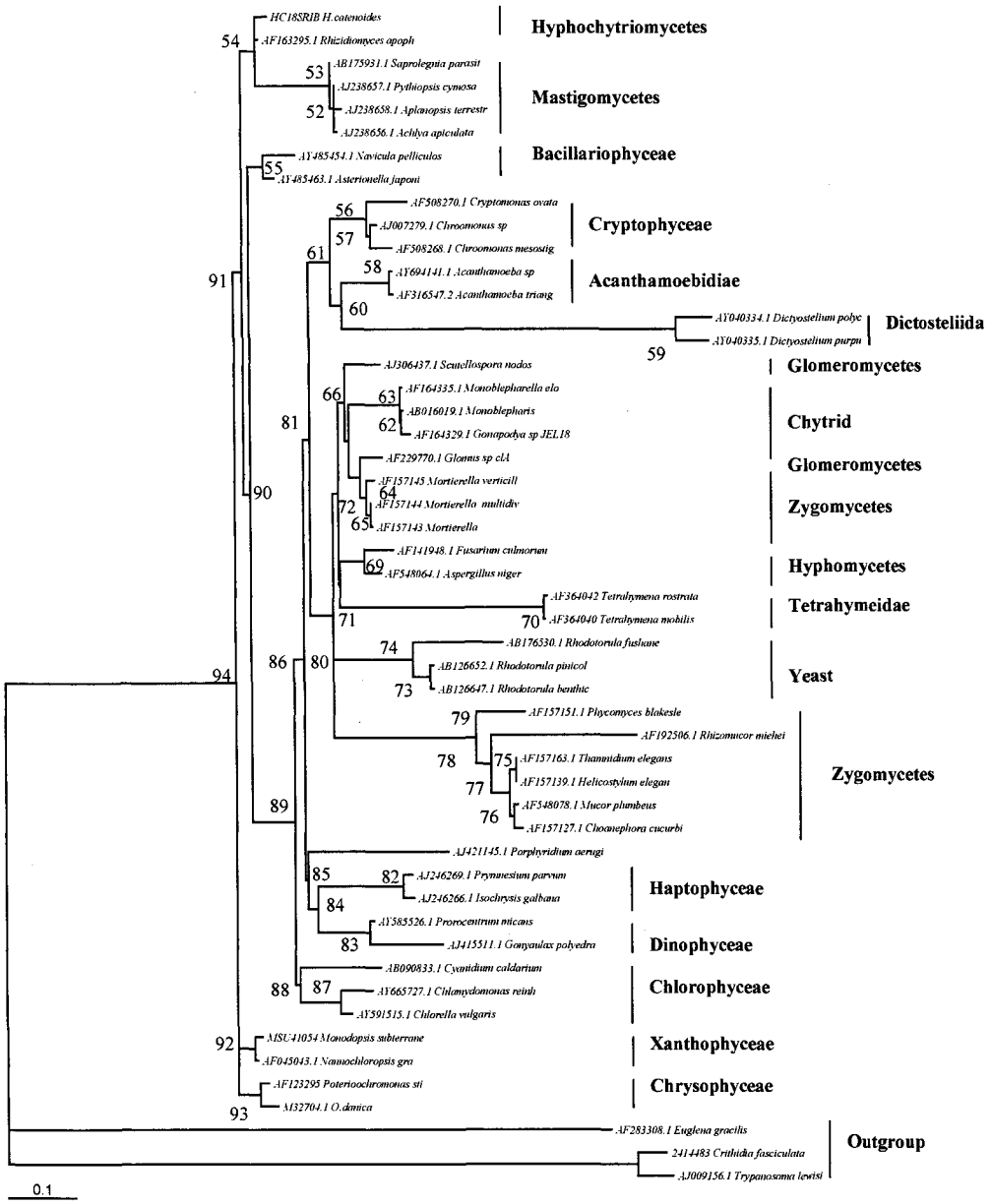
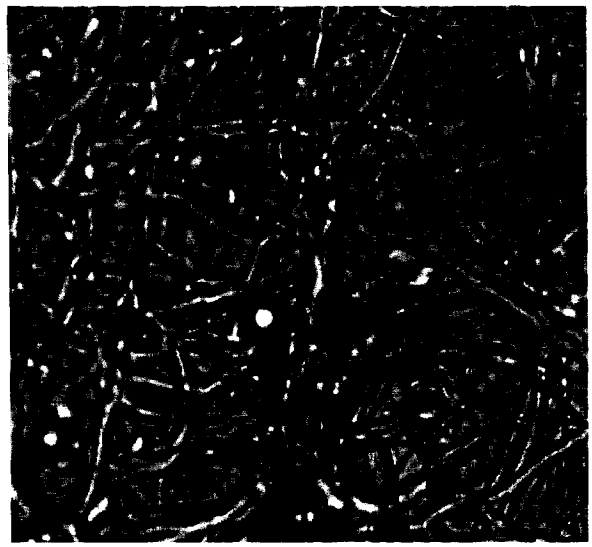


Figure 4.2 18S rRNA gene phylogram.
Tree topology was constructed in PAUP 4.1 using maximum likelihood with GTR-G parameters. Accession numbers refer to GenBank sources for sequences.
(-lnL = 8026)



100 um

Figure 4.3 Brightfield microscopy 400X magnification of wood biofilm from jar microcosms. Top: Oomycete mycelial network and fruiting structures Bottom: Phycomycete fungi

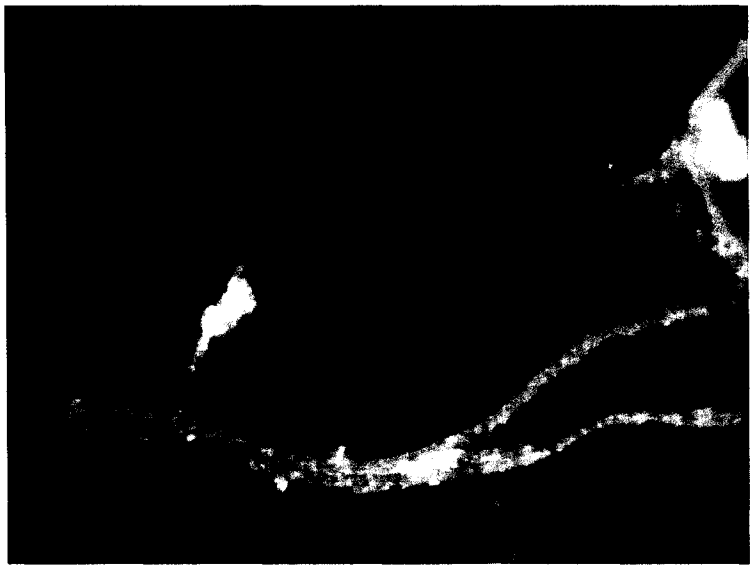
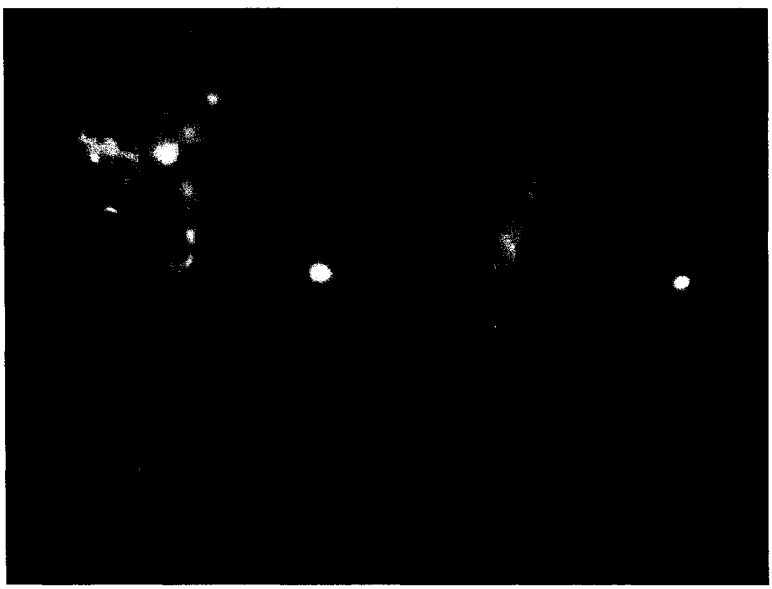


Figure 4.4 DAPI stained microeukaryotes from syringe microcosm samples
Top: 400X magnification of unidentified phycomycete
Bottom: 400X magnification of chytrid upper left and alga



CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.0 Conclusions

Prior to this research, permeable barriers for NO_3^- removal were a microbial black box. The results presented here supply a framework for the characterization of the microbial community, the shifts in structure due to the contributions of soil and wood populations and the pathways of eubacterial NO_3^- reduction selected by the experimental conditions. Excess NO_3^-, of agricultural origin, transported by the Mississippi River threatens fishing and recreation in the Gulf of Mexico and other water bodies. Permeable barriers for NO_3^- reduction are assumed to select for denitrifying bacteria due to denitrification enzyme activity experiments but there are no prior reports of the specific populations involved.

Jar, syringe and flow-through column microcosms were analyzed to determine the microbial populations that contribute to NO_3^- reduction in a permeable barrier and to gain an understanding of how they interact with the total microbial community in order to manage permeable barriers for anthropogenic NO_3^- removal from surface and shallow ground water. A summary of the results of jar microcosm experiments is presented in Table 5.1.

EL-FAME analysis was an economical and time efficient method for the estimation of microeukaryote and eubacterial populations although there were detection limits based on population size. The data and methods described here can be applied to the monitoring and manipulation of microbial communities in permeable barriers constructed with pine wood products. Permeable barriers have been shown to successfully remove NO_3^- from water and have also been used for the removal of heavy metals from acid mine drainage.

5.1 Wood Adds Nitrate Reducer Populations and Microeukaryotes to Permeable Barrier Microcosms

The first two goals of this study were to determine if the addition of wood increases NO_3^- reducing populations and to assess the effect of pine wood on the overall diversity of the microbial community in a permeable barrier microcosm. Jar microcosm mixture samples had a greater percentage of microeukaryotes and significantly more culturable NO_3^- reducers than the soil samples but not the wood. The wood control microcosms had significantly more culturable NO_3^- reducers than the soil which supports rejection of the null hypothesis, that the addition of pine wood shavings to a clay soil will not increase NO_3^- reducer populations found in the soil, and supports the alternative hypothesis that the addition of pine wood shavings to a clay soil increases NO_3^- reducer populations found in the soil through the addition of new NO_3^- reducer populations associated with the wood. (Chapter II)

EL-FAME diversity was significantly different for the wood, soil and the mixture treatments. The mixture treatments were significantly different from the wood and soil

controls as a result of the combination of nutrients and microbial inocula contributed from both. Soil EL-FAME profiles, exclusive of water, reflected a dominant eubacterial population while the wood and mixture EL-FAME profiles were more characteristic of microeukaryote populations. The data supports the rejection of the null hypothesis that wood will not contribute microeukaryote populations to the permeable barrier in favor of the alternate hypothesis that microeukaryotic populations will be introduced into the permeable barrier with wood. (Chapter II) The dominant community is expected to assimilate NO_3^- into microbial biomass while a minority population reduces NO_3^- in respiratory pathways. Accumulation of ammonium may be attributed to release by microeukaryote grazing on eubacteria and microeukaryotic fermentation of NO_3^- as well as activity by DNRAs.

The slope of the regression of NO_3^- on cell number in both the syringe and flow-though column microcosms during incubation was the same at -0.002 which predicts a 2 ppb decrease in NO_3^- concentration per cell in the presence of sawdust suggesting a direct correlation between biomass and NO_3^- concentration. The r^2 for the regressions were 0.64 and 0.57 for syringe and flow-through respectively indicating that more than half the variability in the data is due to microbial assimilation of NO_3^- during growth on organic carbon.

5.2 Higher Odds of Isolating Denitrifiers from Water versus Solid Samples of Saturated Jar Microcosms

Soil and wood water samples were dominated by microeukaryotes but there were some eubacterial populations present in both the soil and wood water as determined by the culturing of NO_3^- reducing isolates. The mixture water had conditions that inhibited the microeukaryotes so that the eubacterial populations were not overshadowed in the EL-FAME analysis. The third goal was to compare denitrifying populations in water versus solid samples. There was no significant difference in the number of NO_3^- reducers cultured from water versus solid samples of saturated mixture jar microcosms and the odds of isolating denitrifiers as a percent of NO_3^- reducers from the water were significantly higher than that from the solid portion of saturated microcosms. Together these results support the rejection of the null hypothesis that the odds of isolating denitrifying NO_3^- reducers will be the same in the water versus the solid samples from saturated microcosms in favor of the alternative hypothesis that the odds of isolating NO_3^- reducers that are capable of denitrification will be greater in water samples than in solid samples due to the solubility of the substrates of the reaction. (Chapter III) Additionally, NO_3^- reducing organisms in the *Pseudomonas* genus were transported with water in flow-through columns.

5.3 The Odds for Isolation of Denitrifiers Increase with Time in the Permeable Barrier Microcosms

The fourth goal was to assess the affect of wood over time on denitrifier populations. The odds of isolating denitrifiers after 24 weeks of incubation were significantly higher in the mixture samples than in the wood or soil samples. The percent of denitrifiers of the total NO_3^- reducers in the soil was consistent from six to 24 weeks at 42%, while the mixture fluctuated from 23% at six weeks to 62% at 24 weeks. At six weeks the odds of isolating denitrifiers was not significantly different in the mixture versus the soil therefore the null hypothesis that the odds of isolating NO_3^- reducers that are capable of denitrification will be the same in the soil as in the mixture is not rejected; however after 24 weeks of incubation the soil is unchanged with respect to the percent denitrifiers while the mixture has a significant increase due to selection for *Azospirillum* and fluorescent pseudomonads. This suggests rejection of the null after 24 weeks in favor of the alternative hypothesis that the addition of wood to soil increases the odds of isolating denitrifiers. (Chapter III)

Denitrification ability was rare in the wood controls (1/128) and may have been due to the low pH, ranging from 4.8-5.1, which can inhibit denitrification. Other causes could be toxic phenols or tannins, lack of nutrients or microbial competition. The pH of the soil and mixture jar microcosms ranged from 7.6-7.7 and 7.6-7.8 respectively.

Low pH microsites, low nutrient availability and leaching of potential toxins around wood particles in the mixture may play a role in the flux. The odds of isolating a denitrifier were significantly lower in the wood than in the mixture or soil. The data

supports rejection of the null hypothesis, that there is no difference in the odds of isolating denitrifiers from the wood versus soil or mixture, in favor of the alternative that the odds of isolating NO_3^- reducers that are capable of denitrification from pure pine wood shavings will be lower than in the soil or mixture due to low pH or other inhibitory substances in the wood and that the odds of isolating NO_3^- reducers that are capable of denitrification will be greater in the mixture (permeable barrier) than in the wood due to favorable pH, nutrients and microbial inocula supplied by the soil. (Chapter III)

5.4 *Azospirillum* and Fluorescent Pseudomonads were the Most Commonly Isolated Denitrifier Genera from Permeable Barrier Jar Microcosms

The fifth goal was to identify denitrifier populations in the permeable barrier microcosms and to identify specific NO_3^- reducing eubacterial species that were favored by the addition of pine wood. Fluorescent pseudomonads and *Azospirillum* were the denitrifying genera most commonly isolated from mixture incubations after 24 weeks. Possible selective advantages in the high C/N environment are saprophytic activity by pseudomonads and nitrogen-fixation by *Azospirillum.*

Additionally, fluorescent pseudomonads lyse fungal hypha which confers an advantage through the reduction of competition for nutrients and they produce siderophores which allow them to scavenge Fe. The pH of the mixture microcosms was 7.6-7.8. Above pH 7, components of the nitrogen oxide reductase metalloenzymes, Fe and Cu, become less soluble while Mo does not. The ability of microbes to scavenge for essential metals used in denitrification enzymes, which become less soluble due to pH changes, also gives

them a competitive advantage over those that cannot. Other NO_3^- reducing genera from the mixture treatment include *Janthinobacterium*, *Citrobacter, Pantoea (Erwinia), Agrobacterium* and *Clostridium*. Organisms in these genera can also degrade wood, fix-nitrogen and evade protozoan grazing which supplies competitive advantages in the permeable barrier.

5.5 Wood Selected for Phycomycetes

The final goal was to identify specific microeukaryote populations affected by the addition of wood. Cluster dendrograms of fatty acid profiles identified dominant populations of phycomycete fungi and algae in the wood containing microcosms as supported by microscopic observation. Phycomycetes are saprophytic or parasitic on each other or algae and were shown to be present in the wood and mixture samples with the exception of the mixture water samples. The activity of these organisms contributes to the short-term sequestering of assimilated NO_3^- in biomass. Their ability to reduce NO_3^- by dissimilatory pathways is unknown.

There was no EL-FAME evidence of protozoan grazing populations in the jar microcosm soil solids possibly related to small pore size in the clay soil which provides protection to the eubacteria from the larger, predatory microeukaryotes. The soil water has EL-FAME evidence for raptoral grazers which may be due to suspended clay particles which provide a surface for eubacterial growth and hunting grounds for raptoral protozoan predators. The presence of raptoral grazers in mixture solids and filter feeders in jar microcosm mixture water was also consistent EL-FAME profiles. Ammonium

concentrations were highest in the wood treatments, with less than 1% of NO_3^- reducers capable of denitrification and supportive of a dominant microeukaryotic population.

5.6 Additional Observations

The addition of NO_3^- had no effect on microbial diversity or total cells counts in short-term syringe microcosm incubations with wood which supports the theory that NO_3^- does not alter the structure of the microbial populations in soil but is used opportunistically by the populations that are already present. However, the three day incubation may not have been long enough to observe shifts in the microbial community. Additionally, because the syringe microcosms were sparged with nitrogen, nitrogen-fixation may have confounded the results of the effects of additional NO_3^-. (Chapter II)

Pseudomonas borealis was the most common isolate from the subsurface soil collected in Colorado from under native grass vegetation and was previously reported to be dominant in subsurface soil under California grassland. This may have implications for wide distribution of this species in subsurface soils under grassland. (Chapter III)
Desulfovibrio vulgaris has a fatty acid distribution most similar to the incubated soil solid samples which is not surprising since the soil inoculum has a high concentration of sulfate. (Chapter IV) Due to the domination of the microeukaryotes, the predominant NO_3^- reduction paths leading to ammonium are likely to be a result of ammonia fermentation by fungi and ammonium release from turnover of nutrients from grazing on bacteria and to a lesser extent the result of activity by DNRAs.

5.7 Recommendations

This study identified dominant populations in the microbial community associated with permeable barrier microcosm incubations and factors that can be used to manipulate permeable barriers for NO_3^- removal. These data can also be applied to the development and management of soil:pine wood permeable barriers for the bioremediation of other pollutants such as acid mine drainage.

5.8 Manipulation of Barriers through Control of Abiotic Factors

Micronutrients are required for nitrogen oxide reductase activity and the availability of micronutrients is dependent upon pH. Therefore, the pH of the system affects the microbial community by selecting for those organisms capable of scavenging low levels of nutrients such as Fe, Cu and Mo, which are required for denitrification. A pH close to neutral favors availability of these nutrients. The pH of pine wood in the jar microcosms was 4.5 for the initial inoculum. Dependent upon the ratio of pine wood used in a barrier and the pH of the native soil, lime may be required to raise the pH to a range suitable for eubacterial denitrification. Barriers also need to be monitored for the presence and availability of metals (Cu, Fe, Mo) that are required for function of the nitrogen oxide reductase metalloenzymes and supplemented when necessary or manipulated through pH to allow for solubility.

The addition of lime (calcium carbonate) has been reported to increase the level of ammonium. This could be due to a stimulation of DNRAs due to an increase in the C/N ratio from carbonate and it could be related to cation exchange. Ammonium competes

for exchange sites in the soil with calcium and may be released upon addition of the lime. High ammonium in the soil will inhibit NO_3^- assimilation into biomass. The addition of lime will flush ammonium from exchange sites in a barrier and may be useful if the barrier extends up into the rooting zone where plant roots could take up the ammonium.

The higher the ratio of wood to soil in a barrier, the greater the inocula of microeukaryotes and non-denitrifying NO_3^- reducers which will out-compete denitrifiers under high NO_3^- conditions in the barrier. There is greater variability in the fatty acid distributions among the jar microcosm mixture reps than in soil or wood controls. This could be due to hot spots in the wood or soil inoculum used to create the mixture which led to the development of different communities during incubation. Uneven concentrations of microbial cells or nutrients in the initial inoculum would contribute to this. These microcosms were homogenized through sterile 4 mm stainless steel sieves and high variability in permeable barriers in the field is expected. Solid samples from within the barrier could be from regions of pure soil, pure wood or mixtures in different ratios and these microsites will develop significantly different microbial communities.

The use of well and effluent water samples is recommended as the results of the syringe, flow-through and jar microcosm experiments indicate that the data are better suited for statistical analysis due to lower variability among reps and there are equivalent numbers of NO_3^- reducers in the water versus the solid samples of jar mixture microcosms. Additionally, water samples from the saturated jar microcosms had higher odds of isolating denitrifiers than did solids. The substrates for denitrification are soluble and

will move with the water table. As the water table recedes, the barrier above it will have microsites of activity in water filled pores. Some of the denitrifiers and other NO_3^- reducers will be lost with water as it leaves the barrier and NO_3^- reduction activity may be dominated by microeukaryotes and non-denitrifying eubacteria. Composting or the addition of nitrogen may be required if the initial conditions in the barrier lead to DOC in the leachate. There was no significant effect of time from six to 24 weeks for mixture and soil treatments. NRCFUs were similar at both time points, suggesting stable populations of NO_3^- reducer numbers which is consistent with reports from the field on NO_3^- removal in barriers. However there was a significant decline in NRCFU population in the wood treatments over time ($p=0.02$). This suggests that competition from microbial populations introduced into a barrier with wood will decrease from six to 24 weeks.

5.9 Microbial Inocula

At high concentrations of NO_3^- the competition between DNRAs and denitrifiers will be dependent on population size. Additionally, high C/N environments, such as those found in saturated permeable barriers without consistently high NO_3^- inputs, are believed to favor DNRA populations. In order to enhance denitrification in permeable barriers at high concentrations of NO_3^-, supplemental inoculation with denitrifiers may be necessary. Native soil inoculum should be used as a source of denitrifying populations and enriched through incubation prior to creation of the barrier with subsequent additions at intervals of high NO_3^- input to increase denitrifier populations. These populations could be introduced into a barrier with irrigation.

5.10 Future Research

Further research is required prior to large scale inoculation in the field. The effect of lime on pine wood microbial communities needs to be addressed. Additionally, permeable barriers could become a breeding ground for the emergence of new pathogens due to the influx of antibiotics, biopesticides and fecal contaminants in the agricultural runoff and isolates should be tested for their sensitivity and antagonism. Careful monitoring of effluent is required since microbial populations from the barrier can then be transported into surface water. Pine wood microcosms can also be used to culture phycomycetes and to study the role of these dominant, but often neglected, organisms in NO_3^- transformations in permeable barriers.

	Clay Soil	Mixture	Pine Wood Shavings
Pores	small	variable	large
Dominant Population - solid	Eubacteria	Microeukaryote	Microeukaryote
Dominant Population - water	Microeukaryote	Eubacteria & Microeukaryote	Microeukaryote
pH	7.7-7.8	7.6-7.8	4.8-5.1
C/N	4 to 60-80	6 to 80-100	350 to 50-75
NH_4-N	1 - 3 ppm	1 - 3 ppm	1- 5 ppm
% Denitrifiers of Nitrate Reducers	42%	23% to 62%	less than 1%
Nitrate Reducer CFUs (ln)	7-9 g^{-1} or ml^{-1}	12-13 g^{-1} or ml^{-1}	11-14 g^{-1} or ml^{-1}
Biofilm in Saturated Microcosms	none	weak	well developed
Saturated Microcosm Solution	cloudy	clear	clear

Table 5.1 Comparison of jar microcosms by soil, mixture and wood treatments. Mixture is 2:1 (v/v) soil to wood. Dominant populations were identified by EL-FAME fatty acid profiles. The % denitrifiers are of nitrate reducers tested in durham tubes with nitrate broth. Pore sizes were estimated by observation of structure at saturation.

APPENDIX I

Appendix I.A. NMS Ordination for Jar Microcosm Fatty Acids and ANOVA on Axis 2

```
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PC-ORD, Version 4.20
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Deletion of    37 columns:
FA1      FA4      FA7      FA16     FA18     FA22     FA23     FA24     FA29
FA30     FA31     FA36     FA37     FA42     FA46     FA49     FA51     FA63
FA71     FA74     FA75     FA76     FA77     FA80     FA82     FA83     FA86
FA92     FA99     FA101    FA102    FA103    FA104    FA109    FA110    FA114
FA115

****************************** Operation completed ******************************


****************************** Data Modification ******************************
PC-ORD, Version 4.20
 4 Oct 2004,  0:27

Deletion of     1 columns:
FA72

****************************** Operation completed ******************************


****************************** Data Modification ******************************
PC-ORD, Version 4.20
 4 Oct 2004,  0:28

Deletion of     1 rows:
8A_105

****************************** Operation completed ******************************


****************************** Data Modification ******************************
PC-ORD, Version 4.20
 4 Oct 2004,  0:28

 General relativization by   ROW : Parameter=    1.00

****************************** Operation completed ******************************


****************************** Data Modification ******************************
PC-ORD, Version 4.20
 4 Oct 2004,  0:29

Columns arcsine-squareroot transformed:
     Transformation applied to all columns.

****************************** Operation completed ******************************


****************************** Data Summarization ******************************
PC-ORD, Version 4.20
 4 Oct 2004,  0:29
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ArcSqRt

Summary of 52 Samples N = 79 Fas

Num.	Name	Mean	Stand.Dev.	Sum	Minimum	Maximum	S	E	H	D`
1	1A_60	0.039	0.062	3.044	0.000	0.252	25	0.983	3.165	0.9553
2	1A_32	0.038	0.062	3.031	0.000	0.244	24	0.988	3.141	0.9550
3	1A_34	0.041	0.060	3.220	0.000	0.256	28	0.984	3.280	0.9601
4	1B_40	0.033	0.065	2.634	0.000	0.271	18	0.988	2.854	0.9400
5	1B_56	0.035	0.064	2.742	0.000	0.272	20	0.984	2.947	0.9447
6	1B_22	0.036	0.063	2.881	0.000	0.228	21	0.993	3.023	0.9502
7	2A_100	0.027	0.070	2.107	0.000	0.365	16	0.911	2.527	0.9023
8	2A_104	0.029	0.068	2.294	0.000	0.358	20	0.911	2.729	0.9180
9	2A_84	0.035	0.065	2.764	0.000	0.331	36	0.894	3.204	0.9441
10	2B_108	0.031	0.067	2.478	0.000	0.336	21	0.934	2.844	0.9308
11	2B_102	0.032	0.066	2.511	0.000	0.309	23	0.921	2.887	0.9329
12	2B_90	0.036	0.064	2.840	0.000	0.291	30	0.929	3.160	0.9479
13	3A_48	0.044	0.059	3.465	0.000	0.384	49	0.934	3.634	0.9644
14	3A_10	0.039	0.063	3.085	0.000	0.426	37	0.936	3.380	0.9544
15	3A_30	0.038	0.063	3.027	0.000	0.364	37	0.924	3.337	0.9535
16	3B_02	0.032	0.066	2.527	0.000	0.319	20	0.952	2.853	0.9338
17	3B_28	0.040	0.061	3.121	0.000	0.292	34	0.944	3.330	0.9571
18	3B_08	0.030	0.067	2.339	0.000	0.291	16	0.961	2.663	0.9227
19	4A_19	0.038	0.062	2.972	0.000	0.263	24	0.981	3.119	0.9530
20	4A_67	0.039	0.061	3.110	0.000	0.263	26	0.984	3.207	0.9572
21	4A_17	0.028	0.068	2.240	0.000	0.328	13	0.984	2.523	0.9159
22	4B_23	0.036	0.063	2.854	0.000	0.264	21	0.990	3.014	0.9491
23	4B_35	0.041	0.060	3.269	0.000	0.253	29	0.984	3.312	0.9613
24	4B_37	0.033	0.065	2.568	0.000	0.277	17	0.988	2.800	0.9367
25	5A_91	0.034	0.066	2.693	0.000	0.321	33	0.898	3.139	0.9410
26	5A_105	0.035	0.065	2.740	0.000	0.313	33	0.905	3.164	0.9432
27	5A_95	0.033	0.066	2.611	0.000	0.292	27	0.910	3.001	0.9379
28	5B_103	0.032	0.066	2.567	0.000	0.304	22	0.941	2.908	0.9359
29	5B_79	0.035	0.065	2.779	0.000	0.341	35	0.903	3.209	0.9446
30	5B_101	0.033	0.066	2.630	0.000	0.300	27	0.915	3.017	0.9388
31	6A_49	0.042	0.061	3.318	0.000	0.381	51	0.915	3.597	0.9611
32	6A_03	0.038	0.063	2.981	0.000	0.338	37	0.914	3.299	0.9524
33	6A_63	0.041	0.062	3.202	0.000	0.407	45	0.922	3.508	0.9579
34	6B_43	0.033	0.066	2.580	0.000	0.306	23	0.932	2.921	0.9367
35	6B_27	0.036	0.064	2.856	0.000	0.345	31	0.927	3.183	0.9480
36	6B_47	0.034	0.065	2.724	0.000	0.324	24	0.949	3.017	0.9433
37	7A_17	0.019	0.073	1.515	0.000	0.415	6	0.954	1.709	0.8050
38	7A_19	0.020	0.073	1.541	0.000	0.405	6	0.968	1.734	0.8133
39	7A_67	0.015	0.075	1.175	0.000	0.426	3	0.998	1.097	0.6654
40	7B_23	0.013	0.113	1.000	0.000	1.000	1	0.000	0.000	0.0000
41	7B_37	0.013	0.079	1.000	0.000	0.534	2	0.997	0.691	0.4977
42	7B_35	0.021	0.072	1.634	0.000	0.397	7	0.963	1.873	0.8349
43	8A_95	0.015	0.076	1.161	0.000	0.473	3	0.976	1.072	0.6500
44	8A_91	0.021	0.072	1.658	0.000	0.385	7	0.973	1.893	0.8412
45	8B_79	0.018	0.074	1.395	0.000	0.435	5	0.947	1.524	0.7651
46	8B_101	0.023	0.072	1.782	0.000	0.379	9	0.950	2.087	0.8617
47	8B_103	0.019	0.074	1.511	0.000	0.455	6	0.951	1.704	0.8021
48	9A_63	0.023	0.071	1.849	0.000	0.361	9	0.971	2.134	0.8736
49	9A_49	0.018	0.075	1.407	0.000	0.520	5	0.951	1.531	0.7634
50	9A_03	0.017	0.074	1.325	0.000	0.375	4	0.992	1.376	0.7449
51	9B_07	0.020	0.072	1.581	0.000	0.354	6	0.988	1.770	0.8262
52	9B_47	0.017	0.075	1.304	0.000	0.495	4	0.970	1.344	0.7272
	AVERAGES:	0.030	0.068	2.378	0.000	0.358	20.7	0.934	2.585	0.8741

		Skewness	Kurtosis
1	1A_60	1.389	1.210
2	1A_32	1.269	0.514
3	1A_34	1.218	0.817
4	1B_40	1.744	2.144
5	1B_56	1.691	2.158
6	1B_22	1.310	0.281
7	2A_100	3.326	11.760
8	2A_104	3.168	10.926
9	2A_84	2.932	9.514
10	2B_108	2.631	7.413
11	2B_102	2.514	6.221
12	2B_90	2.250	4.979
13	3A_48	3.052	13.969
14	3A_10	3.395	17.358
15	3A_30	2.729	9.891
16	3B_02	2.420	6.055
17	3B_28	1.954	4.065
18	3B_08	2.451	5.610
19	4A_19	1.489	1.613
20	4A_67	1.318	1.083
21	4A_17	2.369	5.273
22	4B_23	1.470	1.250
23	4B_35	1.187	0.764
24	4B_37	1.837	2.582
25	5A_91	2.954	9.528
26	5A_105	2.894	9.250
27	5A_95	2.543	6.501
28	5B_103	2.422	5.921
29	5B_79	2.991	10.176
30	5B_101	2.532	6.379
31	6A_49	3.135	13.309
32	6A_03	2.507	7.659
33	6A_63	3.382	16.040
34	6B_43	2.335	5.298
35	6B_27	2.557	8.049
36	6B_47	2.224	5.349
37	7A_17	4.159	17.712
38	7A_19	3.962	15.897
39	7A_67	4.961	23.446
40	7B_23	8.888	79.119
41	7B_37	6.207	37.799
42	7B_35	3.811	14.970
43	8A_95	5.281	27.607
44	8A_91	3.574	12.697
45	8B_79	4.630	22.071
46	8B_101	3.666	14.279
47	8B_103	4.322	19.897
48	9A_63	3.219	10.459
49	9A_49	4.939	27.349
50	9A_03	4.318	17.578
51	9B_07	3.524	11.474
52	9B_47	4.877	24.959
	Averages:	3.037	11.697

Number of cells in main matrix = 4108
Percent of cells empty = 73.807
Matrix total = 0.1236E+03
Matrix mean = 0.3010E-01
Variance of totals of Samples = 0.4988E+00
CV of totals of Samples = 29.70%

S = Richness = number of non-zero elements in row
E = Evenness = H / ln (Richness)
H = Diversity = - sum (Pi*ln(Pi)) = Shannon`s diversity index
D = Simpson`s diversity index for infinite population = 1 - sum (Pi*Pi)
where Pi = importance probability in element i (element i relativized by row total)

ArcSqRt

Summary of 79 Fas N = 52 Samples

Num.	Name	Mean	Stand.Dev.	Sum	Minimum	Maximum	S	E	H	D`
1	FA2	0.002	0.008	0.102	0.000	0.044	3	0.975	1.071	0.6482
2	FA3	0.004	0.014	0.225	0.000	0.060	5	0.968	1.559	0.7817
3	FA5	0.001	0.004	0.046	0.000	0.016	3	0.996	1.095	0.6641
4	FA6	0.004	0.015	0.200	0.000	0.099	5	0.864	1.391	0.6904
5	FA8	0.001	0.005	0.063	0.000	0.024	3	0.985	1.082	0.6561
6	FA9	0.004	0.009	0.187	0.000	0.029	8	0.987	2.053	0.8689
7	FA10	0.005	0.014	0.243	0.000	0.062	6	0.961	1.722	0.8100
8	FA11	0.002	0.007	0.081	0.000	0.037	3	0.969	1.064	0.6428
9	FA12	0.003	0.009	0.155	0.000	0.041	5	0.992	1.596	0.7945
10	FA13	0.002	0.007	0.110	0.000	0.027	5	0.991	1.595	0.7943
11	FA14	0.001	0.005	0.060	0.000	0.027	3	0.961	1.055	0.6392
12	FA15	0.002	0.008	0.100	0.000	0.037	3	0.992	1.090	0.6612
13	FA17	0.002	0.008	0.099	0.000	0.043	3	0.954	1.048	0.6357
14	FA19	0.037	0.042	1.949	0.000	0.123	30	0.960	3.264	0.9576
15	FA20	0.003	0.010	0.140	0.000	0.062	4	0.931	1.290	0.6964
16	FA21	0.004	0.013	0.194	0.000	0.057	4	0.991	1.374	0.7437
17	FA25	0.013	0.022	0.659	0.000	0.089	15	0.983	2.661	0.9261
18	FA26	0.043	0.066	2.246	0.000	0.195	21	0.939	2.860	0.9369
19	FA27	0.041	0.052	2.109	0.000	0.166	27	0.942	3.105	0.9495
20	FA28	0.012	0.023	0.643	0.000	0.083	14	0.965	2.545	0.9147
21	FA32	0.004	0.015	0.233	0.000	0.081	5	0.956	1.539	0.7687
22	FA33	0.040	0.060	2.061	0.000	0.173	21	0.942	2.868	0.9379
23	FA34	0.009	0.026	0.478	0.000	0.116	6	0.988	1.771	0.8257
24	FA35	0.004	0.008	0.183	0.000	0.027	8	0.998	2.074	0.8737
25	FA38	0.007	0.013	0.345	0.000	0.036	12	0.993	2.467	0.9138
26	FA39	0.027	0.053	1.398	0.000	0.153	11	0.998	2.393	0.9081
27	FA40	0.064	0.073	3.309	0.000	0.290	32	0.942	3.264	0.9560
28	FA41	0.037	0.067	1.924	0.000	0.184	13	0.986	2.528	0.9189
29	FA43	0.184	0.074	9.568	0.000	0.328	49	0.988	3.845	0.9777
30	FA44	0.034	0.059	1.785	0.000	0.163	15	0.973	2.635	0.9253
31	FA45	0.016	0.023	0.825	0.000	0.068	17	0.995	2.819	0.9395
32	FA47	0.053	0.041	2.768	0.000	0.120	35	0.990	3.520	0.9694
33	FA48	0.054	0.042	2.816	0.000	0.108	34	0.994	3.504	0.9693
34	FA50	0.008	0.020	0.404	0.000	0.074	8	0.973	2.023	0.8608
35	FA52	0.020	0.040	1.025	0.000	0.153	13	0.955	2.449	0.9043
36	FA53	0.011	0.028	0.595	0.000	0.093	8	0.983	2.044	0.8670
37	FA54	0.003	0.011	0.138	0.000	0.055	3	0.976	1.072	0.6502
38	FA55	0.088	0.085	4.595	0.000	0.222	29	0.988	3.327	0.9633
39	FA56	0.206	0.137	10.727	0.000	0.473	41	0.982	3.646	0.9725
40	FA57	0.015	0.031	0.790	0.000	0.101	11	0.986	2.365	0.9034
41	FA58	0.011	0.035	0.580	0.000	0.165	6	0.928	1.662	0.7968
42	FA59	0.305	0.161	15.840	0.000	1.000	47	0.982	3.783	0.9755
43	FA60	0.041	0.090	2.148	0.000	0.378	10	0.982	2.261	0.8908
44	FA61	0.008	0.024	0.404	0.000	0.085	5	0.999	1.608	0.7995
45	FA62	0.105	0.120	5.454	0.000	0.520	36	0.936	3.354	0.9562
46	FA64	0.066	0.051	3.439	0.000	0.154	36	0.987	3.538	0.9696
47	FA65	0.002	0.008	0.122	0.000	0.036	5	0.930	1.496	0.7647
48	FA66	0.014	0.026	0.703	0.000	0.096	12	0.990	2.461	0.9123
49	FA67	0.003	0.011	0.134	0.000	0.050	3	0.993	1.091	0.6619
50	FA68	0.033	0.053	1.726	0.000	0.149	16	0.982	2.724	0.9319
51	FA69	0.016	0.038	0.820	0.000	0.113	8	0.994	2.067	0.8723
52	FA70	0.035	0.098	1.821	0.000	0.341	6	0.999	1.789	0.8325
53	FA73	0.046	0.051	2.411	0.000	0.125	24	0.998	3.172	0.9578
54	FA78	0.008	0.024	0.434	0.000	0.102	6	0.980	1.757	0.8217
55	FA79	0.026	0.049	1.344	0.000	0.134	12	0.993	2.468	0.9143
56	FA81	0.008	0.016	0.422	0.000	0.050	11	0.994	2.384	0.9065
57	FA84	0.028	0.036	1.442	0.000	0.123	22	0.985	3.045	0.9498
58	FA85	0.067	0.081	3.465	0.000	0.207	22	0.993	3.070	0.9527
59	FA87	0.031	0.045	1.637	0.000	0.116	18	0.995	2.877	0.9430
60	FA88	0.008	0.028	0.409	0.000	0.110	4	0.998	1.384	0.7489
61	FA89	0.011	0.026	0.588	0.000	0.081	9	0.989	2.174	0.8841
62	FA90	0.014	0.025	0.710	0.000	0.077	12	0.996	2.476	0.9152
63	FA91	0.073	0.088	3.777	0.000	0.225	24	0.974	3.094	0.9533

64	FA93	0.018	0.026	0.948	0.000	0.081	18	0.995	2.875	0.9426
65	FA94	0.031	0.049	1.622	0.000	0.174	18	0.971	2.808	0.9348
66	FA95	0.053	0.086	2.780	0.000	0.252	16	0.984	2.728	0.9319
67	FA96	0.009	0.022	0.490	0.000	0.087	9	0.984	2.162	0.8806
68	FA97	0.005	0.017	0.282	0.000	0.068	5	0.993	1.598	0.7954
69	FA98	0.004	0.014	0.201	0.000	0.060	4	0.993	1.377	0.7453
70	FA100	0.028	0.068	1.454	0.000	0.317	9	0.959	2.106	0.8679
71	FA105	0.029	0.062	1.501	0.000	0.211	10	0.987	2.272	0.8938
72	FA106	0.012	0.041	0.604	0.000	0.193	4	0.988	1.369	0.7414
73	FA107	0.036	0.044	1.857	0.000	0.127	24	0.976	3.101	0.9521
74	FA108	0.023	0.070	1.175	0.000	0.259	5	0.998	1.607	0.7988
75	FA111	0.024	0.038	1.268	0.000	0.109	19	0.954	2.810	0.9339
76	FA112	0.006	0.020	0.294	0.000	0.077	4	0.998	1.384	0.7489
77	FA113	0.005	0.013	0.236	0.000	0.073	7	0.952	1.852	0.8238
78	FA116	0.060	0.072	3.117	0.000	0.275	24	0.984	3.126	0.9535
79	FA117	0.008	0.026	0.400	0.000	0.150	5	0.949	1.528	0.7619
AVERAGES:		0.030	0.039	1.565	0.000	0.143	13.6	0.977	2.242	0.8536

		Skewness	Kurtosis
1	FA2	4.265	17.990
2	FA3	3.210	9.381
3	FA5	3.959	14.548
4	FA6	5.353	32.413
5	FA8	4.101	16.040
6	FA9	2.176	3.304
7	FA10	3.128	9.412
8	FA11	4.401	19.733
9	FA12	2.990	7.866
10	FA13	2.970	7.586
11	FA14	4.385	19.154
12	FA15	4.013	15.117
13	FA17	4.402	19.137
14	FA19	0.759	-0.673
15	FA20	4.530	22.659
16	FA21	3.423	10.667
17	FA25	1.557	2.010
18	FA26	1.198	-0.136
19	FA27	1.015	-0.318
20	FA28	1.811	2.430
21	FA32	3.719	14.826
22	FA33	1.153	-0.255
23	FA34	2.758	6.794
24	FA35	2.032	2.516
25	FA38	1.463	0.540
26	FA39	1.506	0.552
27	FA40	1.073	0.666
28	FA41	1.330	0.016
29	FA43	-0.597	0.402
30	FA44	1.292	0.020
31	FA45	0.900	-0.836
32	FA47	-0.181	-1.199
33	FA48	-0.317	-1.381
34	FA50	2.473	5.254
35	FA52	2.103	3.920
36	FA53	2.216	3.472
37	FA54	4.193	16.980
38	FA55	0.038	-1.675
39	FA56	-0.228	-0.739
40	FA57	1.708	1.477
41	FA58	3.237	10.137
42	FA59	0.966	6.302
43	FA60	2.087	3.747
44	FA61	2.835	6.494
45	FA62	1.852	4.033
46	FA64	-0.083	-1.038
47	FA65	3.366	10.380
48	FA66	1.596	1.379
49	FA67	4.002	15.010

```
  50 FA68          1.181    -0.140
  51 FA69          2.062     2.625
  52 FA70          2.507     4.700
  53 FA73          0.223    -1.756
  54 FA78          2.830     7.133
  55 FA79          1.409     0.238
  56 FA81          1.596     1.028
  57 FA84          0.888    -0.115
  58 FA85          0.513    -1.393
  59 FA87          0.791    -1.074
  60 FA88          3.297     9.471
  61 FA89          1.950     2.270
  62 FA90          1.406     0.324
  63 FA91          0.524    -1.376
  64 FA93          0.833    -0.876
  65 FA94          1.389     1.085
  66 FA95          1.234     0.172
  67 FA96          2.151     3.696
  68 FA97          2.955     7.508
  69 FA98          3.385    10.323
  70 FA100         2.605     6.809
  71 FA105         1.880     2.227
  72 FA106         3.483    11.295
  73 FA107         0.791    -0.640
  74 FA108         2.855     6.659
  75 FA111         1.346     0.433
  76 FA112         3.297     9.469
  77 FA113         3.554    14.944
  78 FA116         0.832     0.074
  79 FA117         3.969    17.668
-----------------------------------
   Averages:       2.150     5.682
-------------------------------------------
Number of cells in main matrix =     4108
Percent of cells empty =   73.807
Matrix total =   0.1236E+03
Matrix mean  =   0.3010E-01
Variance of totals of Fas      =   0.6020E+01
CV of totals of Fas      =     156.78%
-------------------------------------------
S = Richness  = number of non-zero elements in row
E = Evenness  = H / ln (Richness)
H = Diversity = - sum (Pi*ln(Pi)) = Shannon`s diversity index
D = Simpson`s diversity index for infinite population = 1 - sum (Pi*Pi)
       where Pi = importance probability in element i (element i
                  relativized by row total)

****************************** Analysis completed ******************************

****************************** Outlier Analysis ******************************
PC-ORD, Version 4.20
 4 Oct 2004,  0:30

fig1aArcSqRt
-----------------------------------------
        ENTITY       AVERAGE     STANDARD
 RANK    NAME        DISTANCE   DEVIATIONS
-----------------------------------------
    1  FA60           0.96952     2.59818
   77  FA64           0.68255    -2.01240
   78  FA48           0.67642    -2.11078
   79  FA47           0.67235    -2.17627
-----------------------------------------

Statistics for average distances for each of N =   79 Fas
Distance measure: Sorensen (Bray-Curtis)
 0.80781E+00 = Grand mean
 0.62243E-01 = Standard deviation
     2.00000 = Cutoff number of standard deviations used to flag outlier

****************************** Analysis completed ******************************
```

```
****************************** Outlier Analysis ******************************
PC-ORD, Version 4.20
 4 Oct 2004,  0:30


fig1aArcSqRt
-----------------------------------------
        ENTITY       AVERAGE     STANDARD
 RANK    NAME        DISTANCE   DEVIATIONS
-----------------------------------------
    1  7B_23          0.81431     3.08802
    2  9B_47          0.79180     2.78397
-----------------------------------------


Statistics for average distances for each of N =   52 Samples
Distance measure: Sorensen (Bray-Curtis)
 0.58576E+00 = Grand mean
 0.74010E-01 = Standard deviation
     2.00000 = Cutoff number of standard deviations used to flag outliers


****************************** Analysis completed ******************************


******************** Nonmetric Multidimensional Scaling ********************
PC-ORD, Version 4.20
 4 Oct 2004,  0:31


fig1aArcSqRt
Ordination of Samples  in Fas      space.         52 Samples        79 Fas

         The following options were selected:
ANALYSIS OPTIONS
         1.    SORENSEN = Distance measure
         2.           6 = Number of axes (max. = 6)
         3.         100 = Maximum number of iterations
         4.      RANDOM = Starting coordinates (random or from file)
         5.           1 = Reduction in dimensionality at each cycle
         6.        1.00 = Step length (rate of movement toward minimum stress)
         7.    USE TIME = Random number seeds (use time vs. user-supplied)
         8.          10 = Number of runs with real data
         9.          20 = Number of runs with randomized data
        10.          NO = Autopilot
        11.    0.000500 = Stability criterion, standard deviations in stress
                          over last 200 iterations.
OUTPUT OPTIONS
        13.          NO = Write distance matrix?
        14.         YES = Write starting coordinates?
        15.          NO = List stress, etc. for each iteration?
        18.         YES = Plot stress vs. iteration?
        17.         YES = Plot distance vs. dissimilarity?
        16.         YES = Write final configuration?
        19.  UNROTATED = Write varimax-rotated or unrotated scores for graph?
        20.         YES = Write run log?
        21.         YES = Write weighted-average scores for Fas     ?
-------------------------------------------------------------------------------

       2548 = Seed for random number generator.
```

Coordinates of starting configuration

Samples No.	Name	Axis 1	2	3	4	5	6
1	1A_60	80.3055	94.5343	74.2332	20.3845	24.7991	0.7674
2	1A_32	34.3505	14.0735	5.6153	91.6658	98.1684	93.1960
3	1A_34	3.9734	98.4345	17.6262	38.8019	88.0087	37.6601
4	1B_40	69.8644	88.2499	12.3148	46.2769	55.1187	55.0122
5	1B_56	12.6993	29.1688	5.2199	17.7505	43.2828	57.4683
6	1B_22	48.5945	86.4670	96.4728	58.9423	89.2317	83.6314
7	2A_100	59.9397	82.4779	53.1642	80.7005	83.4678	66.9176
8	2A_104	22.0051	39.3160	16.6859	86.3224	10.7688	90.5013
9	2A_84	47.5695	82.1939	89.5217	67.7741	15.3787	66.4383
10	2B_108	68.7226	26.6419	23.0700	72.2375	3.5579	13.0948
11	2B_102	68.8438	75.9211	88.4594	67.4664	64.3533	67.8380
12	2B_90	14.5345	31.8554	68.8954	84.5904	69.5932	99.0282
13	3A_48	8.8366	75.1965	97.9635	74.4651	78.2392	31.2670
14	3A_10	7.0478	75.5515	82.9073	85.1910	37.3246	19.4291
15	3A_30	50.7747	56.0030	43.3948	73.4716	65.7534	73.7345
16	3B_02	32.0257	70.3436	65.9708	37.9453	10.8517	53.3836
17	3B_28	42.9534	93.8409	73.5057	65.8119	26.8795	98.9641
18	3B_08	43.6476	30.2321	53.4150	58.0059	78.9352	9.6160
19	4A_19	94.0080	77.2059	3.5212	69.2016	94.8704	61.1087
20	4A_67	35.9676	19.4870	0.0011	48.2881	81.2478	38.3896
21	4A_17	89.4204	91.0287	11.9525	2.1060	50.5642	74.2271
22	4B_23	58.9471	26.7146	99.8932	70.8981	85.8635	49.3944
23	4B_35	95.0357	86.9257	11.6266	46.9987	42.2350	42.7759
24	4B_37	80.3489	93.7355	51.1782	40.4571	80.7607	11.4510
25	5A_91	0.8573	62.4966	18.4632	65.2358	39.0994	88.8778
26	5A_105	91.9487	93.3226	77.4052	52.2220	87.6914	86.8484
27	5A_95	6.8962	34.3311	34.9294	24.9430	87.9196	33.5823
28	5B_103	2.5125	22.9917	90.5150	62.9555	56.9261	12.5318
29	5B_79	64.6207	89.7420	41.2791	0.4930	51.9637	4.4796
30	5B_101	40.3186	63.8073	62.7894	56.8293	71.8562	32.6171
31	6A_49	90.7432	43.0954	57.9925	85.1269	34.4134	12.6900
32	6A_03	64.2775	41.0280	67.6241	82.4790	6.7811	96.7920
33	6A_63	61.7924	19.1609	15.3545	59.1919	46.5551	0.9901
34	6B_43	68.0265	72.7943	94.9125	9.5431	89.9898	12.4250
35	6B_27	53.8519	0.4839	66.1494	97.4316	7.6000	64.7255
36	6B_47	22.7959	87.8087	4.0540	27.8840	98.0661	5.4109
37	7A_17	70.5084	37.2807	92.2948	75.3117	63.9217	61.6589
38	7A_19	63.2103	30.8711	5.0049	39.8720	23.9153	77.8138
39	7A_67	4.5334	37.3104	79.8024	76.4511	52.1487	20.9362
40	7B_23	49.8298	66.1575	65.1502	34.0196	13.3305	38.4014
41	7B_37	21.2638	22.4312	23.9120	66.5206	98.6023	5.6509
42	7B_35	74.9320	96.6414	35.8901	71.5070	36.7465	8.5435
43	8A_95	76.9611	76.6370	84.5498	52.8825	21.9214	33.0714
44	8A_91	21.3664	48.9734	10.8281	37.1079	96.2912	86.2323
45	8B_79	1.2539	39.2866	92.1807	1.4847	25.1196	55.8285
46	8B_101	17.2914	62.6440	33.0488	37.1119	29.1104	37.2955
47	8B_103	72.3537	55.0998	56.4139	46.4668	95.4380	65.0950
48	9A_63	25.9877	96.1905	69.4461	18.5846	53.3336	49.2704
49	9A_49	78.1783	98.9713	23.1408	60.1732	13.8128	76.8461
50	9A_03	61.2244	28.5067	74.9252	71.5098	44.0313	45.5112
51	9B_07	9.3744	36.8931	23.2019	72.5482	99.8752	45.7187
52	9B_47	8.4939	92.5630	71.1549	3.4552	3.2026	2.0525

```
**************** 1-dimensional solution ****************

      41.94727 = final stress for 1-dimensional solution
       0.04905 = final instability
           100 = number of iterations

Final configuration (ordination scores) for this run
    Samples              Axis
Number Name                 1
     1 1A_60           -1.2983
     2 1A_32            1.3736
     3 1A_34           -1.6867
     4 1B_40           -1.2601
     5 1B_56           -1.2420
     6 1B_22           -1.7113
     7 2A_100           0.3600
     8 2A_104           0.3382
     9 2A_84            0.1202
    10 2B_108           0.1531
    11 2B_102           0.2325
    12 2B_90           -0.1466
    13 3A_48           -0.8509
    14 3A_10           -0.6048
    15 3A_30           -0.3024
    16 3B_02           -0.3904
    17 3B_28           -0.4201
    18 3B_08           -0.2595
    19 4A_19           -1.2083
    20 4A_67           -1.7460
    21 4A_17            1.6298
    22 4B_23           -1.3829
    23 4B_35           -1.4107
    24 4B_37           -1.2855
    25 5A_91            0.0938
    26 5A_105           0.0741
    27 5A_95            0.1856
    28 5B_103           0.2096
    29 5B_79           -0.0451
    30 5B_101           0.0297
    31 6A_49           -0.5790
    32 6A_03           -0.3171
    33 6A_63           -0.8229
    34 6B_43           -0.0267
    35 6B_27           -0.1315
    36 6B_47           -0.1915
    37 7A_17            0.6141
    38 7A_19            0.5990
    39 7A_67            1.4013
    40 7B_23            2.1307
    41 7B_37            1.2653
    42 7B_35            0.6230
    43 8A_95            0.9703
    44 8A_91            0.7393
    45 8B_79            0.8231
    46 8B_101           0.5414
    47 8B_103           0.8505
    48 9A_63            1.7676
    49 9A_49           -1.5925
    50 9A_03            0.9119
    51 9B_07            0.9040
    52 9B_47            1.9712
```

STRESS IN RELATION TO DIMENSIONALITY (Number of Axes)

	Stress in real data 10 run(s)			Stress in randomized data Monte Carlo test, 20 runs			
Axes	Minimum	Mean	Maximum	Minimum	Mean	Maximum	p
1	27.772	42.751	56.578	42.369	48.967	56.587	0.0476
2	9.988	11.647	19.782	27.231	30.758	34.197	0.0476
3	6.854	7.194	7.596	20.724	22.266	23.887	0.0476
4	5.208	5.498	6.910	16.474	17.733	18.886	0.0476
5	4.134	6.118	21.692	13.743	14.465	15.226	0.0476
6	3.117	3.424	4.499	11.424	12.253	15.263	0.0476

p = proportion of randomized runs with stress < or = observed stress
i.e., p = (1 + no. permutations <= observed)/(1 + no. permutations)

Conclusion: a 2-dimensional solution is recommended.

Selected file CONFIG2.GPH for the starting configuration for
the final run.

fig1aArcSqRt
Ordination of Samples in Fas space. 52 Samples 79 Fas

The following options were selected:

ANALYSIS OPTIONS

1.	SORENSEN	= Distance measure
2.	2	= Number of axes (max. = 6)
3.	100	= Maximum number of iterations
4.	FROM FILE	= Starting coordinates (random or from file)
5.	2	= Reduction in dimensionality at each cycle
6.	1.00	= Step length (rate of movement toward minimum stress)
7.	USE TIME	= Random number seeds (use time vs. user-supplied)
8.	1	= Number of runs with real data
9.	0	= Number of runs with randomized data
10.	NO	= Autopilot
11.	0.000500	= Stability criterion, standard deviations in stress over last 200 iterations.

OUTPUT OPTIONS

13.	NO	= Write distance matrix?
14.	YES	= Write starting coordinates?
15.	NO	= List stress, etc. for each iteration?
18.	YES	= Plot stress vs. iteration?
17.	YES	= Plot distance vs. dissimilarity?
16.	YES	= Write final configuration?
19.	UNROTATED	= Write varimax-rotated or unrotated scores for graph?
20.	NO	= Write run log?
21.	YES	= Write weighted-average scores for Fas ?

Coordinates of starting configuration

Samples		Axis	
No.	Name	1	2
1	1A_60	95.8359	87.4879
2	1A_32	63.8570	31.4395
3	1A_34	74.7337	15.7777
4	1B_40	95.3520	73.8151
5	1B_56	65.7632	11.9887
6	1B_22	94.7104	9.5265
7	2A_100	77.9059	96.2101
8	2A_104	28.7059	66.6964
9	2A_84	76.9075	68.2149
10	2B_108	72.2434	94.1333
11	2B_102	74.2941	50.1455
12	2B_90	54.4535	52.7791
13	3A_48	17.5894	36.5445
14	3A_10	3.9568	61.7322
15	3A_30	83.5565	40.9202
16	3B_02	10.0762	38.7522
17	3B_28	42.4904	4.0125
18	3B_08	8.0548	6.5049
19	4A_19	9.5085	15.5081
20	4A_67	76.7640	91.3646
21	4A_17	73.2922	99.5732
22	4B_23	21.8937	35.0673
23	4B_35	51.0216	86.6359
24	4B_37	15.8507	38.1414
25	5A_91	84.0484	8.4988
26	5A_105	43.1657	80.7383
27	5A_95	86.0346	11.8055
28	5B_103	51.8183	89.9941
29	5B_79	25.3264	30.0945
30	5B_101	96.5359	22.6781
31	6A_49	50.8695	46.9078
32	6A_03	72.3921	48.8247
33	6A_63	50.3953	1.5010
34	6B_43	92.3280	53.7436
35	6B_27	41.4390	92.2004
36	6B_47	72.2748	87.3974
37	7A_17	82.7854	48.3960
38	7A_19	67.6321	81.3750
39	7A_67	53.6148	14.5953
40	7B_23	30.4813	20.0067
41	7B_37	63.0384	70.4714
42	7B_35	55.5196	93.4198
43	8A_95	93.5256	34.8950
44	8A_91	31.8209	93.9967
45	8B_79	96.7198	73.5052
46	8B_101	4.2958	48.4613
47	8B_103	58.8165	99.6783
48	9A_63	31.8437	85.1347
49	9A_49	12.7618	48.6827
50	9A_03	47.1151	50.0847
51	9B_07	23.3555	71.4764
52	9B_47	21.2420	85.1476

********** 2-dimensional solution **********

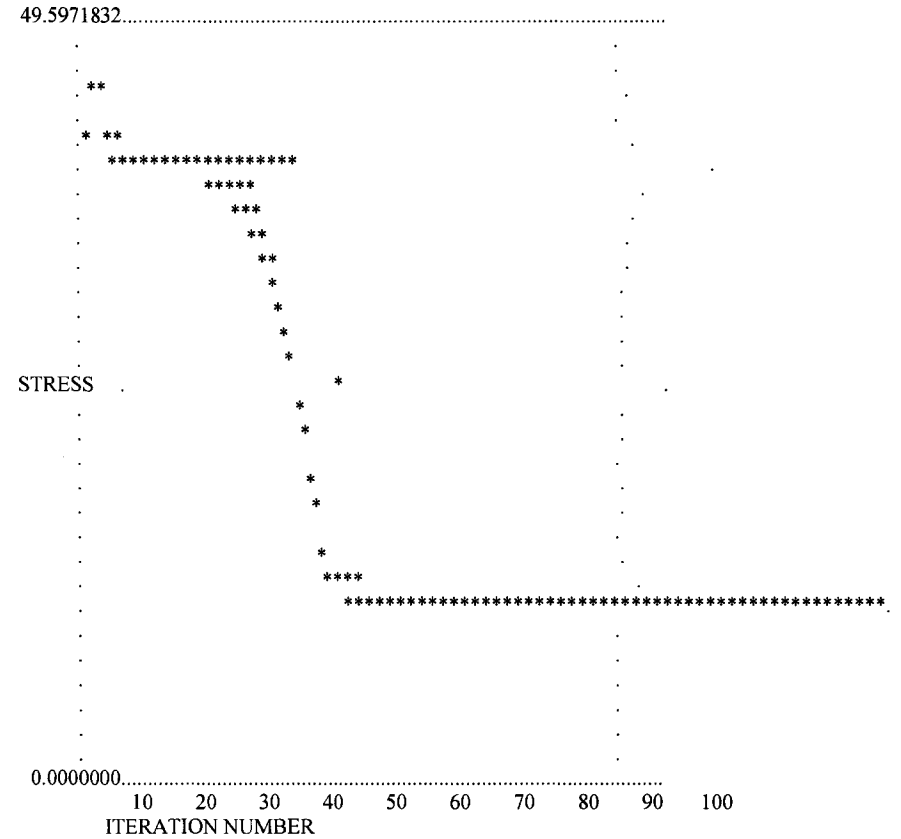
9.98767 = final stress for 2-dimensional solution
0.13836 = final instability
100 = number of iterations

Final configuration (ordination scores) for this run

Samples		Axis	
Number	Name	1	2
1	1A_60	-0.0389	1.0106
2	1A_32	-0.0407	1.4708
3	1A_34	-0.0239	1.4195
4	1B_40	-0.2102	0.9435
5	1B_56	-0.1678	0.9549
6	1B_22	-0.1234	1.4516
7	2A_100	0.2644	-0.6415
8	2A_104	0.3556	-0.7383
9	2A_84	0.7007	-0.5197
10	2B_108	0.4639	-0.5288
11	2B_102	0.5268	-0.6386
12	2B_90	0.6987	-0.2778
13	3A_48	0.7117	0.5499
14	3A_10	0.4184	0.3016
15	3A_30	0.3804	-0.0086
16	3B_02	0.1323	0.0995
17	3B_28	0.4325	0.0697
18	3B_08	0.1239	-0.0511
19	4A_19	-0.0388	0.9701
20	4A_67	-0.0196	1.4794
21	4A_17	-0.5499	1.4466
22	4B_23	-0.1632	1.0638
23	4B_35	0.0510	1.1398
24	4B_37	-0.2718	0.9293
25	5A_91	0.7096	-0.5200
26	5A_105	0.7365	-0.4825
27	5A_95	0.7125	-0.7622
28	5B_103	0.5123	-0.6759
29	5B_79	0.7498	-0.4480
30	5B_101	0.8896	-0.6042
31	6A_49	0.7779	0.2738
32	6A_03	0.6011	-0.0243
33	6A_63	0.7926	0.5111
34	6B_43	0.4218	-0.3015
35	6B_27	0.3720	-0.1672
36	6B_47	0.4525	-0.1866
37	7A_17	-0.1066	-0.8539
38	7A_19	-0.0840	-0.8439
39	7A_67	-0.7781	-1.0902
40	7B_23	-1.3754	-1.2768
41	7B_37	-0.5830	-1.2554
42	7B_35	-0.1781	-0.8142
43	8A_95	-0.6373	-0.7267
44	8A_91	-0.5531	-0.4291
45	8B_79	-0.4687	-0.7856
46	8B_101	-0.2929	-0.5626
47	8B_103	-0.5157	-0.6865
48	9A_63	-1.2850	0.4409
49	9A_49	-1.3417	0.2979
50	9A_03	-0.7097	-0.3345
51	9B_07	-0.7677	-0.1810
52	9B_47	-1.6631	0.5930

Plot of distance vs. dissimilarity was skipped.
The data set is large enough that the plot would be too cluttered. Use coordinates in SHEPARD.TXT to create this plot in a graphics package or spreadsheet program.

PLOT OF STRESS V. ITERATION NUMBER
(to prevent wrapping of wide plots when printing, use small font)




```
Writing weighted average scores on 2 axes for    79 Fas
   into file for graphing.

Calculations completed  4 Oct 2004,  0:33
        2.04 minutes elapsed time.

**************************** Calculations finished ****************************
```

********** Output from Graph **********
PC-ORD Version 4.20
10/4/04, 12:36 AM

fig1aArcSqRt

Pearson and Kendall Correlations with Ordination Axes N= 52

Axis:	1			2			3		
	r	r-sq	tau	r	r-sq	tau	r	r-sq	tau
FA2	.257	.066	.233	.122	.015	.135			
FA3	.340	.115	.328	.152	.023	.170			
FA5	.302	.091	.300	.143	.020	.148			
FA6	.312	.097	.370	-.037	.001	-.068			
FA8	.298	.089	.300	.145	.021	.148			
FA9	.505	.255	.485	-.210	.044	-.118			
FA10	.174	.030	.221	.303	.092	.269			
FA11	.289	.084	.300	.143	.020	.148			
FA12	.288	.083	.244	-.257	.066	-.230			
FA13	.373	.139	.360	.091	.008	.114			
FA14	.289	.083	.300	.145	.021	.148			
FA15	.301	.090	.305	.144	.021	.144			
FA17	.289	.084	.305	.144	.021	.144			
FA19	.168	.028	.119	.871	.758	.650			
FA20	.172	.030	.258	.304	.092	.234			
FA21	.316	.100	.301	.155	.024	.168			
FA25	.492	.242	.451	.033	.001	.062			
FA26	-.053	.003	-.036	.881	.776	.692			
FA27	.041	.002	.067	.888	.789	.696			
FA28	.295	.087	.336	.446	.199	.351			
FA32	.193	.037	.230	.182	.033	.079			
FA33	-.044	.002	-.025	.885	.783	.692			
FA34	.322	.104	.279	.009	.000	.060			
FA35	.463	.214	.428	-.066	.004	.023			
FA38	.547	.299	.482	-.024	.001	.066			
FA39	-.081	.007	-.144	.771	.594	.537			
FA40	-.458	.210	-.186	.781	.610	.709			
FA41	-.116	.013	-.181	.830	.689	.569			
FA43	-.267	.071	-.375	.752	.565	.532			
FA44	-.072	.005	-.038	.867	.751	.648			
FA45	.608	.369	.482	-.388	.151	-.249			
FA47	.543	.295	.153	.630	.397	.567			
FA48	.653	.426	.254	.530	.281	.549			
FA50	.133	.018	.124	.376	.141	.290			
FA52	-.034	.001	.049	.186	.035	.253			
FA53	.028	.001	.008	.566	.320	.417			
FA54	.295	.087	.305	.145	.021	.144			
FA55	.475	.225	.261	-.633	.400	-.526			
FA56	.129	.017	-.052	-.782	.611	-.657			
FA57	.510	.260	.464	-.352	.124	-.266			
FA58	-.084	.007	-.072	.430	.185	.285			
FA59	-.016	.000	-.018	-.761	.579	-.563			
FA60	-.625	.390	-.493	-.198	.039	-.144			
FA61	-.008	.000	-.026	.544	.296	.395			
FA62	-.585	.343	-.318	.510	.261	.655			
FA64	.462	.214	.168	.764	.584	.648			
FA65	.349	.122	.342	-.082	.007	-.009			
FA66	.580	.337	.545	-.130	.017	-.045			
FA67	.301	.091	.305	.143	.021	.144			
FA68	-.009	.000	-.096	.783	.613	.575			
FA69	-.038	.001	-.073	.670	.449	.485			
FA70	.436	.190	.417	-.253	.064	-.172			
FA73	.723	.523	.487	-.359	.129	-.241			
FA78	.356	.126	.330	.143	.020	.179			
FA79	-.058	.003	-.110	.781	.611	.545			
FA81	.552	.305	.488	-.277	.077	-.190			
FA84	.740	.548	.603	-.165	.027	-.062			
FA85	.511	.261	.349	-.305	.093	-.192			
FA87	.688	.473	.589	-.120	.015	.005			

FA88	.241	.058	.199	-.258	.067	-.246
FA89	.457	.209	.405	-.315	.099	-.226
FA90	.527	.278	.423	-.389	.152	-.299
FA91	.518	.268	.380	-.384	.147	-.253
FA93	.680	.462	.533	-.253	.064	-.106
FA94	.107	.011	.162	.366	.134	.345
FA95	.344	.118	.287	-.189	.036	-.104
FA96	.486	.236	.450	-.220	.049	-.130
FA97	.307	.094	.272	-.017	.000	-.005
FA98	.338	.114	.316	-.101	.010	-.055
FA100	.094	.009	.215	.067	.005	.127
FA105	.272	.074	.264	-.082	.007	-.024
FA106	.307	.094	.293	-.229	.052	-.203
FA107	.151	.023	.107	.836	.699	.644
FA108	.392	.154	.360	-.002	.000	.040
FA111	.120	.014	.144	.749	.561	.591
FA112	-.005	.000	-.012	.509	.259	.371
FA113	.264	.070	.297	.037	.001	.003
FA116	.392	.154	.260	.367	.135	.355
FA117	.284	.080	.304	-.140	.020	-.082

fig1aArcSqRt

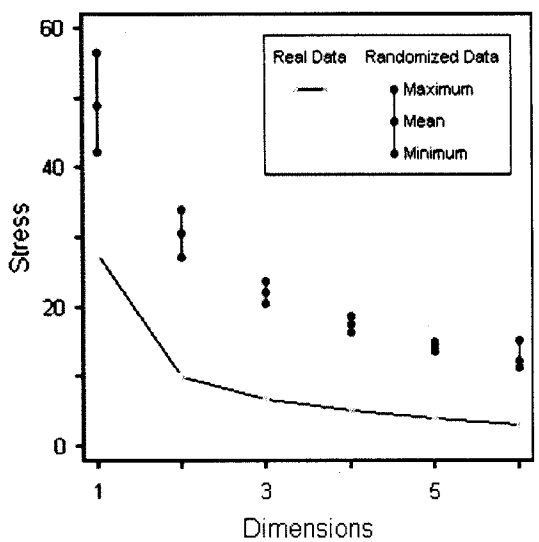
Coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space:

	R Squared	
Axis	Increment	Cumulative
1	.324	.324
2	.594	.918

Increment and cumulative R-squared were adjusted for any lack of orthogonality of axes.

Axis pair	r	Orthogonality,% = 100(1-r^2)
1 vs 2	-0.056	99.7

Number of entities = 52
Number of entity pairs used in correlation = 1326
Distance measure for ORIGINAL distance: Sorensen (Bray-Curtis)



Scree Plot for EL-FAME ordination – Chapter Two

The SAS System
01:19 Monday, October 4, 2004

The GLM Procedure

Dependent Variable: axis2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	30.16565396	1.77445023	32.53	<.0001
Error	34	1.85480693	0.05455314		
Corrected Total	51	32.02046089			

R-Square	Coeff Var	Root MSE	axis2 Mean
0.942074	12145440	0.233566	1.92308E-6

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	17	30.16565396	1.77445023	32.53	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	17	30.16565396	1.77445023	32.53	<.0001

The GLM Procedure

Student-Newman-Keuls Test for axis2

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	34
Error Mean Square	0.054553
Harmonic Mean of Cell Sizes	2.842105

NOTE: Cell sizes are not equal.

Number of Means	2	3	4	5	6
Critical Range	0.3981842	0.4801182	0.5291731	0.5641953	0.5913703
Number of Means	7	8	9	10	11
Critical Range	0.6135284	0.6322047	0.6483252	0.6624917	0.6751165
Number of Means	12	13	14	15	16
Critical Range	0.686495	0.6968454	0.7063338	0.7150892	0.723214

Number of Means	17	18
Critical Range	0.7307906	0.7378864

Means with the same letter are not significantly different.

SNK Grouping			Mean	N	trt
	A		1.3003	3	11 Soil Moist 6 wk
	A				
	A		1.2987	3	41 Soil Saturated Solid 6 wk
	A				
	A		1.1167	3	12 Soil Moist 24 wk
	A				
	A		1.0443	3	42 Soil Saturated Solid 24 wk
	B		0.2810	3	31 Mixture Moist 6 wk
	B				
	B		0.2535	3	61 Mixture Saturated Solid 6 wk
	B				
	B		0.2060	2	92 Mixture Water 24 wk
	B				
	B		0.1348	3	91 Mixture Water 6 wk
	B				
	B		0.0394	3	32 Mixture Moist 6 wk
	B				
C	B		-0.2184	3	62 Mixture Saturated Solid 24 wk
C					
C	D		-0.4817	3	22 Wood Moist 24 wk
C	D				
C	D	E	-0.5760	3	52 Wood Saturated Solid 24 wk
C	D	E			
C	D	E	-0.5779	2	81 Wood Water 6 wk
C	D	E			
C	D	E	-0.5882	3	51 Wood Saturated Solid 6 wk
C	D	E			
C	D	E	-0.6332	3	21 Wood Moist 6 wk
C	D	E			
C	D	E	-0.6782	3	82 Wood Water 24 wk
	D	E			
	D	E	-0.9293	3	71 Soil Water 6 wk
		E			
		E	-1.1155	3	72 Soil Water 24 wk

Appendix I.B Nitrate Reducer Colony Forming Units (NRCFUs) from Jar Microcosms

Nitrate Reducer CFU's
6 week incubation

	Jar #	-2	-2	-3	-3	-4	-4	-5	-5	-6	-6	ln transforms				Jar Avg	Trt Avg	Trt sd	Trt se	Antilog	Exponent
Mixture Moist Solids	48			354	84	85	8					12.78	11.34	13.65	11.29	12.26	12.74	0.56	0.31	341232	3.4×10^5
	30			24	220	480	31					10.09	12.3	15.38	12.64	12.6					
	10			387	366	78	142					12.87	12.81	13.57	14.17	13.35					
Mixture Saturated Solids	3			617	424	97	14					13.33	12.96	13.79	11.85	12.98	12.16	0.99	0.97	191816	1.9×10^5
	49			21	574	7	2					9.95	13.26	11.16	9.9	11.07					
	63			515	16	93	54					13.15	9.68	13.74	13.2	12.44					
Mixture Saturation Water	3			258	181	26	4					12.46	12.11	12.47	10.6	11.91	12.78	0.76	0.57	355161	3.5×10^5
	49			516	447	58	70					13.15	13.01	13.27	13.46	13.22					
	63			587	563	67	40					13.28	13.24	13.42	12.9	13.21					
Soil Moist Solids	60	186	176	10	13							9.83	9.78	9.21	9.47	9.57	9.83	0.23	0.52	18628	1.8×10^4
	34	140	209	34	24							9.55	9.95	10.43	10.09	10					
	32	153	130	54	16							9.64	9.47	10.9	9.68	9.92					
Soil Saturated Solids	17	91	144	6	42							9.12	9.57	6.4	8.34	8.36	9.01	0.83	0.68	8169	8.2×10^3
	19	54	210	64	20							8.59	9.95	8.76	7.6	8.73					
	67	634	399	155	47							11.06	10.59	9.65	8.46	9.94					
Soil Saturation Water	17	17	41	3	0							7.44	8.32	5.7		7.15	6.81	0.46	0.22	909	9.0×10^2
	19	18	23	2	1							7.5	7.74	5.3	4.61	6.28					
	67	38	38	2	5							8.24	8.24	5.3	6.21	7					
Wood Moist Solids	84							8	5	4	0	13.59	13.12	15.2		13.97	13.57	0.48	0.22	785990	7.8×10^5
	100							1	5	2	0	11.51	13.12	14.51		13.05					
	104							2	0	0	4	12.21			15.2	13.7					
Wood Saturated Solids	91							8	13	1	1	13.59	14.08	13.82	13.82	13.83	14.02	0.79	0.62	1223314	1.2×10^6
	105							18	20	5	4	14.4	14.51	15.42	15.2	14.88					
	95							5	1	3	1	13.12	11.51	14.91	13.82	13.34					
Wood Saturation Water	91			55	55	3	7					10.92	10.92	10.31	11.16	10.82	11.49	0.59	0.34	97413	9.7×10^4
	105			95	161	13	27					11.46	11.99	11.78	12.51	11.93					
	95			118	126	12	12					11.68	11.74	11.7	11.7	11.7					

Nitrate Reducer CFU's
24 week incubation

	Jar #	-2	-2	-3	-3	-4	-4	-5	-5	-6	-6	ln transforms				Jar Avg	Trt Avg	Trt sd	Trt se	Antilog	Exponent
Mixture Moist Solids	8			207	82	56	7					12.24	11.31	13.24	11.16	11.99	11.92	0.07	0.005	150242	1.5×10^5
	28			350	25	19	23					12.77	10.13	12.15	12.35	11.85					
	2			27	40	30	158					10.2	10.6	12.61	14.27	11.92					
Mixture Saturated Solids	7			234	91	46	62					12.36	11.42	13.04	13.34	12.54	12.02	0.66	0.44	166043	1.7×10^5
	27			238	271	6	1					12.38	12.51	11	9.21	11.28					
	47			39	45	113	99					10.57	10.71	13.94	13.81	12.26					
Mixture Saturation Water	7			40	36	11	11					10.6	10.49	11.61	11.61	11.08	11.36	0.32	0.1	85819	8.6×10^4
	47			295	52	8	18					12.59	10.86	11.29	12.1	11.71					
	27			137	8	18	20					11.83	8.99	12.1	12.21	11.28					
Soil Moist Solids	56	34	64	13	16							8.13	8.76	9.47	9.68	9.01	8.9	0.43	0.19	7332	7.3×10^3
	22	39	54	4	5							8.27	8.59	8.29	8.52	8.42					
	40	41	89	18	19							8.32	9.09	9.8	9.85	9.27					
Soil Saturated Solids	35	209	103	19	17							9.95	9.24	9.85	9.74	9.7	9.56	0.39	0.16	14618	1.5×10^4
	37	94	85	9	186							9.15	9.05	9.1	12.13	9.86					
	23	90	43	16	11							9.1	8.37	9.68	9.31	9.11					
Soil Saturation Water	23	0	11	2	1								7	7.6	6.91	7.17	6.61	0.64	0.41	743	7.4×10^2
	37	1	5	0	1							4.61	6.21		6.91	5.91					
	35	16	4	1	0							7.38	5.99	6.91		6.76					
Wood Moist Solids	90					96	24	2	7			13.77	12.39	12.21	13.46	12.96	11.9	1.5	2.25	147267	1.5×10^5
	108					1	26	0	0			9.21	12.47			10.84					
	102					0	0	0	0												
Wood Saturated Solids	101					1	4	0	0			9.21	10.6			9.9	11.8	1.69	2.88	133252	1.3×10^5
	79					36	27	37	2			12.79	12.51	15.12	12.21	13.16					
	103					57	24	2	1			13.25	12.39	12.21	11.51	12.34					
Wood Saturation Water	103			21	33	4	1					9.95	10.4	10.6	9.21	10.04	10.74	0.61	0.37	46166	4.6×10^4
	101			256	TNTC	1	10					12.45		9.21	11.51	11.06					
	79			38	72	8	10					10.55	11.18	11.29	11.51	11.13					

APPENDIX I.C Water Agar Colony Forming Units from Jar Microcosms

Water Agar CFUs - Log Transformation		
Treatment	6 wks	24 wks
Mixture Moist	4.77	6.48
Mixture Water	TNTC	5.33
Mixture Saturation Solid	5.31	5.95
Soil Moist	4.42	5.40
Soil Saturated Solid	5.01	3.91
Soil Water	4.53	4.19
Wood Moist	TNTC	6.34
Wood Water	TNTC	5.27
Wood Saturated Solid	TNTC	6.12

Appendix I.D nmol EL-FAME from Jar Microcosms

Treatment	Jar	Total nmole	Sample Size (g or ml)	nmol g or ml	Add 1 Constant	ln nmole	Avg Trt	Trt sd	Trt se	% solids	nmole dry wt
wood control	Saw1	7676.06	1.5	5117.37	5118.37	8.54	8.45	0.13			
wood control	Saw2	6414.69	1.5	4276.46	4277.46	8.36					
soil control	Soil1	39.82	3	13.27	14.27	2.66	2.92	0.37		0.865	11.48
soil control	Soil2	69.50	3	23.17	24.17	3.19				0.865	20.04
6 week incubations											
Mixture Moist Solids	48	459.48	3	153.16	154.16	5.04	5.50	0.51	0.26	0.327	50.08
	30	1271.70	3	423.90	424.90	6.05				0.327	138.62
	10	659.03	3	219.68	220.68	5.40				0.327	71.83
Mixture Saturated Solids	3	1355.56	3	451.85	452.85	6.12	5.67	0.41	0.17	0.305	137.81
	49	791.38	3	263.79	264.79	5.58				0.305	80.46
	63	603.67	3	201.22	202.22	5.31				0.305	61.37
Mixture Saturation Water	3	5.07	20	0.25	1.25	0.23	0.33	0.12	0.01		
	49	7.06	20	0.35	1.35	0.30					
	63	11.75	20	0.59	1.59	0.46					
Soil Moist Solids	60	38.65	3	12.88	13.88	2.63	2.61	0.20	0.04	0.315	4.06
	34	45.99	3	15.33	16.33	2.79				0.315	4.83
	32	30.02	3	10.01	11.01	2.40				0.315	3.15
Soil Saturated Solids	19	46.52	3	15.51	16.51	2.80	2.64	0.15	0.02	0.328	5.09
	67	36.91	3	12.30	13.30	2.59				0.328	4.04
	17	34.05	3	11.35	12.35	2.51				0.328	3.72
Soil Saturation Water	17	10.38	20	0.52	1.52	0.42	0.33	0.21	0.05		
	19	12.52	20	0.63	1.63	0.49					
	67	1.99	20	0.10	1.10	0.09					
Wood Moist Solids	84	5058.34	3	1686.11	1687.11	7.43	7.74	0.27	0.08	0.306	515.95
	100	7753.01	3	2584.34	2585.34	7.86				0.306	790.81
	104	8379.53	3	2793.18	2794.18	7.94				0.306	854.71
Wood Saturated Solids	91	3281.02	3	1093.67	1094.67	7.00	7.20	0.40	0.16	0.287	313.88
	105	3071.09	3	1023.70	1024.70	6.93				0.287	293.80
	95	6387.11	3	2129.04	2130.04	7.66				0.287	611.03
Wood Saturation Water	91	7.50	20	0.38	1.38	0.32	0.27	0.07	0.004		
	95	5.07	20	0.25	1.25	0.23					
24 week incubations											
Mixture Moist Solids	8	195.94	3	65.31	66.31	4.19	4.33	0.51	0.26	0.286	18.68
	28	400.01	3	133.34	134.34	4.90				0.286	38.13
	2	145.34	3	48.45	49.45	3.90				0.286	13.86
Mixture Saturated Solids	7	424.56	3	141.52	142.52	4.96	5.38	0.37	0.14	0.327	46.28
	27	843.42	3	281.14	282.14	5.64				0.327	91.93
	47	761.89	3	253.96	254.96	5.54				0.327	83.05
Mixture Saturation Water	7	5.59	20	0.28	1.28	0.25	0.21	0.05	0.002		
	47	3.92	20	0.20	1.20	0.18					
Soil Moist Solids	56	29.92	3	9.97	10.97	2.40	2.46	0.46	0.21	0.357	3.56
	22	54.38	3	18.13	19.13	2.95				0.357	6.47
	40	19.89	3	6.63	7.63	2.03				0.357	2.37
Soil Saturated Solids	35	34.64	3	11.55	12.55	2.53	2.28	0.25	0.06	0.288	3.33
	37	19.79	3	6.60	7.60	2.03				0.288	1.90
	23	26.48	3	8.83	9.83	2.28				0.288	2.54
Soil Saturation Water	23	0.48	20	0.02	1.02	0.02	0.18	0.22	0.05		
	37	1.82	20	0.09	1.09	0.09					
	35	10.99	20	0.55	1.55	0.44					
Wood Moist Solids	90	3537.80	3	1179.27	1180.27	7.07	6.87	0.24	0.06	0.327	385.62
	108	2209.11	3	736.37	737.37	6.60				0.327	240.79
	102	3070.83	3	1023.61	1024.61	6.93				0.327	334.72
Wood Saturated Solids	101	5884.79	3	1961.60	1962.60	7.58	7.18	0.73	0.53	0.302	592.40
	79	6158.77	3	2052.92	2053.92	7.63				0.302	619.98
	103	1706.47	3	568.82	569.82	6.35				0.302	171.79
Wood Saturation Water	103	13.67	20	0.68	1.68	0.52	0.50	0.04	0.001		
	101	13.68	20	0.68	1.68	0.52					
	79	11.66	20	0.58	1.58	0.46					

APPENDIX I.E. ANOVA TABLES
1. NRCFU ANOVA TABLES – Jar Microcosms

Mixture versus Soil is significant at p=0.003
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Mixture	18	218.95	12.16389	0.53539
Soil	19	176.16	9.271579	14.52904

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	77.32395	1	77.32395	10.00035	0.003228	4.121338
Within Groups	270.6243	35	7.732122			
Total	347.9482	36				

Mixture versus Wood - not significant
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Wood	17	208.65	12.27353	2.196762
Mixture	18	218.95	12.16389	0.53539

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.105098	1	0.105098	0.078379	0.781254	4.139252
Within Groups	44.24982	33	1.340904			
Total	44.35491	34				

Mixture Treatments - no significant difference for time or moisture
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Moist 6 week	3	38.21	12.74	0.31
Moist 24 Week	3	35.76	11.92	0.00
Saturated 6 Week	3	36.49	12.16	0.97
Saturated 24 Week	3	36.08	12.03	0.44
Water 6 Week	3	38.34	12.78	0.57
Water 24 Week	3	34.07	11.36	0.10

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.312761	5	0.862552	2.161394	0.127207	3.105875
Within Groups	4.788867	12	0.399072			
Total	9.101628	17				

Soil Treatments significant difference among treatments
Anova: Single Factor water treaments were lower than solids

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Moist 6 week	3	29.49	9.83	0.05
Moist 24 Week	3	26.70	8.90	0.19
Saturated 6 Week	3	27.03	9.01	0.68
Saturated 24 Week	3	28.67	9.56	0.16
Water 6 Week	3	20.43	6.81	0.22
Water 24 Week	3	19.84	6.61	0.41

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	29.12427	5	5.824853	20.4349	1.72E-05	3.105875
Within Groups	3.420533	12	0.285044			
Total	32.5448	17				

Soil solids versus water significant at p =0.04

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Solids	13	135.89	10.45308	16.89499
Water	6	40.27	6.711667	0.263337

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	57.46609	1	57.46609	4.787514	0.042922	4.451322
Within Groups	204.0566	17	12.00333			
Total	261.5227	18				

Soil Treatments Block for Time is not significant
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
6 Weeks	9	76.95	8.55	2.07
24 Weeks	9	75.21	8.36	1.98

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1682	1	0.1682	0.083122	0.776812	4.493998
Within Groups	32.3766	16	2.023538			
Total	32.5448	17				

Wood Treatments are significantly different
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Moist 6 week	3	40.72	13.57	0.22
Moist 24 Week	2	23.80	11.90	2.25
Saturated 6 Week	3	42.05	14.02	0.62
Saturated 24 Week	3	35.40	11.80	2.88
Water 6 Week	3	34.45	11.48	0.34
Water 24 Week	3	32.23	10.74	0.37

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	24.03352	5	4.806704	4.757115	0.014696239	3.203874
Within Groups	11.11467	11	1.010424			
Total	35.14819	16				

Wood Treatments block for time is significant at p=0.02
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
6 Weeks	9	117.22	13.02444	1.669278
24 Weeks	8	91.43	11.42875	1.572841

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.78408	1	10.78408	6.639322	0.02105277	4.543077
Within Groups	24.36411	15	1.624274			
Total	35.14819	16				

Wood Treatments Solids versus Water is significant at p=0.01
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Solids	11	141.97	12.90636	2.041425
Water	6	66.68	11.11333	0.450467

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12.4816	1	12.4816	8.259911	0.011589414	4.543077
Within Groups	22.66659	15	1.511106			
Total	35.14819	16				

APPENDIX I.E ANOVA TABLES
2. nmol EL-FAME ANOVA TABLES – Jar Microcosms

Mixture versus Wood is not significantly different
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Wood	17	89.03419	5.237305	10.49418
Mixture	17	64.0428	3.767223	5.686447

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18.3697	1	18.3697	2.27058	0.14166	4.149097
Within Groups	258.89	32	8.090312			
Total	277.2597	33				

Mixture versus Soil is significant at p=0.003
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Mixture	17	64.0428	3.767223	5.686447
Soil	18	31.48842	1.749357	1.244269

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	35.59904	1	35.59904	10.47631	0.002752	4.139252
Within Groups	112.1357	33	3.398052			
Total	147.7348	34				

Mixture Solids versus Water is significant at p=7.38E-11
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Solid	12	62.62695	5.218913	0.450417
Water	5	1.415843	0.283169	0.011638

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	85.98201	1	85.98201	257.887	7.38E-11	4.543077
Within Groups	5.001145	15	0.33341			
Total	90.98316	16				

Soil Solids versus Water is significant p=1.4E-11
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Solid	12	29.94	2.495	0.082955
Water	6	1.548421	0.258	0.04493

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	20.015421	1	20.01542	281.6225	1.4E-11	4.493998
Within Groups	1.1371489	16	0.071072			
Total	21.15257	17				

Wood Solids versus Water is significant p=1.24E-14
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Solids	12	86.98	7.248333	0.257652
Water	5	2.05	0.410838	0.016976

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	165.0047	1	165.0047	852.8642	1.24E-14	4.543077
Within Groups	2.902069	15	0.193471			
Total	167.9068	16				

Wood Block for Time is not significant

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
6 Weeks	8	45.37	5.6713	11.22561
24 Weeks	9	43.66	4.8516	10.81025

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.845544	1	2.845544	0.25859	0.618488	4.543077
Within Groups	165.0613	15	11.00408			
Total	167.9068	16				

Soil Block for Time is not significant
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
6 Weeks	9	16.72	1.857778	1.333969
24 Weeks	9	14.77	1.640936	1.283653

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.2115923	1	0.211592	0.161668	0.692946	4.493998
Within Groups	20.940978	16	1.308811			
Total	21.15257	17				

Mixture Block for Time is not significant
Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
6 Weeks	9	34.48695	3.8319	7.0156
24 Weeks	8	29.55584	3.6945	4.968342

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.079961	1	0.079961	0.013194	0.910074	4.543077
Within Groups	90.9032	15	6.060213			
Total	90.98316	16				

APPENDIX I.E ANOVA TABLES
3. ANOVA TABLES – Syringe Microcosm Cell Counts (ln) g^{-1} or ml^{-1}

A. Syringe Solids	Wood Nitrate+	Wood Nitrate-	No Wood Nitrate+	No Wood Nitrate-
Mean	9.09	9.11	5.78	5.43
Standard Error	0.64	0.45	0.39	0.52
Standard Deviation	0.91	0.78	0.68	0.90
Sample Variance	0.82	0.61	0.46	0.82

B. Syringe Water	Wood Nitrate+	Wood Nitrate-	No Wood Nitrate+	No Wood Nitrate-
Mean	8.61	8.07	7.48	6.65
Standard Error	0.28	0.25	0.11	0.28
Standard Deviation	0.40	0.43	0.20	0.48
Sample Variance	0.16	0.19	0.04	0.23

Syringe Microcosms Block for Wood Difference is significant

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
wood	10	86.94	8.694	0.508937778
no wood	12	76.03	6.335833333	0.970517424

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	30.3324547	1	30.3324547	39.76428017	3.72E-06	4.351243
Within Groups	15.25613167	20	0.762806583			
Total	45.58858636	21				

Syringe Microcosms Block for Nitrate Difference is not significant

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Nitrate	10	75.16	7.516	2.040271111
No Nitrate	12	87.81	7.3175	2.455565909

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.214921364	1	0.214921364	0.094733967	0.761425	4.351243
Within Groups	45.373665	20	2.26868325			
Total	45.58858636	21				

Syringe Microcosms Solids versus Water Difference is not significant

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Solids	11	79.15	7.195454545	3.810167273
Water	11	83.82	7.62	0.64956

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.991313636	1	0.991313636	0.444562448	0.512548	4.351243
Within Groups	44.59727273	20	2.229863636			
Total	45.58858636	21				

Appendix I.F Subsurface Clay Soil Analyses Results

Texture	Clay
pH	7.8
EC (mmhos/cm)	1.7
% OM	1.9
NO_3^-N (pppm)	15.7
NO_2^-N (pppm)	<0.01
NH_4^-N (ppm)	1.11
Phosphorus (ppm)	2.5
Potassium (ppm)	162
Zinc (ppm)	0.99
Iron (ppm)	7.9
Manganese (ppm)	1.6
Copper (ppm)	2
Boron (ppm)	0.07
Molybdenum (ppm)	0.05
Cadmium (ppm)	0.05
Lead (ppm)	1.5
SO_4^-S (ppm)	255

Appendix I.G Regression Statistics
1. Flow-Through Columns

Regression Statistics	
Multiple R	0.762677257
R Square	0.581676599
Adjusted R Squ	0.567732486
Standard Error	3.705204094
Observations	32

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	572.6838787	572.6839	41.71485	3.8977E-07
Residual	30	411.8561213	13.72854		
Total	31	984.54			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*Upper 95.0%*
Intercept	32.11144346	1.502901058	21.36631	1.05E-19	29.04211318	35.18077	29.04211	35.18077373
X Variable 1	-0.0023339	0.000361357	-6.458703	3.9E-07	-0.003071889	-0.001596	-0.003072	-0.00159591

Control sample#	**RepA Nitrate**	**RepB Nitrate**	**RepA TOC**	**RepB TOC**	**RepA DAPI**	**RepB DAPI**
1					69	
2				22	657	894
3				13.96	494	1123
4				6.72	308	1734
5				5.08	596	2293
6			9.44	8.14	757	1470
7			3.62		2251	
8			2.62	2.66	1200	1303
9			3.4	1.42	1483	1367
10			3.14	1.3	1425	959

Trt1 sample#	**RepA Nitrate**	**RepB Nitrate**	**RepA TOC**	**RepB TOC**	**RepA DAPI**	**RepB DAPI**	**Avg Nitrate**	**Avg TOC**	**Avg DAPI**
1					19				
2	28.5	24.5		8.04	825	2765	26.5		1795
3	30.2	19.2		5.44	905	3470	24.7		2188
4		12.2		3.92	1529	6436			3983
5	27.7	10		4.32	1724	8093	18.85		4909
6	29	19.1	8.88	6.52	1770	5012	24.05	7.7	3391
7	26.9		2.82		3334				
8	28	22.9	1.78	1.56	2484	2201	25.45	1.67	2343
9	27.4	20.9	1.92	1.1	3837	4105	24.15	1.51	3971
10	23.9	18.9	2.42	1.14	6095	4224	21.4	1.78	5160

Trt 2 sample#	**RepA Nitrate**	**RepB Nitrate**	**RepA TOC**	**RepB TOC**	**RepA DAPI**	**RepB DAPI**	**Avg Nitrate**	**Avg TOC**	**Avg DAPI**
1					113				
2	26.6	25.9		8.12	1800	4519	26.25		3160
3	28.9	19.5		4.52	1660	3845	24.2		2753
4	27.8	11.6		4.18	2134	5967	19.7		4051
5	29.7	6.3		4.74	2695	5832	18		4264
6	28.1	13.2	4.56	5.8	2794	7330	20.65	5.18	5062
7	26.4 NS		2.44		2213				
8	25.7	21	1.84	1.82	4530	4440	23.35	1.83	4485
9	28	21	1.8	1.18	5728	4139	24.5	1.49	4934
10	24.8	20.5	1.82	1.2	5433	3277	22.65	1.51	4355

Appendix I.G Regression Statistics
2. Syringe Microcosms

Regression Statistics	
Multiple R	0.811381963
R Square	0.65834069
Adjusted R Squ	0.641257724
Standard Error	3.103431145
Observations	22

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	371.1689704	371.169	38.53785	4.60212E-06
Residual	20	192.6256974	9.631285		
Total	21	563.7946678			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*ower 95.0%*	*Upper 95.0%*
Intercept	19.35735579	0.84127359	23.00958	7.32E-16	17.60249065	21.11222	17.60249	21.11222093
# cells/ml	-0.002225423	0.000358483	-6.207886	4.6E-06	-0.002973205	-0.001478	-0.002973	-0.00147764

Saturated Syringe Permeable Barrier Microcosms

Treatment 1: sand, sawdust, river sediment & nitrate
Treatment 2: no nitrate control for 1
Treatment 3: sand, river sediment & nitrate
Treatment 4: no nitrate control for 3

Experiment #1

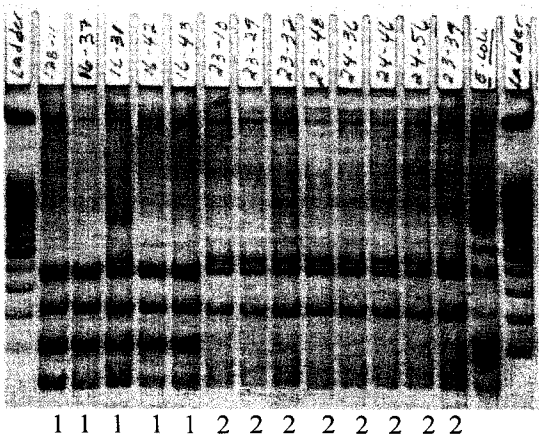
Starting conditions:
blank: pH=6.93, alk=23
Nitrate spike: pH=6.94, alk=24, NO3=19

Trt	Time	pH	NO3	NO2	DAPI # cells/ml
1	0	5.25	18.70	<0.03	7
1	0	5.77	18.93	<0.03	0
1	0	5.28	19.24	<0.03	0
1	24	5.09	15.91	0.70	1057
1	24	5.06	17.53	1.06	654
1	24	5.01	15.46	<0.03	779
1	48				16432
1	48	5.07	16.05	<0.03	2746
1	48	5.14	15.73	<0.03	5303
1	70	4.97			n/a
1	70	5.09	<0.045	<0.03	7275
1	70	5.23	1.47	1.47	4102
2	0	5.82	<0.045	<0.03	9
2	0	5.14	<0.045	<0.03	15
2	0	5.37	<0.045	<0.03	0
2	70	5.03	<0.045	<0.03	3863
2	70	5.10	<0.045	<0.03	4349
2	70	5.00	<0.045	<0.03	1952
3	0	7.25	17.48	<0.03	128
3	0	7.17	21.39	<0.03	167
3	0	7.21	17.51	<0.03	128
3	24	6.97	17.51	<0.03	327
3	24	7.03	17.51	<0.03	841
3	24	7.01	19.17	<0.03	367
3	48	7.05	19.01	<0.03	384
3	48	7.11	18.23	<0.03	948
3	48	7.14	17.87	<0.03	1303
3	70	7.06	17.05	<0.03	2036
3	70	7.09	16.09	<0.03	1405
3	70	7.14	17.05	<0.03	1929
4	0	7.31	<0.045	<0.03	49
4	0	7.21	<0.045	<0.03	76
4	0	7.37	<0.045	<0.03	61
4	70	7.02	<0.045	<0.03	448
4	70	7.18	<0.045	<0.03	1004
4	70	7.13	<0.045	<0.03	1038

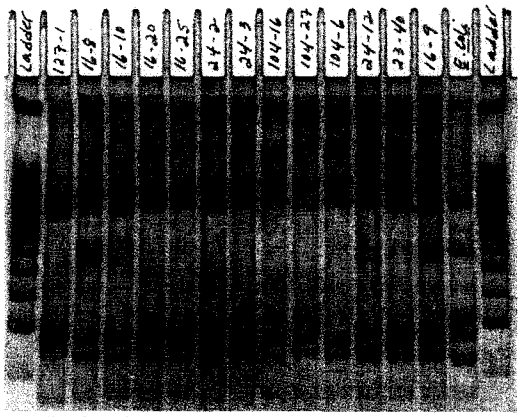
Trt	Time	NO3	DAPI # cells/ml
1	0	18.70	7
1	0	18.93	0
1	0	19.24	0
1	24	15.91	1057
1	24	17.53	654
1	24	15.46	779
1	48	16.05	2746
1	48	15.73	5303
1	70	0.00	7275
1	70	1.47	4102
3	0	17.48	128
3	0	21.39	167
3	0	17.51	128
3	24	17.51	327
3	24	17.51	841
3	24	19.17	367
3	48	19.01	384
3	48	18.23	948
3	48	17.87	1303
3	70	17.05	2036
3	70	16.09	1405
3	70	17.05	1929

APPENDIX II

Appendix II.A RFLP Patterns 1 - 14



1 1 1 1 1 2 2 2 2 2 2 2 2



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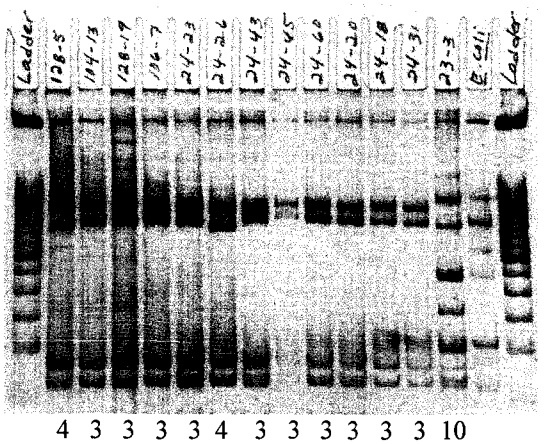
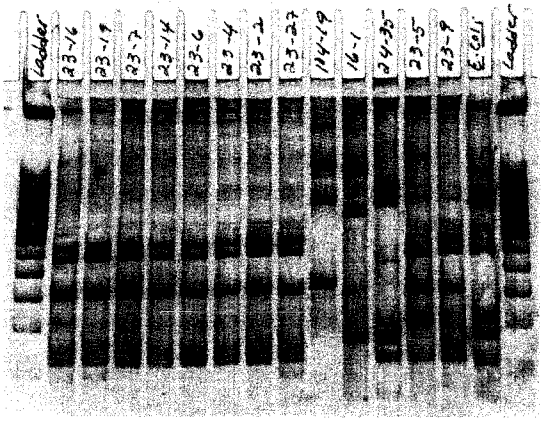
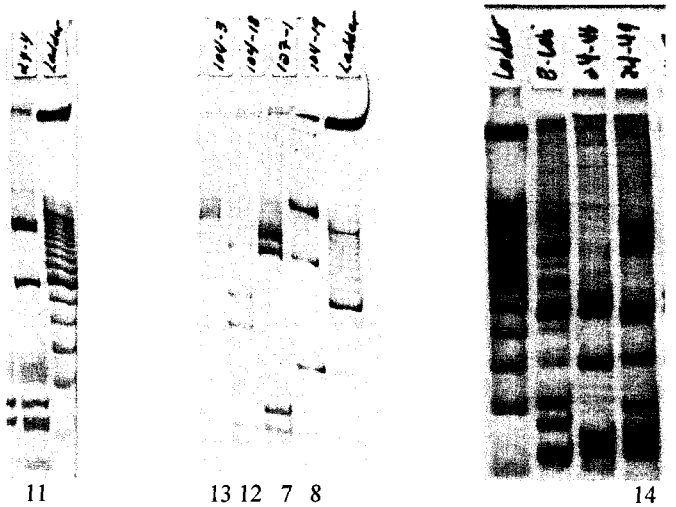


4 3 3 3 3 4 3 3 3 3 3 3 10



2 2 2 2 2 2 2 2 8 9 3 1 2



11 13 12 7 8 14

Note: The presence of extra bands in some of the #3 patterns is artifactual and due to cross-contamination during gel loading.

APPENDIX II.B 24 Week Denitrifying Isolates by RFLP Pattern

RFLP Pattern	Isolates from 24 week Mixture Treatments
1	16-37, 16-31, 16-42, 16-43, 23-5
2	23-2, 23-4, 23-6, 23-7, 23-9, 23-10, 23-14, 23-16, 23-19, 23-27, 23-29, 23-32, 23-34,23-39, 23-42, 23-48, 24-36, 24-46, 24-56,
3	16-8, 16-20, 16-25, 24-3, 24-12, 24-23, 24-43, 24-45, 24-60, 24-20, 24-18, 24-31, 24-35, 23-31
4	16-10, 24-2, 23-40,16-9,24-26,
9	16-1
10	23-3
11	24-4
14	24-49

23 = saturation water
24 = moist solids
16 = saturated solids

Appendix II.C Isolate Partial 16S rRNA Gene Sequences

1. Jar Microcosm Denitrifiers

>106_23 Mixture moist solids 6
TGGAGAGTTTGATCCTGGCTCAGAATGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGA
GAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGACGCTAATACCGCATACGTCCTA
CGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC
GACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAkCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG
CATTAACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>106_02 Mixture moist solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTG
GCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCAGTTACCTAATACGTAATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>106_07 Mixture moist solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTG
GCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCAGTTACCTAATACGTAwyTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>128_05 Mixture saturated Solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGA
GAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTA
CGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGC
GACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG
TTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>128_19 Mixture saturation water 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTG
GCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>128_11 Mixture saturation water 6
TGGAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGAGGGCTTCGGCCCTAGTGGCGCACG
GGTGAGTAACACGTGGGAACCTGCCTTATGGTTCGGGATAACGTCTGGAAACGGACGCTAACACCGGATGTGCCCTTCGGGGGAAAGTT
TACGCCATGAGAGGGGCCCGCGTCCGATTAGGTAGTTGGTGGGGTAATGGCCCACCAAGCCGACGATCGGTAGCTGGTCTGAGAGGATG
ATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAG
CAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGCACGCGACGATGATGACGGTAGCGTGAGAAGAAGCCCCGGCT
AACTTCGTGCCAGCAGCCGCGGTA

>126_11 Soil moist solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGA
GAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTA
CGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGC
GACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG
TTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>126_19 Soil moist solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTG
GCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>126_07 Soil moist solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTG
GCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCAGTTACCTAATACGTAwCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>127_01 Soil saturated solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTAGCGGGATGCCTTACACATGCAAGTCGAACGGCAGCACGGACTTCGGTCTGGTGGC
GAGTGGCGAACGGGTGAGTAATGTATCGGAACGTGCCTAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTA
CGGGGGAAAGCAGGGGATCGCAAGACCTTGCACTATTAGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAyGGCTCACCAAGGC
GACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATT
TTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTTTGGCAGGAAAGAAACG
TCATGGGCTAATACCCCGTGAAACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTA

>127_10 Soil saturated solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGA
GAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCCTA
CGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC
GACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG
CATTAACCTAATACGTTGGTGTCTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>127_03 Soil saturated solids 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTG
GCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGyCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCATTTACCTAATACGTAAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>104_21_Soil saturation water 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTAGCGGGATGCCTTACACATGCAAGTCGAACGGCAGCACGGACTTCGGTCTGGTGGC
GAGTGGCGAACGGGTGAGTAATGTATCGGAACGTGCCTAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTA
CGGGGGAAAGCAGGGGATCGCAAGACCTTGCACTATTAGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAyGGCTCACCAAGGC
GACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATT
TTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTTTGGCAGGAAAGAAACG
TCATGGGCTAATACCCCGTGAAACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTA

>104_6 Soil saturation water 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGTAACAGGTCTTCGGATGCTGACGA
GTGGCGAACGGGTGAGTAATACATCGGAACGTGCCCGATCGTGGGGGATAACGCAGCGAAAGCTGTGCTAATACCGCATACGATCTACG
GATGAAAGCGGGGGATCGCAAGACCTCGCGCGGACGGAGCGGCCGATGGCAGATTAGGTAGTTGGTGGGATAAAAGCTTACCAAGCCGA
CGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTT
GGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAAGT
CTTCTCTAATACAGGGGGCGAATGACGGTACCGTAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTA

>104_27 Soil saturation water 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGCAGCACGGAGCTTGCTCTGGTGGC
GAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCCTAGAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATACGATCTA
AGGATGAAAGTGGGGGATCGCAAGACCTCATGCTCGTGGAGCGGCCGATATCTGATTAGCTAGTTGGTAGGGTAAAAGCCTACCAAGGC
ATCGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATT
TTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGAAACG
GTGAGAGCTAATATCTCTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTA

>104_19 Soil saturation water 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGCAGCACGGGAGCAATCCTGGTGGC
GAGTGGCGAACGGGTGAGTAATGTATCGGAACGTACCTTTCAGTGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATTCTGTG
AGCAGGAAAGCAGGGGATCGCAAGACCTTGCGCTGATTGAGCGGCCGATATCAGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGC
GACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATT
TTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCGGCCGGGAAGAAATC
GCATGGGTTAATACCCT

>104_18 Soil saturation water 6
TGGAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGG
TGAGTAACGCGTGGGAATCTACCCTTTTCTACGGAATAACGCATGGAAACGTGTGCTAATACCGTATGAGCCCTTCGGGGGAAAGATTT
ATCGGGAAAGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATG
ATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAG
CCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCT
AACTTCGTGCCAGCAGCCGCGGTA

>104_16 Soil saturation water 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGAGAGGTGCTTGCACCTCTTGA
GAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCCTA
CGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC
GACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG
CAGTTACCTAATACGTATCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>104_13 Soil saturation water 6
TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTG
GCGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGCTCGGAAACGGACGCTAATACCGCATACGTCC
TACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCAGTTACCTAATACGTAwyTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>104_10 Soil saturation water 6
TGGAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGCCCCGCAAGGGGAGCGGCAGACGGG
TGAGTAACGCGTGGGAATCTACCCTTTTCTACGGAATAACGCAGGGAAACTTGTGCTAATACCGTATACGCCCTTCGGGGGAAAGATTT
ATCGGGAAAGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATG
ATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAG
CCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCT
AACTTCGTGCCAGCAGCCGCGGTA

>104_03 Soil saturation water 6

TGGAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTwGAsAGGAGCTTGCTyCTswTGw
sAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTrGTrGTGGGGGAyAACGTTysGAAAsGrACGCTAATACCGCATACGTCCTA
CGGGAGAAAGCrGGGGACCTTCGGGCCTyGCGCyATyAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAAyGGCTCACCAAGGC
GACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGmAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG
CAGTAAsCkAATACsTTrsTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA

>108_19 Wood saturated solids 6

TGGAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGCCTCGCAAGAGGAGCGGCAGACGGG
TGAGTAACACGTGGGAATCTACCCATCCCTGCGGAACAACTCCGGGAAACTGGAGCTAATACCGCATACGCCCTACGGGGGAAAGATTT
ATCGGGGATGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATG
ATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAG
CCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGATGAAGATAATGACGGTAGTCGGAGAAGAAGCCCCGGCT
AACTTCGTGCCAGCAGCCGCGGTA

>108_17 Wood saturated solids 6

TGGAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGCCTCGCAAGAGGAGCGGCAGACGGG
TGAGTAACACGTGGGAATCTACCCATCCCTGCGGAACAACTCCGGGAAACTGGAGCTAATACCGCATACGCCCTACGGGGGAAAGATTT
ATCGGsGATGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAmGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATG
ATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAG
CCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGATGAAGATAATGACGGTAGTCGGAGAAGAAGCCCCGGCT
AACTTCGTGCCAGCAGCCGCGGTA

>24_36 Mixture moist solids 24

GGGCAGTGGCGCACGGGTGAGTAACACGTGGGAATCTGCCTTTCGGTTCGGAATAACGTCTGGAAACGGACGCTAACACCGGATACGCC
CCCCAGCGATGGGTGGGGAAAGTTCACGCCGAGAGAGGAGCCCGCGTCCGATTAGGTAGTTGGTGAGGTAATGGCTCACCAAGCCTGCG
ATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGTCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
GACAATGGGCGCAAGCATGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGCACGTGACGATGATGAC
GGTAACGTGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTA
AAGGGCGCGTAGGCGGCCTGTTTAGTCGGAAGTGAAAGCCCCGGGCTCAACCTGGGAATTGCTTTCGATACTGGCAGGCTTGAGTTCCG
GAGAGGATGGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCCATCTGGACGGACAC
TGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGACGTCGGGGT
GCATGCACTTCGGTGTCGCCGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAACCCTTGACATGTCCACTATCGGCACCAGAGA
TGGTGCTTTCGGTTCGGCCCGGGTGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCCTACTGCCAGTTGCCATCATTCAGTTGGGCACTCTGGTGGAACTGCCGGTGACAAGCCGGAGGAAGGCGGGGATGACG
TCAAGTCCTCATGGCCCTTATGGGTTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGACGCGAAGCCGCGAGGTGGAGCCAATC
CCCAAAAGCCGTCTCAGTTCGGATCGCACTCTGCAACTCGAGTGCGTGAAGTTGGAATCGCTAGTAATCGCGGATCAGCACGCCGC

>24_56 Mixture moist solids 24

GGGTCAGTGGCGCACGGGTGAGTAACACGTGGGAATCTGCCTTTCGGTTCGGAATAACGTCTGGAAACGGACGCTAACACCGGATACGC
CCCCCAGCGATGGGTGGGGAAAGTTCACGCCGAGAGAGGAGCCCGCGTCCGATTAGGTAGTTGGTGAGGTAATGGCTCACCAAGCCTGC
GATCCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGCACGTGACGATGATGA
CGGTAACGTGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGT
AAAGGGCGCGTAGGCGGCCTGTTTAGTCGGAAGTGAAAGCCCCGGGCTCAACCTGGGAATTGCTTTCGATACTGGCAGGCTTGAGTTCC
GGAGAGGATGGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCCATCTGGACGGACA
CTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGACGTCGGGG
TGCATGCACTTCGGTGTCGCCGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAACCCTTGACATGTCCACTATCGGCACCAGAG
ATGGTGCTTTCGGTTCGGCCGGGTGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCCTACTGCCAGTTGCCATCATTCAGTTGGGCACTCTGGTGGAACTGCCGGTGACAAGCCGGAGGAAGGCGGGGATGACG
TCAAGTCCTCATGGCCCTTATGGGTTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGACGCGAAGCCGCGAGGTGGAGCCAATC
CCCAAAAGCCGTCTCAGTTCGGATCGCACTCTGCAACTCGAGTGCGTGAAGTTGGAATCGCTAGTAATCGCGGATCAGC

>23_7 Mixture saturation water 24

GTGGCGCACGGGTGAGTAACACGTGGGAACCTGCCTTTCGGTTCGGAATAACGTCTGGAAACGGACGCTAACACCGGATACGTCCCCCA
AGCGATTGGGTGGAGAAAGTTTACGCCGAGAGAGGGGCCCGCGTCCGATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGATC
GGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGCACGTGACGATGATGACGGTA
ACGTGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGG
GCGCGTAGGCGGTCCGCTCAGTCAGAAGTGAAAGCCCCGGGCTCAACCTGGGAATTGCTTTTGATACTGGTGGACTTGAGTTCCGGAGA
GGATGGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCCATCTGGACGGACACTGAC
GCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGACGTCGGGGTGCAT
GCACTTCGGTGTCGCCGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAACCCTTGACATGTCCACAGTCGGCTTCAGAGATGGA
GCTTTCGGTTCGGCCGGGTGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCCTACCGCCAGTTGCCATCATTCAGTTGGGCACTCTGGTGGAACTGCCGGTGACAAGCCGGAGGAAGGCGGGGATGACGTCAAG
TCCTCATGGCCCTTATGGGTTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGATGCGAAGCCGTGAGGTGGAGCCAATCCCCAA
AAGCCGTCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGC

>23_2 Mixture saturation water 24

GGCCAGTGGCGCACGGGTGAGTAACACGTGGGAACCTGCCTTTCGGTTCGGAATAACGTCTGGAAACGGACGCTAACACCGGATACGTC
CCCCAAGCGATTGGGTGGAGAAAGTTTACGCCGAGAGAGGGGCCCGCGTCCGATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGA
CGATCGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCGCACGCGACGATGATGA
CGGTAACGTGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGT
AAAGGGCGCGTAGGCGGTCCGCTCAGTCAGAAGTGAAAGCCCCGGGCTCAACCTGGGAATTGCTTTTGATACTGGTGGACTTGAGTTCC
GGAGAGGATGGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCCATCTGGACGGACA
CTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGACGTCGGGG
TGCATGCACTTCGGTGTCGCCGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAACCCTTGACATGTCCACAGTCGGCTTCAGAG
ATGGAGCTTTCGGTTCGGCCGGGTGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCCTACCGCCAGTTGCCATCATTCAGTTGGGCACTCTGGTGGAACTGCCGGTGACAAGCCGGAGGAAGGCGGGGATGACG
TCAAGTCCTCATGGCCCTTATGGGTTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGATGCGAAGCCGTGAGGTGGAGCCAATC
CCCAAAAGCCGTCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCACGCC

>16_1 Mixture saturated solids 24

GGTGAGTAATACATCGGAACGTGCCCTGTAGTGGGGGATAACTAGTCGAAAGATTAGCTAATACCGCATACGACCTGAGGGTGAAAGTG
GGGGACCGCAAGGCCTCATGCTATAGGAGCGGCCGATGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCCACCAAGGCGACGATCAGTAG
CTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGG
CGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATGGCTCTGGTTAAT
ACCTGGGGTCGATGACGGTACCGGAAGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTNCCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGACCGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTGGTGACT
GCACGGCTAGAGTGTGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCA
GCCCCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGT
CAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
AAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCA
CTAACGAAGCAGAGATGCATTAGGTGCTCGAAAGAGAAAGTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCNCTTGTCTCTAGTTGCTACGAAAGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTGCATACAGAGGGTTGCCAAGCCGCGAG
GTGGAGCTAATCCCAGAAAATGCATCGTAGTCCGGATCGTAGTCTGCAACTCGACTACGTGAAGC

>24_4 Mixture moist solids 24

GCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGACGCTAATACCG
CATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGG
CTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTNGAGACACGGTCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCCTCGGATTGTAAAGCACTTTAAGT
TGGGAGGAAGGGCATTAACCTANATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGT
AATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTC
AACCTGGGAACTGCATTCAAAACTGACAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAG
GAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAG
GAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGG
TCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTG
AAGTCGGAATCGCTAGTAATCGCGAATCAGA

>24_29 Mixture moist solids 24

GCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGACGCTAATACCG
CATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGG
CTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTT
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2. Jar Microcosm Isolates - Other Nitrate Reducers

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>**127_12**
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>**104-17**
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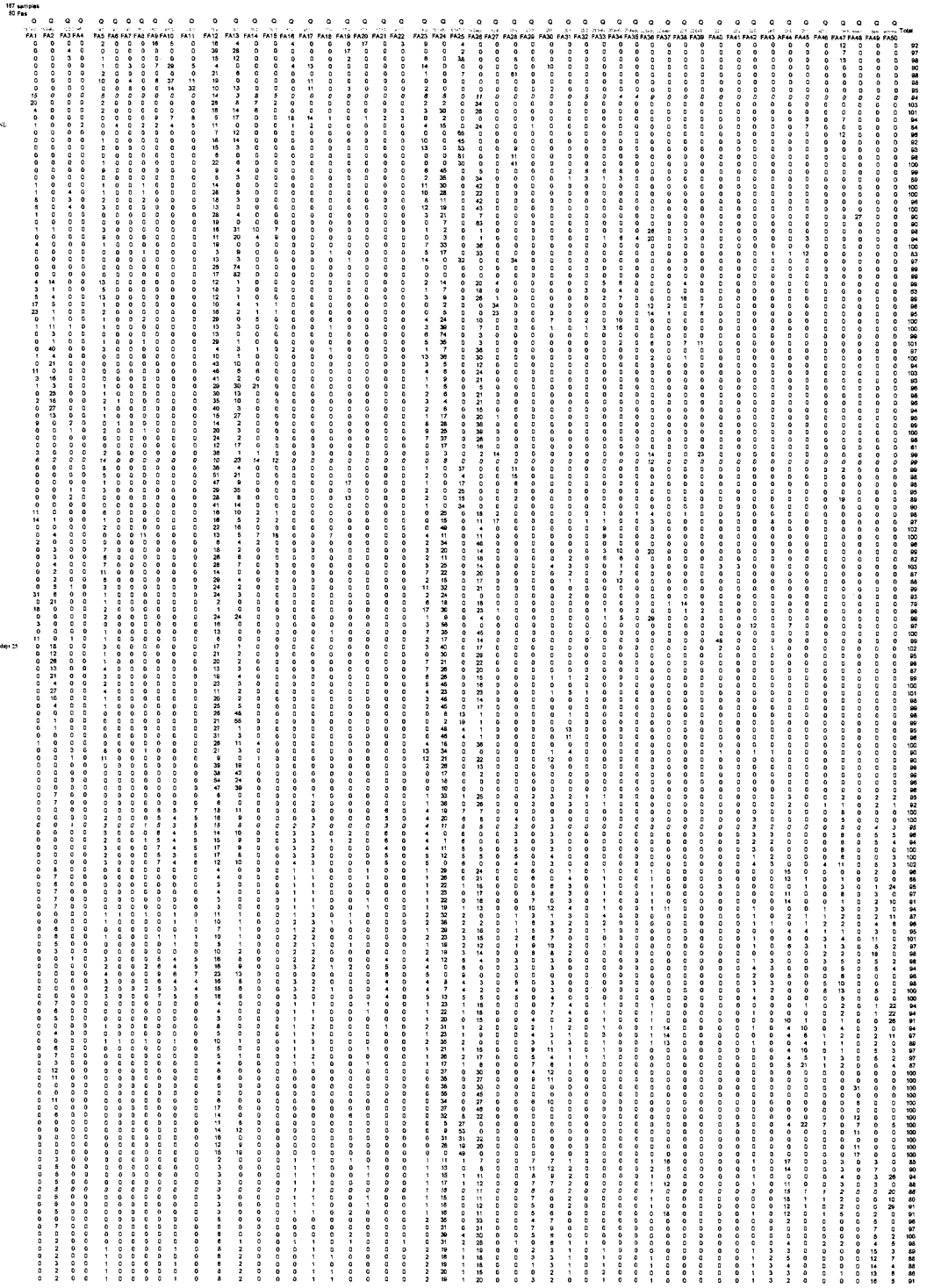
APPENDIX III

Appendix III.A Fatty Acid Database



Appendix III.B NMS Ordination and Cluster Analysis

********** Data Summarization **********
PC-ORD, Version 4.20
20 Sep 2005, 11:55

FinalDendroStats

Summary of 167 samples N = 50 Fas

Num.	Name	Mean	Stand.Dev.	Sum	Minimum	Maximum	S	E	H	D`
1	Desulfov	1.840	4.386	92.000	0.000	17.000	11	0.907	2.176	0.8686
2	ThioDeni	1.940	7.107	97.000	0.000	39.000	6	0.799	1.431	0.7170
3	ThioFerr	1.960	6.233	98.000	0.000	38.000	9	0.814	1.788	0.7818
4	Staphylo	1.800	5.071	90.000	0.000	29.000	10	0.859	1.977	0.8244
5	Lactobac	1.960	9.102	98.000	0.000	61.000	6	0.620	1.111	0.5573
6	Listeria	1.960	6.279	98.000	0.000	37.000	7	0.879	1.711	0.7788
7	Legionel	1.900	5.552	95.000	0.000	32.000	9	0.859	1.888	0.8126
8	EuGracHe	1.880	3.745	94.000	0.000	15.000	13	0.951	2.440	0.9022
9	CvulgAu	2.060	6.622	103.000	0.000	34.000	9	0.782	1.719	0.7775
10	CvulgHe	2.020	6.209	101.000	0.000	30.000	8	0.843	1.753	0.7948
11	Sporocyt	1.880	4.331	94.000	0.000	18.000	13	0.880	2.257	0.8760
12	Streptom	1.686	4.307	84.310	0.000	23.990	20	0.761	2.279	0.8521
13	Azospiri	1.920	9.452	96.000	0.000	65.000	4	0.703	0.975	0.5050
14	Caulobac	1.840	7.063	92.000	0.000	45.000	6	0.786	1.409	0.6912
15	Prosteco	1.860	7.980	93.000	0.000	53.000	5	0.762	1.227	0.6193
16	Nitrobac	1.960	11.540	98.000	0.000	81.000	3	0.522	0.574	0.3005
17	Agrobact	2.000	7.696	100.000	0.000	41.000	5	0.792	1.275	0.6898
18	Acantham	1.988	6.760	99.400	0.000	45.400	10	0.797	1.834	0.7534
19	Physarum	1.778	6.884	88.900	0.000	35.000	10	0.634	1.460	0.6862
20	Fusarium	2.000	7.484	100.000	0.000	41.600	7	0.717	1.395	0.7055
21	F_solani	2.000	6.371	100.000	0.000	28.100	9	0.770	1.692	0.7811
22	Neurospo	1.926	6.703	96.300	0.000	42.300	9	0.766	1.684	0.7426
23	Penicill	2.000	6.973	100.000	0.000	43.100	7	0.824	1.604	0.7418
24	Clavicep	1.800	6.101	90.000	0.000	27.800	8	0.752	1.564	0.7548
25	Chaetomi	1.805	9.347	90.230	0.000	63.480	4	0.581	0.805	0.4542
26	Navicula	1.960	6.170	98.000	0.000	31.000	11	0.737	1.767	0.7858
27	Astrione	1.880	4.525	94.000	0.000	20.000	13	0.869	2.230	0.8665
28	Rhizocto	2.000	7.280	100.000	0.000	35.800	6	0.791	1.418	0.7203
29	Lactariu	1.656	5.504	82.800	0.000	33.000	16	0.637	1.765	0.7635
30	Achromob	1.940	6.950	97.000	0.000	34.000	6	0.802	1.437	0.7285
31	Nitrosol	1.980	10.978	99.000	0.000	74.000	2	0.815	0.565	0.3775
32	Nitrosom	1.980	11.795	99.000	0.000	82.000	2	0.662	0.459	0.2845
33	Ochromon	1.980	4.569	99.000	0.000	20.000	12	0.899	2.234	0.8756
34	Poterioc	1.260	3.746	63.000	0.000	18.000	10	0.826	1.903	0.8068
35	O_malham	1.980	5.020	99.000	0.000	26.000	12	0.858	2.131	0.8540
36	Cryptomo	1.910	5.726	95.500	0.000	34.000	12	0.779	1.936	0.8038
37	Chroomon	1.890	5.352	94.500	0.000	23.000	12	0.780	1.937	0.8228
38	Allomyce	1.990	5.553	99.500	0.000	28.500	13	0.791	2.029	0.8274
39	Blastocl	1.998	6.331	99.900	0.000	39.100	14	0.719	1.898	0.7832
40	Rhizophl	1.980	10.597	99.000	0.000	74.000	5	0.538	0.866	0.4185
41	Traustoc	2.012	6.529	100.600	0.000	34.600	12	0.726	1.805	0.7736
42	Tetrahym	1.932	7.460	96.600	0.000	39.600	10	0.642	1.479	0.6878
43	Trypanos	1.996	6.898	99.800	0.000	36.200	11	0.677	1.625	0.7459
44	Spirulin	1.880	6.986	94.000	0.000	43.000	6	0.815	1.460	0.7094
45	Anabaena	2.060	7.457	103.000	0.000	46.000	7	0.802	1.561	0.7232
46	Snyechoc	1.860	6.851	93.000	0.000	41.000	7	0.754	1.468	0.7141
47	S_6910	1.920	6.483	96.000	0.000	30.000	9	0.736	1.616	0.7565
48	Aphanoca	1.960	6.363	98.000	0.000	30.000	7	0.825	1.606	0.7734
49	A_6805	1.920	6.292	96.000	0.000	35.000	9	0.773	1.699	0.7695
50	Microcys	1.880	7.038	94.000	0.000	40.000	7	0.735	1.431	0.7053
51	Microcha	1.900	5.776	95.000	0.000	27.000	8	0.818	1.701	0.7989
52	Aspergil	1.986	6.699	99.300	0.000	35.600	8	0.776	1.614	0.7570
53	Sporobol	2.000	7.091	100.000	0.000	39.100	8	0.728	1.514	0.7336
54	Papulasp	1.928	7.073	96.400	0.000	36.800	9	0.636	1.398	0.7163
55	Acremoni	1.228	4.240	61.380	0.000	16.800	4	0.994	1.378	0.7462
56	Gonyaula	1.980	6.467	99.000	0.000	36.000	10	0.748	1.723	0.7709
57	Prorocen	1.980	4.926	99.000	0.000	23.000	9	0.935	2.054	0.8587
58	Campylob	1.980	7.389	99.000	0.000	37.000	7	0.739	1.438	0.7071

59	Clostrid	1.960	7.979	98.000	0.000	51.000	6	0.736	1.319	0.6551
60	Serratia	1.960	7.442	98.000	0.000	47.000	7	0.742	1.444	0.6974
61	Erwinia	1.900	7.178	95.000	0.000	35.000	6	0.737	1.320	0.7003
62	Pseudomo	1.780	5.452	89.000	0.000	28.000	8	0.840	1.747	0.7961
63	P_syntha	1.800	7.656	90.000	0.000	41.000	4	0.769	1.065	0.6254
64	Prymnesi	1.960	5.147	98.000	0.000	25.000	13	0.812	2.082	0.8449
65	Isochrys	1.940	4.537	97.000	0.000	17.000	14	0.846	2.232	0.8728
66	Hyphochy	2.040	7.869	102.000	0.000	49.000	6	0.777	1.392	0.6884
67	Rhizidio	2.000	4.267	100.000	0.000	18.000	11	0.959	2.299	0.8908
68	Dictoste	1.916	8.024	95.800	0.000	46.400	8	0.611	1.271	0.6363
69	Saproleg	1.980	5.135	99.000	0.000	20.000	10	0.880	2.027	0.8482
70	P_debary	1.642	4.611	82.100	0.000	25.500	10	0.859	1.978	0.8255
71	Pythium	2.062	5.682	103.100	0.000	28.400	12	0.830	2.062	0.8312
72	Phytopho	1.730	4.928	86.500	0.000	22.100	8	0.890	1.851	0.8210
73	P_erythr	1.752	5.344	87.600	0.000	28.800	9	0.818	1.797	0.7976
74	Phycomyc	1.984	6.393	99.200	0.000	32.100	9	0.764	1.680	0.7765
75	Chlamydo	1.860	6.379	93.000	0.000	31.000	8	0.753	1.567	0.7495
76	Crithidi	1.582	4.852	79.100	0.000	21.300	8	0.817	1.699	0.7956
77	Leishman	1.980	6.137	99.000	0.000	29.800	16	0.654	1.814	0.7917
78	Monodops	1.980	6.268	99.000	0.000	29.000	9	0.781	1.717	0.7836
79	Cryptoco	1.932	8.279	96.610	0.000	55.600	10	0.568	1.307	0.6201
80	Candida	2.002	8.074	100.100	0.000	44.700	7	0.641	1.246	0.6612
81	Rhodotor	1.986	7.494	99.300	0.000	48.000	8	0.715	1.487	0.7009
82	Thamnidi	2.040	6.833	102.000	0.000	39.700	10	0.723	1.665	0.7601
83	Rhizopus	1.896	6.564	94.800	0.000	30.000	6	0.821	1.470	0.7451
84	Chaoanep	1.976	6.165	98.800	0.000	26.400	7	0.844	1.643	0.7892
85	Helicost	1.738	5.273	86.900	0.000	26.400	7	0.895	1.742	0.7996
86	M_inaequ	1.982	5.762	99.100	0.000	26.400	10	0.805	1.854	0.8143
87	Mucor_mi	1.996	7.700	99.800	0.000	47.800	7	0.734	1.428	0.6883
88	Mucor_py	2.010	6.025	100.500	0.000	27.200	10	0.790	1.819	0.8039
89	Cunningh	1.962	7.583	98.090	0.000	48.150	9	0.656	1.442	0.6872
90	Mortiere	1.972	7.524	98.610	0.000	45.090	7	0.728	1.417	0.6947
91	S_hetero	1.951	7.839	97.560	0.000	48.060	11	0.550	1.318	0.6636
92	Scutello	1.979	8.652	98.940	0.000	55.240	6	0.629	1.126	0.6053
93	G_gigant	1.901	7.884	95.030	0.000	48.300	10	0.553	1.274	0.6428
94	Gigaspor	1.924	7.855	96.180	0.000	45.640	9	0.587	1.289	0.6532
95	Chlorell	2.000	6.776	100.000	0.000	36.000	7	0.808	1.573	0.7550
96	Neocalli	1.804	5.883	90.200	0.000	34.300	15	0.673	1.823	0.7715
97	Monoblep	1.800	5.104	90.000	0.000	22.100	11	0.770	1.847	0.8224
98	Chlorogl	1.988	7.191	99.400	0.000	39.000	6	0.782	1.401	0.7236
99	Mastigoc	1.980	8.213	99.000	0.000	42.000	4	0.803	1.113	0.6428
100	Hapalosi	1.920	8.609	96.000	0.000	54.000	3	0.896	0.984	0.5859
101	Anacysti	1.920	8.626	96.000	0.000	47.000	3	0.866	0.951	0.5844
102	Wood	1.896	5.819	94.800	0.000	32.820	26	0.652	2.125	0.7954
103	Wood	1.846	6.248	92.290	0.000	35.860	19	0.633	1.863	0.7555
104	Soil	2.000	4.439	100.000	0.000	19.200	11	0.949	2.275	0.8834
105	Soil	2.000	4.015	100.020	0.000	19.610	16	0.916	2.540	0.9011
106	Soil6M	1.904	3.060	95.180	0.000	14.890	22	0.929	2.870	0.9294
107	Soil6M	1.927	3.095	96.360	0.000	14.000	19	0.951	2.799	0.9294
108	Soil6M	1.882	2.996	94.110	0.000	15.180	22	0.928	2.870	0.9304
109	Soil24M	2.000	3.535	99.990	0.000	17.020	17	0.944	2.675	0.9188
110	Soil24M	2.000	3.472	100.020	0.000	17.140	19	0.931	2.740	0.9209
111	Soil24M	2.046	3.144	102.300	0.000	12.300	19	0.962	2.831	0.9337
112	Wood6M	1.919	5.745	95.970	0.000	29.390	15	0.714	1.933	0.8044
113	Wood6M	1.765	5.103	88.250	0.000	26.210	16	0.726	2.013	0.8162
114	Wood6M	1.897	5.164	94.850	0.000	24.330	25	0.674	2.171	0.8348
115	Wood24M	1.933	4.628	96.660	0.000	23.350	16	0.822	2.278	0.8677
116	Wood24M	1.810	4.497	90.520	0.000	21.760	18	0.772	2.232	0.8591
117	Wood24M	1.872	4.064	93.590	0.000	19.120	23	0.788	2.470	0.8876
118	Mix6M	1.743	4.839	87.130	0.000	31.750	29	0.723	2.435	0.8289
119	Mix6M	1.928	5.601	96.420	0.000	38.460	30	0.718	2.442	0.8146
120	Mix6M	1.900	4.729	95.020	0.000	29.040	32	0.725	2.512	0.8587
121	Mix24M	2.011	4.380	100.550	0.000	22.800	20	0.836	2.504	0.8870
122	Mix24M	1.938	3.690	96.890	0.000	19.480	30	0.811	2.758	0.9089
123	Mix24M	1.962	4.457	98.080	0.000	19.420	15	0.864	2.340	0.8788
124	Soil6S	1.968	3.218	98.400	0.000	16.100	22	0.923	2.854	0.9276
125	Soil6S	1.884	3.011	94.190	0.000	15.900	21	0.939	2.860	0.9299
126	Soil6S	1.920	4.324	95.980	0.000	23.070	12	0.929	2.308	0.8806
127	Soil24S	1.963	3.258	98.140	0.000	16.260	19	0.946	2.784	0.9260
128	Soil24S	2.002	3.156	100.100	0.000	14.610	23	0.928	2.908	0.9313
129	Soil24S	2.000	3.652	100.010	0.000	17.710	16	0.948	2.630	0.9147

130	Wood6S	1.874	5.126	93.700	0.000	23.350	22	0.689	2.129	0.8334
131	Wood6S	1.875	5.045	93.760	0.000	22.320	22	0.702	2.169	0.8381
132	Wood6S	1.824	5.096	91.200	0.000	25.700	19	0.702	2.066	0.8270
133	Mix6S	1.880	5.021	93.990	0.000	31.320	31	0.700	2.405	0.8402
134	Mix6S	1.950	4.169	97.490	0.000	22.650	30	0.765	2.604	0.8904
135	Mix6S	1.788	5.340	89.390	0.000	34.850	30	0.675	2.297	0.8051
136	Mix24S	1.930	4.285	96.520	0.000	21.440	20	0.809	2.423	0.8834
137	Mix24S	1.947	4.539	97.360	0.000	26.280	28	0.765	2.548	0.8735
138	Mix24S	1.733	4.140	86.630	0.000	21.430	21	0.785	2.390	0.8681
139	Soil6W	2.000	6.938	100.000	0.000	36.800	6	0.855	1.532	0.7442
140	Soil6W	2.000	6.634	99.990	0.000	35.240	6	0.892	1.598	0.7643
141	Soil6W	2.000	8.048	100.010	0.000	38.430	3	0.995	1.093	0.6627
142	Soil24W	2.000	9.956	100.000	0.000	55.340	2	0.992	0.687	0.4943
143	Soil24W	2.000	6.439	100.000	0.000	34.080	7	0.870	1.694	0.7768
144	Wood6W	2.000	8.548	100.000	0.000	45.800	3	0.933	1.025	0.6220
145	Wood6W	2.000	6.057	100.000	0.000	32.290	7	0.906	1.762	0.8002
146	Mix6W	2.000	5.308	100.000	0.000	27.410	10	0.896	2.063	0.8419
147	Mix6W	2.000	8.062	100.000	0.000	53.130	5	0.830	1.335	0.6615
148	Mix6W	2.000	7.081	100.000	0.000	30.900	4	0.976	1.353	0.7343
149	Mix24W	2.000	5.917	99.990	0.000	27.820	6	0.959	1.719	0.8084
150	Mix24W	2.007	7.938	100.340	0.000	49.160	5	0.787	1.267	0.6733
151	Wood24Bi	1.692	3.772	84.610	0.000	16.620	22	0.789	2.438	0.8826
152	Wood24Bi	1.804	3.671	90.190	0.000	14.360	25	0.789	2.538	0.8988
153	Wood24Bi	1.875	4.647	93.760	0.000	25.960	23	0.746	2.338	0.8596
154	Wood24Bi	1.769	3.765	88.460	0.000	16.710	26	0.768	2.501	0.8912
155	Wood24S	1.719	4.389	85.960	0.000	20.260	18	0.759	2.195	0.8523
156	Wood24S	1.603	3.839	80.170	0.000	17.570	19	0.783	2.305	0.8677
157	Wood24S	1.818	5.095	90.920	0.000	28.780	17	0.738	2.090	0.8261
158	Wood24S	1.821	4.067	91.070	0.000	17.860	23	0.775	2.432	0.8823
159	Wood24W	1.915	6.815	95.766	0.000	34.830	9	0.723	1.588	0.7318
160	Wood24W	1.937	6.324	96.870	0.000	30.830	7	0.863	1.680	0.7712
161	Wood24W	2.000	6.944	100.010	0.000	38.770	9	0.759	1.668	0.7438
162	Wood24W	1.970	5.597	98.490	0.000	30.730	19	0.751	2.211	0.8217
163	SYRC	1.783	4.357	89.160	0.000	19.160	22	0.766	2.368	0.8630
164	SYRC	1.763	3.860	88.160	0.000	17.500	24	0.791	2.515	0.8861
165	SYRCN	1.752	4.156	87.580	0.000	18.550	24	0.762	2.422	0.8697
166	SYRCN	1.716	4.046	85.790	0.000	19.550	23	0.767	2.406	0.8710
167	SYRCN	1.830	4.501	91.480	0.000	20.370	20	0.782	2.343	0.8614
	AVERAGES:	1.907	5.997	95.362	0.000	32.565	12.3	0.788	1.838	0.7728

		Skewness	Kurtosis
1	Desulfov	2.620	6.169
2	ThioDeni	4.254	18.706
3	ThioFerr	4.508	23.635
4	Staphylo	3.890	17.621
5	Lactobac	5.991	38.274
6	Listeria	4.269	20.895
7	Legionel	3.930	18.133
8	EuGracHe	2.167	4.419
9	CvulgAu	3.810	14.697
10	CvulgHe	3.541	12.574
11	Sporocyt	2.634	6.652
12	Streptom	3.793	16.226
13	Azospiri	6.365	42.822
14	Caulobac	5.150	29.780
15	Prosteco	5.752	36.191
16	Nitrobac	6.836	47.768
17	Agrobact	4.150	17.316
18	Acantham	5.689	36.323
19	Physarum	4.609	20.878
20	Fusarium	4.343	19.634
21	F_solani	3.544	12.028
22	Neurospo	5.012	28.278
23	Penicill	4.775	25.861
24	Clavicep	3.711	13.169
25	Chaetomi	6.237	41.001
26	Navicula	3.756	14.340
27	Astrione	3.035	9.571

28	Rhizocto	4.027	15.999
29	Lactariu	4.512	22.829
30	Achromob	3.983	15.881
31	Nitrosol	6.193	40.181
32	Nitrosom	6.677	45.928
33	Ochromon	2.561	6.156
34	Poterioc	3.874	15.569
35	O_malham	3.221	11.529
36	Cryptomo	4.295	21.201
37	Chroomon	3.222	9.908
38	Allomyce	3.714	14.699
39	Blastocl	4.687	25.148
40	Rhizophl	6.691	46.234
41	Traustoc	4.237	18.449
42	Tetrahym	4.664	21.460
43	Trypanos	4.170	17.707
44	Spirulin	4.853	26.136
45	Anabaena	4.918	26.595
46	Snyechoc	4.620	23.427
47	S_6910	3.800	13.878
48	Aphanoca	3.488	11.735
49	A_6805	4.059	17.634
50	Microcys	4.469	20.880
51	Microcha	3.146	9.468
52	Aspergil	4.047	17.010
53	Sporobol	4.136	17.877
54	Papulasp	3.981	15.737
55	Acremoni	3.277	9.471
56	Gonyaula	4.074	17.805
57	Prorocen	2.686	7.281
58	Campylob	4.372	19.076
59	Clostrid	5.304	30.825
60	Serratia	5.068	28.740
61	Erwinia	3.920	14.599
62	Pseudomo	3.523	12.895
63	P_syntha	4.509	20.226
64	Prymnesi	3.146	10.103
65	Isochrys	2.482	5.133
66	Hyphochy	4.983	27.464
67	Rhizidio	2.173	4.257
68	Dictoste	4.910	24.337
69	Saproleg	2.786	6.940
70	P_debary	3.779	16.128
71	Pythium	3.701	14.311
72	Phytopho	3.165	9.814
73	P_erythr	3.725	14.953
74	Phycomyc	3.674	13.484
75	Chlamydo	3.787	13.749
76	Crithidi	3.182	9.297
77	Leishman	3.495	11.847
78	Monodops	3.552	12.042
79	Cryptoco	5.918	37.958
80	Candida	4.569	21.176
81	Rhodotor	5.199	30.345
82	Thamnidi	4.244	20.019
83	Rhizopus	3.644	12.720
84	Chaoanep	3.148	8.876
85	Helicost	3.472	12.413
86	M_inaequ	3.219	9.837
87	Mucor_mi	5.001	27.349
88	Mucor_py	3.388	10.933
89	Cunningh	5.166	29.611
90	Mortiere	4.734	24.158
91	S_hetero	4.961	26.599
92	Scutello	5.339	31.027
93	G_gigant	5.040	27.032
94	Gigaspor	4.800	23.707
95	Chlorell	3.937	16.046
96	Neocalli	4.416	21.212
97	Monoblep	3.005	8.707
98	Chlorogl	4.029	16.916

99	Mastigoc	4.370	18.804
100	Hapalosi	5.192	29.175
101	Anacysti	4.730	22.108
102	Wood	4.557	21.375
103	Wood	4.665	22.471
104	Soil	2.588	7.052
105	Soil	2.839	9.274
106	Soil6M	2.393	7.134
107	Soil6M	1.950	4.409
108	Soil6M	2.320	7.242
109	Soil24M	2.295	6.412
110	Soil24M	2.474	7.657
111	Soil24M	1.645	2.571
112	Wood6M	3.787	14.738
113	Wood6M	3.758	14.614
114	Wood6M	3.533	12.248
115	Wood24M	3.167	11.021
116	Wood24M	3.110	9.893
117	Wood24M	2.730	7.521
118	Mix6M	5.305	31.636
119	Mix6M	5.952	38.843
120	Mix6M	4.544	23.692
121	Mix24M	3.187	11.460
122	Mix24M	3.002	10.706
123	Mix24M	2.778	7.521
124	Soil6S	2.508	8.024
125	Soil6S	2.476	8.808
126	Soil6S	3.085	11.732
127	Soil24S	2.327	7.094
128	Soil24S	2.396	7.025
129	Soil24S	2.459	7.455
130	Wood6S	3.431	11.452
131	Wood6S	3.417	11.306
132	Wood6S	3.534	12.783
133	Mix6S	4.699	25.313
134	Mix6S	3.390	13.318
135	Mix6S	5.319	31.533
136	Mix24S	2.951	9.596
137	Mix24S	4.075	18.938
138	Mix24S	3.544	13.737
139	Soil6W	4.123	17.590
140	Soil6W	3.971	16.621
141	Soil6W	3.905	14.264
142	Soil24W	4.933	23.782
143	Soil24W	4.006	16.890
144	Wood6W	4.472	19.927
145	Wood6W	3.671	14.645
146	Mix6W	3.542	13.755
147	Mix6W	5.572	34.552
148	Mix6W	3.519	11.631
149	Mix24W	3.111	9.526
150	Mix24W	4.885	26.673
151	Wood24Bi	2.898	8.522
152	Wood24Bi	2.344	4.744
153	Wood24Bi	3.712	15.826
154	Wood24Bi	2.618	6.591
155	Wood24S	3.109	9.393
156	Wood24S	2.966	8.665
157	Wood24S	3.911	17.186
158	Wood24S	2.825	7.863
159	Wood24W	4.395	19.388
160	Wood24W	4.060	16.956
161	Wood24W	4.524	21.103
162	Wood24W	4.363	19.751
163	SYRC	3.336	10.857
164	SYRC	3.007	9.155
165	SYRCN	3.287	10.733
166	SYRCN	3.216	10.482
167	SYRCN	3.324	10.797
	Averages:	3.923	17.360

```
---------------------------------------------
Number of cells in main matrix =      8350
Percent of cells empty =   75.377
Matrix total =   0.1593E+05
Matrix mean  =   0.1907E+01
Variance of totals of samples  =   0.3660E+02
CV of totals of samples  =       6.34%
---------------------------------------------
S = Richness  = number of non-zero elements in row
E = Evenness  = H / ln (Richness)
H = Diversity = - sum (Pi*ln(Pi)) = Shannon`s diversity index
D = Simpson`s diversity index for infinite population = 1 - sum (Pi*Pi)
       where Pi = importance probability in element i (element i
                  relativized by row total)
```

FinalDendroStats

Summary of 50 Fas N = 167 samples

Num.	Name	Mean	Stand.Dev.	Sum	Minimum	Maximum	S	E	H	D`
1	FA1	1.447	4.435	241.700	0.000	31.000	36	0.850	3.045	0.9381
2	FA2	3.682	6.446	614.890	0.000	39.600	86	0.902	4.016	0.9758
3	FA3	0.200	0.689	33.400	0.000	4.100	30	0.832	2.830	0.9233
4	FA4	0.026	0.140	4.420	0.000	1.610	12	0.866	2.152	0.8273
5	FA5	1.672	2.755	279.190	0.000	14.000	114	0.878	4.157	0.9778
6	FA6	0.051	0.322	8.440	0.000	3.800	9	0.809	1.778	0.7525
7	FA7	0.092	0.727	15.290	0.000	8.000	5	0.682	1.097	0.6191
8	FA8	0.196	0.915	32.680	0.000	11.000	35	0.772	2.746	0.8638
9	FA9	0.762	2.262	127.260	0.000	16.000	28	0.891	2.970	0.9416
10	FA10	0.968	3.948	161.590	0.000	37.000	41	0.751	2.790	0.8949
11	FA11	0.812	3.027	135.650	0.000	32.000	28	0.845	2.816	0.9113
12	FA12	15.981	11.317	2668.810	0.000	54.000	165	0.955	4.878	0.9910
13	FA13	7.136	12.280	1191.660	0.000	82.000	133	0.856	4.188	0.9764
14	FA14	0.623	2.434	103.980	0.000	21.000	27	0.793	2.613	0.9030
15	FA15	0.341	1.922	57.000	0.000	18.000	10	0.801	1.844	0.8053
16	FA16	0.558	1.620	93.140	0.000	18.000	48	0.894	3.462	0.9438
17	FA17	0.754	1.989	125.840	0.000	14.000	53	0.887	3.523	0.9525
18	FA18	0.160	0.750	26.720	0.000	7.000	26	0.785	2.557	0.8631
19	FA19	0.477	2.210	79.710	0.000	17.000	24	0.759	2.411	0.8665
20	FA20	0.179	1.325	29.820	0.000	17.000	25	0.628	2.022	0.6663
21	FA21	0.416	1.357	69.540	0.000	6.410	17	0.960	2.719	0.9308
22	FA22	0.044	0.340	7.320	0.000	3.000	4	0.780	1.081	0.6353
23	FA23	2.918	3.444	487.260	0.000	17.000	129	0.928	4.508	0.9857
24	FA24	18.715	14.605	3125.480	0.000	74.000	141	0.963	4.764	0.9904
25	FA25	5.011	12.765	836.860	0.000	81.000	69	0.815	3.450	0.9554
26	FA26	13.805	13.006	2305.391	0.000	63.480	128	0.953	4.622	0.9887
27	FA27	0.583	3.588	97.400	0.000	34.000	9	0.754	1.658	0.7687
28	FA28	1.509	6.510	252.040	0.000	61.000	28	0.787	2.624	0.8833
29	FA29	1.272	2.614	212.480	0.000	11.280	40	0.963	3.552	0.9689
30	FA30	1.762	3.137	294.250	0.000	12.000	58	0.943	3.830	0.9751
31	FA31	0.698	1.634	116.510	0.000	12.750	49	0.921	3.585	0.9614
32	FA32	0.151	0.690	25.250	0.000	5.700	14	0.868	2.291	0.8699
33	FA33	0.491	1.330	82.070	0.000	9.000	46	0.884	3.384	0.9504
34	FA34	0.636	2.292	106.150	0.000	16.400	17	0.923	2.614	0.9166
35	FA35	0.066	0.461	11.100	0.000	4.000	5	0.845	1.360	0.7074
36	FA36	1.140	4.212	190.320	0.000	29.000	36	0.769	2.757	0.9127
37	FA37	0.640	2.873	106.810	0.000	17.860	12	0.868	2.158	0.8739
38	FA38	0.280	1.750	46.700	0.000	16.000	8	0.793	1.650	0.7610
39	FA39	0.291	2.082	48.600	0.000	23.000	5	0.837	1.348	0.6893
40	FA40	0.340	3.728	56.840	0.000	48.000	5	0.396	0.637	0.2800
41	FA41	0.048	0.447	8.000	0.000	4.900	2	0.963	0.668	0.4747
42	FA42	0.396	1.186	66.060	0.000	11.800	39	0.885	3.243	0.9405
43	FA43	0.309	0.948	51.630	0.000	8.000	34	0.863	3.044	0.9381
44	AF44	1.436	3.663	239.880	0.000	17.570	33	0.933	3.261	0.9553
45	FA45	0.696	2.875	116.160	0.000	21.710	23	0.805	2.525	0.8923
46	FA46	0.265	0.941	44.230	0.000	6.930	22	0.894	2.763	0.9189
47	FA47	1.520	3.160	253.770	0.000	19.000	59	0.903	3.682	0.9683
48	FA48	0.736	3.793	122.980	0.000	31.350	8	0.930	1.933	0.8361
49	FA49	1.300	3.139	217.136	0.000	17.540	41	0.924	3.431	0.9593

50 FA50	1.772	5.144	295.970	0.000	28.780	40	0.856	3.159	0.9439
AVERAGES:	1.907	3.385	318.508	0.000	23.171	41.1	0.847	2.804	0.8685

	Skewness	Kurtosis
1 FA1	4.047	18.223
2 FA2	2.699	8.547
3 FA3	4.304	18.920
4 FA4	9.198	99.884
5 FA5	2.559	6.810
6 FA6	9.995	112.846
7 FA7	9.199	91.475
8 FA8	10.212	118.650
9 FA9	3.518	14.673
10 FA10	7.109	57.071
11 FA11	7.263	68.852
12 FA12	1.086	1.030
13 FA13	3.421	14.621
14 FA14	5.523	36.285
15 FA15	6.993	53.689
16 FA16	7.911	81.560
17 FA17	4.925	27.130
18 FA18	7.868	66.417
19 FA19	6.391	42.950
20 FA20	12.481	159.288
21 FA21	3.205	9.044
22 FA22	8.257	69.092
23 FA23	1.772	3.061
24 FA24	0.626	0.321
25 FA25	3.478	13.046
26 FA26	0.890	0.607
27 FA27	7.316	57.468
28 FA28	6.876	53.085
29 FA29	1.979	2.873
30 FA30	1.831	2.429
31 FA31	4.429	26.406
32 FA32	6.117	41.301
33 FA33	4.404	22.354
34 FA34	4.265	20.107
35 FA35	7.885	64.177
36 FA36	4.705	23.285
37 FA37	4.679	21.087
38 FA38	7.666	61.875
39 FA39	9.022	90.439
40 FA40	12.745	163.871
41 FA41	9.708	96.874
42 FA42	6.189	52.363
43 FA43	4.535	27.511
44 AF44	2.876	7.661
45 FA45	5.614	34.950
46 FA46	4.811	25.679
47 FA47	2.740	8.364
48 FA48	6.128	40.845
49 FA49	3.230	11.165
50 FA50	3.760	14.208
Averages:	5.569	43.289

Number of cells in main matrix = 8350
Percent of cells empty = 75.377
Matrix total = 0.1593E+05
Matrix mean = 0.1907E+01
Variance of totals of Fas = 0.4226E+06
CV of totals of Fas = 204.10%

S = Richness = number of non-zero elements in row
E = Evenness = H / ln (Richness)
H = Diversity = - sum (Pi*ln(Pi)) = Shannon`s diversity index
D = Simpson`s diversity index for infinite population = 1 - sum (Pi*Pi)

where Pi = importance probability in element i (element i relativized by row total)

********** Analysis completed **********

********** Outlier Analysis **********
PC-ORD, Version 4.20
20 Sep 2005, 11:55

FinalDendroStats

RANK	ENTITY NAME	AVERAGE DISTANCE	STANDARD DEVIATIONS
1	Nitrobac	87.48218	4.33498
2	Nitrosom	85.27192	4.10634
3	Nitrosol	78.74461	3.43113
4	Azospiri	73.79134	2.91875
5	Lactobac	72.71838	2.80776
6	Rhizophl	65.01134	2.01051

Statistics for average distances for each of N = 167 samples
Distance measure: Euclidean (Pythagorean)
0.45576E+02 = Grand mean
0.96671E+01 = Standard deviation
2.00000 = Cutoff number of standard deviations used to flag outliers

********** Analysis completed **********

********** Outlier Analysis **********
PC-ORD, Version 4.20
20 Sep 2005, 11:56

FinalDendroStats

RANK	ENTITY NAME	AVERAGE DISTANCE	STANDARD DEVIATIONS
1	FA24	300.68805	4.02337
2	FA12	248.59268	3.05948
3	FA26	243.14882	2.95875

Statistics for average distances for each of N = 50 Fas
Distance measure: Euclidean (Pythagorean)
0.83238E+02 = Grand mean
0.54047E+02 = Standard deviation
2.00000 = Cutoff number of standard deviations used to flag outliers

********** Analysis completed **********

********** Nonmetric Multidimensional Scaling **********
PC-ORD, Version 4.20
20 Sep 2005, 11:57

FinalDendroNMS

```
Ordination of samples  in Fas      space.        167 samples        50 Fas

         The following options were selected:
ANALYSIS OPTIONS
         1.    SORENSEN = Distance measure
         2.           6 = Number of axes (max. = 6)
         3.         400 = Maximum number of iterations
         4.      RANDOM = Starting coordinates (random or from file)
         5.           1 = Reduction in dimensionality at each cycle
         6.        0.20 = Step length (rate of movement toward minimum stress)
         7.    USE TIME = Random number seeds (use time vs. user-supplied)
         8.          40 = Number of runs with real data
         9.          50 = Number of runs with randomized data
        10.         YES = Autopilot
        11.    0.000010 = Stability criterion, standard deviations in stress
                          over last  15 iterations.
        12.    THOROUGH = Speed vs. thoroughness
OUTPUT OPTIONS
        13.          NO = Write distance matrix?
        14.          NO = Write starting coordinates?
        15.          NO = List stress, etc. for each iteration?
        18.          NO = Plot stress vs. iteration?
        17.          NO = Plot distance vs. dissimilarity?
        16.          NO = Write final configuration?
        19.  UNROTATED = Write varimax-rotated or unrotated scores for graph?
        20.         YES = Write run log?
        21.          NO = Write weighted-average scores for Fas     ?
-------------------------------------------------------------------------------

      2925 = Seed for random number generator.


STRESS IN RELATION TO DIMENSIONALITY (Number of Axes)
-------------------------------------------------------------------
          Stress in real data          Stress in randomized data
               40 run(s)               Monte Carlo test,   50 runs
      -------------------------  ---------------------------------
Axes  Minimum     Mean  Maximum  Minimum     Mean  Maximum      p
-------------------------------------------------------------------
   1   30.429   47.668   57.413   46.132   50.433   57.383   0.0196
   2   18.051   20.332   41.656   25.114   27.173   41.559   0.0196
   3   13.173   15.451   32.724   18.508   19.356   20.801   0.0196
   4   10.388   12.723   27.266   14.565   15.015   15.657   0.0196
   5    8.672   11.073   23.684   11.900   12.388   12.923   0.0196
   6    7.443    9.553   21.138   10.288   10.672   11.130   0.0196
-------------------------------------------------------------------
p = proportion of randomized runs with stress < or = observed stress
i.e., p  = (1 + no. permutations <= observed)/(1 + no. permutations)


Conclusion:  a 2-dimensional solution is recommended.
Now rerunning the best ordination with that dimensionality.

Selected file CONFIG2.GPH  for the starting configuration for
   the final run.


FinalDendroNMS
Ordination of samples  in Fas      space.        167 samples        50 Fas

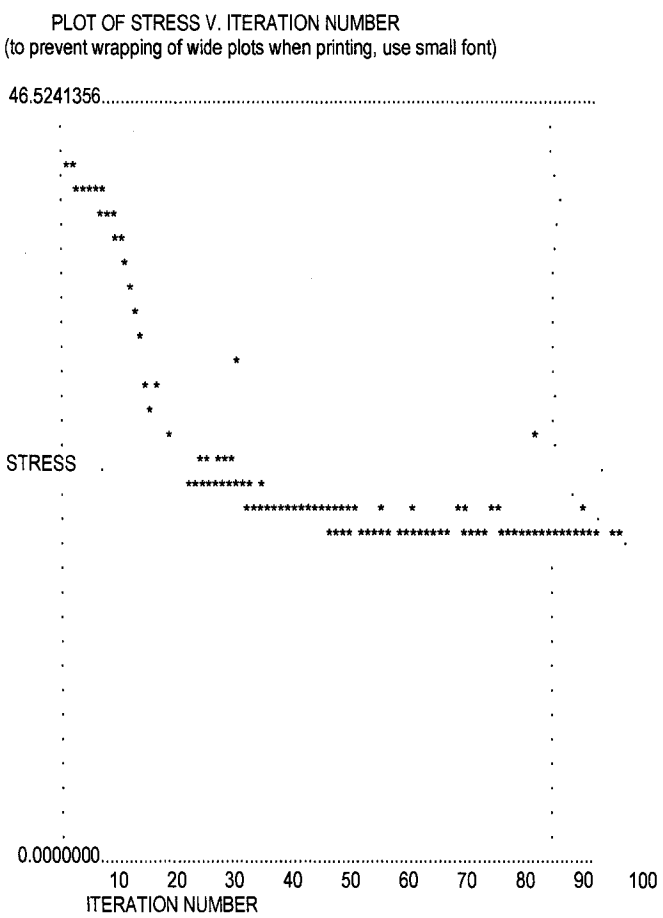
         The following options were selected:
ANALYSIS OPTIONS
         1.    SORENSEN = Distance measure
         2.           2 = Number of axes (max. = 6)
         3.         400 = Maximum number of iterations
         4.   FROM FILE = Starting coordinates (random or from file)
         5.           2 = Reduction in dimensionality at each cycle
         6.        0.20 = Step length (rate of movement toward minimum stress)
         7.    USE TIME = Random number seeds (use time vs. user-supplied)
         8.           1 = Number of runs with real data
         9.           0 = Number of runs with randomized data
```

```
        10.         YES = Autopilot
        11.    0.000010 = Stability criterion, standard deviations in stress
                          over last  15 iterations.
        12.    THOROUGH = Speed vs. thoroughness
OUTPUT OPTIONS
        13.          NO = Write distance matrix?
        14.          NO = Write starting coordinates?
        15.         YES = List stress, etc. for each iteration?
        18.         YES = Plot stress vs. iteration?
        17.          NO = Plot distance vs. dissimilarity?
        16.         YES = Write final configuration?
        19.  UNROTATED = Write varimax-rotated or unrotated scores for graph?
        20.          NO = Write run log?
        21.         YES = Write weighted-average scores for Fas     ?
-------------------------------------------------------------------------------


Final configuration (ordination scores) for this run
    samples            Axis
Number Name              1          2
     1 Desulfov     -1.1957    -0.6327
     2 ThioDeni     -1.1864     0.7785
     3 ThioFerr     -1.3117    -0.0283
     4 Staphylo     -0.7748    -1.3224
     5 Lactobac     -1.4696     0.4544
     6 Listeria     -1.1100     1.3162
     7 Legionel     -1.3483    -1.0192
     8 EuGracHe      0.1624     0.9924
     9 CvulgAu       0.0442     0.7916
    10 CvulgHe       0.1907     0.1383
    11 Sporocyt     -1.2875    -1.1614
    12 Streptom      0.2993    -0.3128
    13 Azospiri     -1.7923    -0.2686
    14 Caulobac     -1.3583     0.1152
    15 Prosteco     -1.4976    -0.0784
    16 Nitrobac     -2.4854    -0.5460
    17 Agrobact     -1.5308     0.2788
    18 Acantham      0.6257     0.3468
    19 Physarum      0.7084     0.0457
    20 Fusarium      0.5579     0.1302
    21 F_solani      0.1526     0.1270
    22 Neurospo      0.3709     0.4453
    23 Penicill      0.5978     0.2660
    24 Clavicep     -0.1464     0.2303
    25 Chaetomi      0.6009     0.7169
    26 Navicula     -0.7881     1.1088
    27 Astrione     -0.6306     1.3063
    28 Rhizocto      0.4507     0.1679
    29 Lactariu      0.8088     0.1388
    30 Achromob     -1.5497    -0.1385
    31 Nitrosol     -1.2315     1.0837
    32 Nitrosom     -1.2936     1.1337
    33 Ochromon      0.5813     0.4054
    34 Poterioc      0.1713     0.4490
    35 O_malham      0.6196     0.5507
    36 Cryptomo      0.0401     1.8361
    37 Chroomon     -0.1623     1.5233
    38 Allomyce      0.0780     0.0801
    39 Blastocl      0.3336    -0.0726
    40 Rhizophl      0.8023     0.3806
    41 Traustoc      0.0649     0.3938
    42 Tetrahym      1.0624     0.5132
    43 Trypanos      0.6205     0.0388
    44 Spirulin     -0.2823     0.5237
    45 Anabaena     -0.0517     0.6542
    46 Snyechoc      0.1663     0.5097
    47 S_6910       -0.5894     0.7185
    48 Aphanoca     -0.1143     0.4430
    49 A_6805       -0.1211     0.4837
    50 Microcys     -0.0332     0.5979
    51 Microcha      0.0786     0.1772
```

52	Aspergil	0.4944	0.1747
53	Sporobol	0.3490	0.1768
54	Papulasp	0.3019	0.1155
55	Acremoni	0.0268	0.0318
56	Gonyaula	-0.3856	1.3819
57	Prorocen	-0.5204	1.2785
58	Campylob	-1.2923	0.3186
59	Clostrid	-1.1212	0.7008
60	Serratia	-1.2923	0.5057
61	Erwinia	-1.1841	0.5492
62	Pseudomo	-1.2367	0.2423
63	P_syntha	-1.2882	0.4440
64	Prymnesi	0.2001	0.2434
65	Isochrys	0.2432	0.6792
66	Hyphochy	-0.1963	0.1643
67	Rhizidio	0.3485	0.7756
68	Dictoste	0.7784	0.1205
69	Saproleg	0.2834	0.3301
70	P_debary	-0.0216	0.2926
71	Pythium	0.0732	0.0375
72	Phytopho	0.4385	0.0612
73	P_erythr	0.0925	0.2760
74	Phycomyc	0.2878	0.0718
75	Chlamydo	0.0629	0.5181
76	Crithidi	1.0904	-0.0503
77	Leishman	1.0642	0.1800
78	Monodops	-0.5108	0.7191
79	Cryptoco	-0.0397	-0.6056
80	Candida	0.6307	0.1086
81	Rhodotor	0.3694	-0.9998
82	Thamnidi	0.4004	0.0178
83	Rhizopus	0.4032	0.1102
84	Chaoanep	0.3513	0.1266
85	Helicost	0.3769	0.0073
86	M_inaequ	0.2407	0.0233
87	Mucor_mi	0.2534	0.0516
88	Mucor_py	0.5343	0.0035
89	Cunningh	0.3266	0.0069
90	Mortiere	0.2259	0.0648
91	S_hetero	-0.8711	0.5284
92	Scutello	-1.1615	0.6239
93	G_gigant	-0.1705	0.0133
94	Gigaspor	-0.2111	0.1084
95	Chlorell	0.1041	0.2392
96	Neocalli	-0.1065	-0.0600
97	Monoblep	0.5825	-0.0700
98	Chlorogl	-0.0884	0.2081
99	Mastigoc	-0.5344	0.5395
100	Hapalosi	-0.5541	0.5329
101	Anacysti	-0.7615	0.7596
102	Wood	0.6826	-0.2775
103	Wood	0.7258	-0.1947
104	Soil	-0.3799	-0.1805
105	Soil	-0.2837	-0.2753
106	Soil6M	-0.4709	-0.4216
107	Soil6M	-0.9856	-0.6457
108	Soil6M	-0.9307	-0.6171
109	Soil24M	-0.5475	-0.3112
110	Soil24M	-0.4797	-0.3427
111	Soil24M	-1.0076	-0.7455
112	Wood6M	0.8625	-0.2961
113	Wood6M	0.8641	-0.3349
114	Wood6M	0.8444	-0.5054
115	Wood24M	0.7405	-0.5574
116	Wood24M	0.8598	-0.5638
117	Wood24M	0.7477	-0.7251
118	Mix6M	0.1294	-0.6682
119	Mix6M	0.2094	-0.6536
120	Mix6M	0.5764	-0.4105
121	Mix24M	0.2913	-0.4377
122	Mix24M	0.4824	-0.6974

123	Mix24M	0.3109	-0.5020
124	Soil6S	-0.4876	-0.4109
125	Soil6S	-0.9614	-0.6005
126	Soil6S	-1.1049	-0.1105
127	Soil24S	-0.6657	-0.4104
128	Soil24S	-0.6639	-0.6093
129	Soil24S	-0.4901	-0.2881
130	Wood6S	0.7940	-0.4616
131	Wood6S	0.7901	-0.4968
132	Wood6S	0.9651	-0.6011
133	Mix6S	0.2921	-0.8396
134	Mix6S	0.5914	-0.7886
135	Mix6S	0.1607	-0.7340
136	Mix24S	0.6689	-0.6202
137	Mix24S	0.5902	-0.4724
138	Mix24S	0.5554	-0.8948
139	Soil6W	0.8199	-0.1168
140	Soil6W	0.8359	-0.1575
141	Soil6W	1.2986	0.0882
142	Soil24W	1.2833	0.1407
143	Soil24W	0.8212	-0.1423
144	Wood6W	0.6099	0.1836
145	Wood6W	0.4027	-0.0714
146	Mix6W	-1.2794	-0.4049
147	Mix6W	-1.1664	0.0469
148	Mix6W	0.0912	-0.1179
149	Mix24W	0.0182	-0.1145
150	Mix24W	-1.5791	0.1814
151	Wood24Bi	0.5964	-1.1821
152	Wood24Bi	0.6744	-1.0650
153	Wood24Bi	0.7387	-0.8980
154	Wood24Bi	0.7085	-0.7974
155	Wood24S	0.9252	-0.8052
156	Wood24S	0.8952	-0.8434
157	Wood24S	0.9651	-0.8136
158	Wood24S	0.6975	-0.9164
159	Wood24W	0.7209	-0.1587
160	Wood24W	0.7922	-0.1997
161	Wood24W	0.7082	-0.2104
162	Wood24W	0.6258	-0.3484
163	SYRC	0.4793	-0.3931
164	SYRC	0.4628	-0.4806
165	SYRCN	0.4578	-0.3915
166	SYRCN	0.3990	-0.4291
167	SYRCN	0.4891	-0.3682




```
Writing weighted average scores on 2 axes for    50 Fas
   into file for graphing.

Calculations completed 20 Sep 2005, 12:24
       26.98 minutes elapsed time.

**************************** Calculations finished
****************************
```

********** Output from Graph **********
PC-ORD Version 4.20
9/20/2005, 12:38 PM

FinalDendroNMS

Pearson and Kendall Correlations with Ordination Axes N= 167

Axis:	1			2			3	
	r	r-sq	tau	r	r-sq	tau	r	r-sq
tau								
FA1	.086	.007	.036	.362	.131	.321		
FA2	.347	.120	.413	.024	.001	-.086		
FA3	-.072	.005	.016	.069	.005	-.090		
FA4	.159	.025	.286	-.194	.038	-.280		
FA5	-.060	.004	-.156	.313	.098	.155		
FA6	.046	.002	-.002	-.073	.005	-.099		
FA7	-.189	.036	-.095	-.101	.010	-.111		
FA8	.043	.002	.095	.046	.002	-.194		
FA9	-.348	.121	-.225	-.276	.076	-.327		
FA10	-.264	.070	-.128	-.118	.014	-.411		
FA11	-.303	.092	-.227	-.233	.054	-.330		
FA12	-.465	.216	-.477	.555	.308	.471		
FA13	-.522	.273	-.568	.416	.173	.305		
FA14	-.066	.004	.021	.349	.122	.185		
FA15	-.040	.002	-.079	.320	.103	.303		
FA16	-.145	.021	.113	-.419	.176	-.532		
FA17	-.189	.036	.088	-.382	.146	-.530		
FA18	.032	.001	.096	.009	.000	-.180		
FA19	-.258	.067	-.111	.059	.004	-.218		
FA20	-.075	.006	.334	-.157	.025	-.416		
FA21	-.285	.081	-.240	-.243	.059	-.258		
FA22	-.153	.023	-.012	-.181	.033	-.152		
FA23	.006	.000	-.014	-.080	.006	-.069		
FA24	.651	.424	.464	-.192	.037	-.127		
FA25	-.635	.404	-.359	-.033	.001	-.222		
FA26	.683	.467	.555	.025	.001	.042		
FA27	.003	.000	.030	.384	.148	.232		
FA28	-.396	.156	-.338	.032	.001	-.151		
FA29	.423	.179	.403	-.507	.257	-.450		
FA30	.285	.081	.219	-.499	.249	-.495		
FA31	.217	.047	.331	-.237	.056	-.339		
FA32	.118	.014	.136	.125	.016	.044		
FA33	.180	.032	.309	.004	.000	-.188		
FA34	.079	.006	.044	.162	.026	.197		
FA35	.007	.000	.043	.222	.049	.131		
FA36	-.070	.005	.200	.403	.162	-.092		
FA37	.158	.025	.162	-.311	.097	-.162		
FA38	.126	.016	.108	.100	.010	.136		
FA39	-.026	.001	-.011	.294	.086	.189		
FA40	.044	.002	.044	-.139	.019	-.083		
FA41	-.107	.011	-.076	-.044	.002	-.036		
FA42	-.100	.010	.000	-.312	.097	-.432		
FA43	-.046	.002	.044	-.173	.030	-.346		
AF44	.327	.107	.327	-.456	.208	-.424		
FA45	.056	.003	.223	-.215	.046	-.316		

FA46	-.104	.011	.079	-.268	.072	-.341
FA47	-.307	.094	-.004	-.362	.131	-.484
FA48	-.003	.000	-.039	.019	.000	.000
FA49	.167	.028	.203	-.370	.137	-.452
FA50	.263	.069	.230	-.380	.144	-.494

FinalDendroNMS

Coefficients of determination for the correlations between ordination distances and distances in the original n-dimensional space:

	R Squared	
Axis	Increment	Cumulative
1	.567	.567
2	.242	.810

Increment and cumulative R-squared were adjusted for any lack of orthogonality of axes.

Axis pair	r	Orthogonality, % = 100(1-r^2)
1 vs 2	-0.211	95.5

Number of entities = 167
Number of entity pairs used in correlation = 13861
Distance measure for ORIGINAL distance: Sorensen (Bray-Curtis)

********** Hierarchical Cluster Analysis
****
PC-ORD, Version 4.20
20 Sep 2005, 12:40

FinalDendroNMS

Linkage method: FARTHEST NEIGHBOR
Distance measure: Sorensen (Bray-Curtis)

Percent chaining = 1.46

********** Cluster analysis completed
****